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RESEARCH ARTICLE

Cytokine gene polymorphisms are associated with risk of urinary bladder cancer and recurrence after BCG immunotherapy

Dinesh K. Ahirwar, Anita Agrahari, Anil Mandhani, and Rama D. Mittal

Department of Urology and Renal Transplantation, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, Uttar Pradesh, India

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Abstract

The association of interleukin-1β (IL-1B) -511C>T and IL-1 receptor antagonist (IL-1RN) VNTR, transforming growth factor-β (TGF-B1) +28C>T and interferon-γ (IFN-G) + 874T>A polymorphisms with bladder cancer (CaB) susceptibility and risk of recurrence in *Bacillus* Calmette–Guérin (BCG)-treated patients was analyzed in 287 controls and 213 CaB patients (73 BCG treated). Increased risk was observed with the IL-1RN*2 allele (odds ratio (OR) 5.01) and the IFN-G +874 A allele (OR 1.78). TGF-B TT and IFN-G +874 A carriers were associated with reduced (hazard ratio (HR) 0.37) and enhanced (HR 2.24) risk of recurrence after BCG immunotherapy, respectively. The study suggests that cytokine gene variants may modulate CaB susceptibility and risk of recurrence after BCG immunotherapy.

Keywords: Bacillus *Calmette–Guérin; urothelial bladder cancer; haplotype; polymorphism*

Introduction

Urinary bladder cancer (UBC) is a major problem worldwide with incidence rate of 20–40 per 100 000 in developed countries; however, it is low in the Asian population and ranges from 3 to 6 per 100 000 individuals (Parkin 2008). Despite the advances in management of non-muscle invasive (NMI) bladder cancer (advances in techniques and better instrumentation/improved vision and thermal energy for resecting the tumour) and therapies (microwave chemothermotherapy and electromotive chemotherapeutic drug delivery), the probability of tumour recurrence (50–70%) and progression (20–30%) remains high (O'Donnell 2007). Intravesical instillation of *Bacillus* Calmette–Guérin (BCG) after transurethral resection is the most effective treatment for NMI bladder cancers (Lamm et al. 2005). It is arguably one of the most effective treatments to reduce the rate of recurrence as well as progression of NMI tumours from a long time. Although the precise mechanism of action is unknown, several studies suggest that it leads to increased production of cytokines which may enhance cell-mediated immunity against cancerous cells (Bohle & Brandau 2003). Despite its efficacy, significant proportions (30–35%) of patients either fail to respond or relapse within the first 5 years of treatment (Nadler et al. 1994).

Cytokines function in a highly coordinated manner and participate in immunological response. Malignancies alter cytokine milieu in a manner facilitating cancerous cells to combat immune surveillance. Increased production of proinflammatory interleukin-1β (IL-1B) has been reported to be associated with tissue damage and more aggressive tumours (Apte et al. 2006). Interleukin-1 receptor antagonist (IL-1RN) binds to the IL-1 receptor competitively and inhibits IL-1-mediated signalling. The regulatory cytokine transforming growth factor-β (TGF-B) exerts tumour suppressive effects that cancer cells elude for malignant development. Methylation-specific inactivation of TGF-B-related genes has been

Address for Correspondence: Rama Devi Mittal, Department of Urology, SGPGIMS, Raebareli Road, Lucknow-226014, India. Tel.: 091-522-2668004-8, ext. 2116. Fax: 091-522-2668017. E-mail: ramamittal@yahoo.com; rmittal@sgpgi.ac.in

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observed in UBC (Suzuki et al. 2005). Interferon-γ (IFN-G) is an immunoregulatory cytokine which regulates the mode of generated immune response. Apart from participating in cancer development, these cytokines together determine the effectiveness of BCG immunotherapy in non-NMI tumours. Increased urinary level of many cytokines, including IFN-G strongly correlates with the anticancer activity of BCG immunotherapy (Bhole & Brandau 2003).

Single nucleotide polymorphisms (SNPs) are major genetic determinants associated with interindividual variations of cytokine production and subsequent predisposition to UBC**.** The IL-1RN gene with a penta-allelic variable number tandem repeat (VNTR) polymorphism and allele 2 (IL-1RN*2), encoding for only two repeats, was observed to be associated with higher risk of bladder cancer than other alleles encoding for longer (L) repeats (Bid et al. 2006). Various studies have reported the functional role of TGF-B1 + 28C > T polymorphism in expression of TGF-B1 (Grainger et al. 1999). Similarly, the polymorphism in IFN-G gene at the + 874 position (T allele) creates a binding site for transcription factor nuclear factor (NF)-κB and participates in regulation of IFN-G expression (Pravica et al. 2000). Therefore, keeping in mind the importance of these cytokines in UBC, we studied the association of IL-1B -511C > T, IL-1RN intron-2 repeat polymorphism, TGF-B1 + 28 C > T and IFN-G +874 T > A polymorphisms with UBC susceptibility, tumour stage, tobacco use and risk of recurrence after giving BCG immunotherapy**.** Moreover the contributions of these polymorphisms may jointly affect bladder cancer risk through gene–gene and gene–smoking interactions.

Materials and methods

Study subjects

The present case–control study of bladder cancer was conducted in samples collected from Lucknow, North India, between May 2003 and June 2007. The study population has been detailed previously (Mittal et al. 2008). Briefly, 213 patients with histologically confirmed transition cell carcinoma of urinary bladder (M:F 186:27; mean age 60.67 ± 12.1 years) who had not received radiotherapy were included in this study from a tertiary care hospital. Simultaneously, 287 age-, gender- and similar ethnicity-matched controls (M: F, 252:35; mean age 60.06 ± 8.7 years) were recruited randomly from the unrelated individuals visiting hospital, in the health-awareness camp and hospital employees. Detailed information on tobacco use, family history of bladder cancer, demographic background, medication use and lifetime occupational history was recorded following a standard clinical proforma (Mittal et al. 2008). Of the controls, 80% provided demographic details including smoking status. The remainder either decline to provide information or could not complete the interview. Informed consent was obtained from each participant in the study. The Institute Ethical Review Board approved the study

Epidemiology and clinical data collection

The clinical information about tumour size, number, stage and grade, intravesical BCG immunotherapy, date of recurrence and histopathological findings, were obtained from the participating urologist in the department. The tumour stage was classified as per norms laid down by American Joint Committee on Cancer's TNM staging system (Sobin & Wittekind 1997). After initial transurethral resection of the bladder tumour (TURBT), all the patients underwent cystoscopy every 3 months in first 2 years and twice yearly thereafter as long as there was no tumour recurrence. Seventy-three high-risk patients (high grade/multiple/ > 3 cm size tumour) (34.27%) of the 213 patients were treated with live attenuated Danish 1331 strain (Guindi lab, Chennai, India) of BCG. Intravesical BCG treatment consisted of either BCG six weekly instillations (induction BCG (iBCG = 64)) or BCG induction + monthly instillations (maintenance (mBCG = 9)). Because the number of patients receiving mBCG was too low, we included them in the BCG-treated group for statistical calculations. Recurrence was defined as a newly found bladder tumour following a previous negative cystoscopy. The remainder of the patients were treated with cystoscopic surveillance and/or mitomycin-C chemotherapy.

Genotyping

Genomic DNA from all subjects was extracted from peripheral blood mononuclear cells using the salting out method (Miller et al. 1988). Detection of genotype variants in the promoter region of the IL-1B gene at the position of C-511T was analyzed by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method after digesting the PCR product with the *Ava*I restriction enzyme. The primer sequence and PCR conditions used were as described earlier (Machado et al. 2001). The genotyping of TGF-B +28 and IFN-G polymorphisms was done as described previously (Pravica et al. 2000, Kruit et al. 2006,). Various alleles of IL-1RN VNTR polymorphism were detected as short (allele 240 bp *2) and long (allele 410, 500 and 325 bp L). The genotypes pattern were classified as L/L (wild-type), L/*2 and *2/*2 in accordance with the report by Machado et al (2001). Validation of the results was done by re-genotyping of 5% of the samples by other laboratory personnel who were blinded to the sample

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identity. The results were reproducible with no discrepancy in genotyping. These samples were further reconfirmed by DNA sequencing.

Statistical analysis

The power of the study was calculated using Quanto software version 1.0 (http://hydra.usc.edu/gxe). The present study achieved 80% of the statistical power for odds ratio (OR) ≥ 1.65 at significance level (α) < 0.05. Hardy–Weinberg equilibrium was checked in control by the goodness of fit χ^2 test. Pearson χ^2 test was used to compare controls and patients. Multiple logistic regression analysis was also performed to assess the risk (odds ratio) associated with UBC after adjusting for relevant biological variables (age, gender and smoking). Pair-wise linkage disequilibrium analysis and haplotypes of each individual consisting of two alleles (IL-RN repeat allele and C-511T allele) constructed by expectation–maximization algorithm using Arlequin software version 2.1 (Excoffier & Slatkin 1995). Further, multiple Cox regression analysis adjusted for age, gender and smoking was used to assess the effect of individual SNPs on the risk of recurrence in BCG-treated patients. Bonferroni's correction was applied in case of multiple comparisons using the formula $pc = p \times n$ (pc represents corrected value; p is χ^2 p value and n is the number of comparisons performed). In case of haplotypes, the p-value was corrected according to number of haplotypes compared (n, 4). A two-tailed p and p_c value of less than 0.05 was considered statistically significant. All the statistical analysis was performed using SPSS software (version 11.5).

Results

A total of 500 individuals were analyzed in the present study. Table 1 represents demographical details of the 287 controls and 213 patients. No significant differences were identified between the controls and patients in reference to age and gender (Table 1). A significantly higher percentage of smokers among cases (49.7%) compared with the controls (10.5%, $p < 0.001$) was observed. All the polymorphisms followed the Hardy–Weinberg equilibrium in control and patients.

Association of gene polymorphisms with CaB susceptibility

The associations of cytokine gene polymorphism with bladder cancer (CaB) risk are presented in Table 2. Multiple logistic regression analysis in CaB patients revealed that the IL-1RN polymorphism was associated with increased risk of CaB and a trend of increasing risk was observed with increasing number of the *2 allele. The heterozygous genotype (L/*2) demonstrated a five-fold increased risk (OR 5.06, $p < 0.001$, 95% confidence interval (CI) 3.06–8.35). Similarly, the homozygous

Table 1. Demographical details of study subjects.

Characteristics	Controls *n* (%)	Patients *n* (%)	*p*-Value
Age (years), mean ± SD	60.06 ± 8.7	60.67 ± 12.1	0.515
Female	35 (12.2)	27 (12.7)	
Male	252 (87.8)	186 (87.3)	0.922
Tobacco use[a]			
Non-users	188 (67.8)	64 (35.0)	
Smokers	29 (10.5)	91 (49.7)	<0.001
Chewers	34 (12.3)	13 (7.1)	0.730
Chewer + Smoker	26 (9.4)	15 (8.2)	0.380
Tumour stage			
Superficial	–	147 (69.0)	
Invasive	–	66 (31.0)	
Tumour grade			
Low grade (G1)	–	129 (60.6)	
High grade (G1+G2)	–	84 (39.4)	

[a]Missing data

Table 2. Association of cytokine gene polymorphisms with urinary bladder cancer risk.

Genotype	Controls *n* (%)	Patient *n* (%)	*p*-Value	OR (95% CI)
IL-1RN				
L/L	215 (47.9)	66 (31.0)	–	
L/*2	47 (16.4)	79 (37.1)	<0.001	5.06 (3.06–8.35)
*2/*2	25 (8.7)	68 (31.9)	<0.001	9.46 (5.14–17.39)
*2 carrier	72 (25.1)	147 (69.0)	<0.001	6.44 (4.16–9.96)
Allele L	477 (83.1)	211 (49.5)	–	
Allele *2	97 (16.9)	215 (50.5)	<0.001	5.01 (3.75–6.69)
IL-1B C-511T				
CC	102 (35.6)	106 (49.8)	–	
CT	131 (45.6)	77 (36.2)	0.837	0.94 (0.52–1.67)
TT	54 (18.8)	30 (14.0)		0.69 (0.31–1.53)
T carriers	185 (64.5)	107 (50.2)	0.606	0.86 (0.94–1.53)
Allele C	335 (58.4)	289 (67.8)	–	
Allele T	239 (41.6)	137 (32.2)	0.002	0.66 (0.51–0.86)
TGF-B				
CC	71 (24.7)	48 (22.5)	–	
CT	126 (43.9)	84 (39.4)	0.966	1.01 (0.49–2.06)
TT	90 (31.4)	81 (38.0)	0.491	1.29 (0.62–2.69)
T carriers	216 (75.3)	165 (77.5)	0.599	1.18 (0.63–2.23)
Allele C	268 (46.7)	180 (43.3)		
Allele T	306 (53.3)	246 (57.7)	1.163	1.19 (0.93)
IFN-G				
TT	116 (40.4)	73 (34.3)	–	
TA	131 (45.6)	63 (29.6)	0.164	0.63 (0.33–1.20)
AA	40 (13.9)	77 (36.1)	0.017	2.32 (1.16–4.63)
A carriers	171 (59.6)	140 (65.7)	0.954	1.01 (0.58–1.75)
Allele T	363 (63.2)	209 (49.1)		
Allele A	211 (36.8)	217 (50.9)	<0.001	1.78 (1.38–2.30)

OR, age, gender and smoking adjusted odds ratio; CI, confidence interval.

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genotype (*2/*2) was imposing more than ninefold risk s compared with the wild-type genotype (L/L) (OR 9.46, $p < 0.001$, 95% CI 5.14–17.39). Likewise, a *2 carrier (L/*2 + *2/*2) was associated with an increased risk of CaB (OR 6.44, $p < 0.001$, 95% CI 4.16–9.96). However, IL-1B C-511T genotypes were not associated with risk of CaB (Table 2). Although, the allele frequency distribution of IL-1B was significantly different between patients and controls ($p < 0.002$), the association with UBC risk was not observed as the OR was not in the significant limits (OR ≥ 1.65 and < 0.50) to demonstrate association (OR 0.66, 95% CI 0.51–0.86). The IFN-G AA genotype and A allele were associated with an enhanced risk of UBC (AA OR 2.32; 95% CI 1.16–4.63 and A allele OR 1.78; 95% CI 1.38–2.30). Further, we constructed IL1-B/RN haplotypes which revealed that haplotypes with the *2 allele conferred increased risk (C/*2; OR 5.76, $p < 0.001$, 95% CI 3.93–8.43 and T/*2 OR 2.48, $p < 0.001$, 95% CI 1.61–3.82), while a haplotype without the *2 allele (T/L) did not demonstrate association with CaB (OR 0.75, $p < 0.376$, 95% CI 0.53–1.05) (Table 3).

Interaction of genotypes with tumour stage and tobacco use

To analyze the association of these genotypes, the patient groups were stratified into NMI and muscle invasive tumours. The frequency distribution suggested no association between genotypes and tumour stage (Table 4). Further, we compared the genotype distribution among tobacco users to analyze the interaction of genotypes with tobacco use and CaB susceptibility. None of the polymorphisms demonstrated interaction with the use of tobacco to modify risk of CaB (data not shown).

Influence of genotypes on risk of recurrence after BCG immunotherapy

Further, we analyzed the association of genotypes with the risk of recurrence in NMI patients with UBC who were underwent BCG immunotherapy. Cox regression analysis showed that TGF-B and IFN-G polymorphisms were associated with recurrence after BCG immunotherapy (Table 5). TGF-B TT demonstrated protective association (hazard ratio (HR) 0.37; 95% CI 0.14–0.98), while the IFN-G TA genotype was associated with an elevated risk of recurrence after BCG immunotherapy (HR 2.80; 95% CI 1.13–6.97). Similarly, A carrier was associated with 2.2-fold risk of recurrence (HR 2.24; 95% CI 1.06–5.80).

Table 3. Distribution of the IL-1B/IL-1RN haplotype frequency with risk of bladder cancer development in patients compared with controls.

IL-1B/IL-1RN	Controls *n* (%)	Patients *n* (%)	pc	OR (95% CI)
C/L	275 (47.9)	136 (31.9)	–	Reference
C/*2	60 (10.5)	153 (35.9)	< 0.001	5.15 (3.583–7.40)
T/L	202 (35.2)	75 (17.6)	0.376	0.75 (0.53–1.05)
T/*2	37 (6.4)	62 (14.6)	< 0.001	3.38 (2.14–5.34)

pc, Bonferoni's corrected *p*-value; OR, age, gender and smoking adjusted odds ratio; CI, confidence interval.

Table 4. Cytokine genotypes and risk estimates, a stratified analysis of bladder cancer risk for the genotypes by tumour stage.

Genotype	Superficial *n* (%)	Invasive *n* (%)	*p*-Value	OR (95% CI)
IL-1B				
CC	71 (48.3)	35 (53.0)	–	Reference
CT	58 (39.5)	19 (28.8)	0.315	0.68 (0.32–1.43)
TT	18 (12.2)	12 (18.2)	0.563	1.32 (0.50–3.48)
IL-1Ra				
L/L	40 (27.2)	26 (39.4)	–	Reference
L/*2	58 (39.5)	21 (31.8)	0.076	0.48 (0.21–1.08)
*2/*2	49 (32.3)	19 (28.8)	0.212	0.59 (0.25–1.35)
TGF-B				
CC	33 (22.4)	15 (22.7)		
CT	53 (36.1)	31 (47.0)	0.320	1.99 (0.51–7.79)
TT	61 (41.5)	20 (30.3)	0.271	0.39 (0.07–2.06)
IFN-G				
TT	52 (35.4)	21 (31.8)		
AT	42 (28.6)	21 (31.8)	0.274	2.07 (0.56–7.62)
AA	53 (36.1)	24 (36.4)	0.796	1.19 (0.31–4.47)

OR, age, gender and smoking adjusted odds ratio; CI, confidence interval.

Table 5. Cox regression analysis of cytokine gene polymorphisms and risk of recurrence in patients treated with BCG immunotherapy.

Genotype	Non-recurrence (*n* = 43) *n* (%)	Recurrence (*n* = 30) *n* (%)	*p*-Value	HR (95% CI)
IL-1B C-511T				
CC	22 (51.2)	14 (46.7)	–	Reference
CT	15 (34.8)	12 (40.0)	0.811	1.09 (0.50–2.37)
TT	6 (14.0)	2 (13.3)	0.883	1.08 (0.35–3.32)
T carriers	21 (48.8)	14 (53.3)	0.873	1.06 (0.47–2.41)
IL-1 RN				
L/L	13 (30.2)	8 (26.7)	–	Reference
L/*2	15 (34.9)	8 (26.7)	0.982	0.98 (0.37–2.64)
*2/*2	15 (34.9)	14 (46.6)	0.797	1.12 (0.46–2.69)
*2 Carriers	30 (69.8)	22 (73.3)	0.803	1.09 (0.53–2.24)
TGF-B				
CC	7 (16.3)	10 (33.3)	–	Reference
CT	13 (30.2)	13 (43.4)	0.364	0.681 (0.29–1.56)
TT	23 (53.5)	7 (23.3)	0.046	0.37 (0.14–0.98)
T carriers	36 (83.7)	20 (66.7)	0.100	0.52 (0.24–1.13)
IFN-G				
TT	20 (46.5)	7 (23.3)	–	Reference
TA	9 (20.9)	14 (46.7)	0.024	2.80 (1.13–6.97)
AA	14 (32.6)	9 (30.0)	0.114	2.09 (0.77–5.66)
A carriers	23 (53.5)	23 (76.7)	0.036	2.24 (1.06–5.80)

HR, age gender and smoking adjusted hazards ratio; CI confidence interval.

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Discussion

Cytokine milieu at the site of chronic inflammation plays a critical role in cancer development and it is observed that IL-1B cross-talk between tumour cells and the chronic environment to facilitate tumour progression. There are many reports describing the association of IL-1B -511C > T and IL-1RN VNTR polymorphisms with oesophageal, prostate and cervical cancer (Upadhyay et al. 2008, Cheng et al. 2007, Santtila et al. 1998). Our group too reported association of the IL-1RN allele *2 with an increased risk of CaB development (Bid et al. 2006). In this subsequent study, we have validated our previous results in a comparatively large cohort and reported the association of the IL-1RN allele *2 with an increased risk of CaB while no association was observed with the IL-1B -511C > T polymorphism. In addition to the previous report, we observed that the haplotypes carrying *2 allele (C/*2 and T/*2) were associated with an increased risk for CaB development. On the other hand, haplotype T/L was not associated with the risk for CaB. This observation suggests that the *2 allele is a potential marker for determining genetic susceptibility to UBC. This observation can further be elucidated with the study conducted by Santtila et al. (1998). The author stated that production of IL-1B was regulated by IL-1RN VNTR polymorphism regardless of IL-1B -511C > T polymorphism which suggested that allele T (position -511) had a slight but non- significant elevated capacity to produce IL-1B. However, IL-1RN*2 strongly increased the production of IL-1B (Nazarenko et al. 2008). In addition, it has been observed that IL-1B can induce angiogenesis via upregulation of COX-2 or inducing nitric oxide and vascular endothelial growth factor, which may favour further tumour growth (Rahman et al. 2001, Ben-Av et al. 1995). These observations support our finding and suggest that enhanced IL-1B production due to the presence of the *2 allele may explain the increased risk of CaB observed in the present study.

Similarly IFN-G is considered to be an immunoregulatory cytokine which determines T helper type 1 (Th1)/Th2 immune response. IFN-G +874 A > T is functionally active and associated with increased production (Pravica et al. 2000). The nature of the association of IFN-G + 874 polymorphism is ambiguous in various types of cancers. As illustrated by the fact that the IFN-G AA genotype is a risk factor for cervical cancer, whereas the TT genotype is at risk in breast cancer, while no association was observed with nasophanryngeal carcinoma (Rahman et al. 2001, Ben-Av et al. 1995, Schamhart et al. 2000). To the best of our knowledge we are, for the first time, reporting in the present study that the IFN-G + 874 TT genotype confers an enhanced risk of CaB. Further, no association of polymorphisms with tumour stage and tobacco use was observed suggesting no role of tobacco carcinogen-induced malignancy.

As recurrence is a common feature in CaB we wanted to determine if any of the genes studied were associated with the BCG outcome. Further we analyzed the association of these polymorphisms with the risk of recurrence in patients who underwent BCG immunotherapy. The precise molecular mechanism involved in the tumour anticancerous activity of BCG immunotherapy is complex and vague so far.

Although elevated levels of IL-1B have been reported after BCG treatment compared with controls, the Th1 cytokines (TNF-A, IFN-G and IL-12) remain the major molecules associated with effectiveness of BCG immunotherapy (Schamhart et al. 2000, Luo et al. 2003). Although, IFN-G is a late responsive cytokine to BCG, it has been reported that BCG successfully enhances the Th1 type of immune response against cancerous cells by upregulating IFN-G (O'Donnell et al. 1999). A number of early cytokines endogenously produced upon BCG stimulation appear to be functionally involved in its production (Luo et al. 2003). The reduced production of IFN-G due to the presence of the + 874AA genotype may lead to a suboptimal immune response after BCG immunotherapy, which may perhaps assist in explaining the increased risk of recurrence observed with the + 874 AA genotype. Basturk et al. (2006) conducted a similar study demonstrating an association of different cytokine gene polymorphisms with the risk of progression in BCG-treated patients (Basturk et al. 2006). The authors observed that TGF-B codon 25 was associated with a risk of progression while IL-1B, IL1RN and IFN-G polymorphisms had no effect. This observation can be explained by the activity of TGF-B to regulate cell proliferation and apoptosis, subsequently preventing incipient tumours from progressing down the path to malignancy (Massague 2008). It is possible that a higher production of TGF-B due to the + 28 T allele (Awad et al. 1998) enhances the apoptosis of cancerous cells in addition to BCG, which may explain the protective association observed with risk of recurrence after BCG in our study. These observations strongly suggest the hypothesis that the alteration in immune response due to cytokine gene polymorphism may modulate the clinical outcome of BCG treatment.

The involvement of other regulatory factors apart from genetic factors is also a determining factor in complex mechanism of recurrence and progression of tumours. Although we observed a strong association of the IL-1RN allele *2 with CaB, the results remain to be validated in a large population and different ethnic groups; simultaneously functional studies are required to corroborate the results.

Our study from a North Indian population provides evidence that the IL-1RN 86 bp VNTR and IFN-G + 874

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polymorphisms might contribute to the aetiology of bladder cancer. However tumour stage or smoking had no profound effect on CaB risk. The study also suggests that TGF-B C+28T and IFN-G T+874A polymorphisms may modulate outcome after BCG immunotherapy. Hence the present study may provide an important lead to determine the genetic mechanism underlying in the process of tumour development and recurrence for future studies. Further validation of the functionality of these polymorphisms and its association with risk of bladder and other cancers in other ethnic populations is warranted.

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