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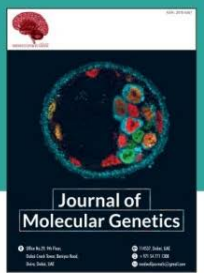

Impact of IL1, IL6, IL8, IL17 and TGF Gene Polymorphisms in Bladder Cancer Patients

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Abstract: Bladder cancer is one of the most common cancers worldwide and the most frequent malignancy of the urinary tract recently, several Single Nucleotide Polymorphisms (SNPs) have been identified in cytokine gene sequences, particularly within the promoter region of these genes and have been shown to be associated with the development of bladder cancer. The present study investigates the association of IL1, 6, 8, 17 and TGF polymorphisms with the incidence of bladder cancer. Blood samples were collected from 50 histologically confirmed adult patients with bladder cancer and 28 apparently healthy individuals. DNA was extracted from each blood sample and the genes were amplified using Polymerase Chain Reaction (PCR) with gene-specific primers target. Systemic IL1β, 6, 8, 17 and TGFβ, concentration was assessed in serum samples from each participant by Enzyme-Linked Immunosorbent Assay (ELISA). A appositive association between the allele T of the SNP rs 16944 of IL 1 gene (p = 0.005, OR = 4.263, 95% CI = 1.566-11.603) and the allele G of the SNP rs 1800469 of TGFβ gene (OR = 0.19, 95% CI = 0.035-1.02) were found. These data indicate an etiological role of IL-1β and TGF gene polymorphism in bladder cancer.

INTRODUCTION

Bladder Cancer (BC) which is the most common malignancy of the urinary tract commonly affects the elderly and men with an estimated 73,510 new cases and 14,880 deaths in the United States in 2012 (Chan *et al*., 2009). Major risk factors for bladder cancer include male gender, older age, tobacco smoking and occupational exposure to aromatic amines (Chen *et al*., 2013). It is increasingly recognized that genetic susceptibility may contribute to bladder cancer (Abdulamir *et al*., 2009). Therefore, identifying individuals susceptible to cancer with the aid of genetic markers can reduce health care costs, increase the cost-benefit of screening and surveillance and improve the treatment and survival of cancer patients.

Inflammation seems to play a critical role in the development of several types of cancers (Edge *et al*., 2010). The presence of chronic inflammation can cause cellular damage resulting in many disease including cancer (Abdulmohymen *et al*., 2010). The functional association between chronic inflammation and cancer dates back to 1863 by Viroch who hypothesis that cancer arise in sites of inflammation because of prolonged irritation and tissue injury. The precise molecular mechanism events leading to malignant transformation have not been fully eliuctated (Burger *et al*., 2000).

Genetic variants of several pathways critical for the inflammatory response have been studied and a number of single nucleotide polymorphisms SNPs in several genes from different pathways have been associated with bladder cancer including IL1, 6, 8, 17 and TGF (Brookes *et al*., 1999). However, there are no reports on the association between the polymorphism of cytokine genes and the development of bladder cancer in the Iraqi population.

Interleukin-1 (IL-1) is a pro-inflammatory cytokine with multiple biological effects. Interleukin-1 plays a central role in the regulation of immune and inflammatory responses to infections. This cytokine is mainly produced by tissue macrophages, monocytes, fibroblasts and dendritic cells. Large number of Single Nucleotide Polymorphisms (SNPs) have been identified in the IL-1ß gene (Camargo *et al*., 2006).

IL6 is produced primarly by mononecular phagocyte, fibroblast, endothelial cells, T-cells and B lymphocyte granulocytes, smooth muscle cells, esinophilis, mast cells, glial cells and keratinocytes, it is a multifunctional cytokine and play roles in adaptive immune response, inflammation and endocrine system (Belluco *et al*., 2010). Although, IL-6 was originally considered to be pro-inflammatory cytokine but several discoveries indicated anti-inflammatory properties, for instances, IL-6 inhibited neutrophil accumulation after lipopolysaccharide injection and antagonized the action of IL-1 and TNF (Cardiolo and Lippolit, 2006).

Interlukin 8 is a type of neutrophil-activating cytokine, IL-8 is a chemotactic factor that attracts neutrophils, basophils and T-cells but not monocytes. It is also involved in neutrophil activation. It is released from several cell types in response to an inflammatory stimulus. It was found that IL-8 have a 5-10 fold higher activity on neutrophil (Dorneles *et al*., 2016). Interleukin 8 have many biological activities including angiogenesis, calcium-mediated signaling, cell cycle arrest, cell motility, chemotaxis, embryonic gut development, G-protein coupled receptor protein signaling pathway, immune response, induction of positive chemotaxis, inflammatory response, negative regulation of cell proliferation, negative regulation of G-protein coupled receptor protein signaling pathway, neutrophil activation, neutrophil chemotaxis, positive regulation of angiogenesis, receptor internalization, regulation of cell adhesion, regulation of retroviral genome replication, response to molecule of bacterial origin and signal transduction (Landi *et al*., 2003).

IL-17 Interleukin 17 is a pro-inflammatory cytokine produced by T-helper cells and is induced by IL-23. To elicit its functions, IL-17 binds to a type I cell surface receptor (Dorneles *et al*., 2016). In promoting inflammation, IL-17 has been demonstrated to act synergistically with tumor necrosis factor and interleukin-1. This activity can also be redirected towards the host and result in various autoimmune disorders that involve chronic inflammation (Landi *et al*., 2003).

TGF-ß Transforming growth factor beta (TGF-ß) is a multifunctional cytokine belonging to the transforming growth factor super family (Chen *et al*., 2014).

In Iraq addressing the impact of cytokine gene polymorphism with bladder cancer, therefore, this study aimed to investigate the association of SNPs in five cytokine genes with bladder cancer. TGF-ß is a potent inhibitor of proliferation in epithelial cells and acts as a tumor suppressor. Additionally, continuous expression of TGF-ß by cancerous cells helps cancer to progress further (Wang *et al*., 2012).

MATERIALS AND METHODS

Two hospitals in Iraq, Al-Kadimiya Teaching Hospital and a Private Nursing Home Hospital near Baghdad were included in this study. Patients attending these hospitals to undergo surgical resection of bladder cancer from March, 2015 to November, 2015 were included in this study. Ethical clearance to conduct the research was obtained from these hospitals separately. Selection of patients was accomplished after the assistance of surgeons in the hospitals sixty patients were selected for this study. All patients had bladder tumors of different grade and stages (38 men and 22 women) and with a mean age of 65 years (range between 43-80). The 28 controls were selected randomly from apparently healthy individuals (18 men and 10 women) with a mean age of 51 years (range between 21-75). Individuals previously diagnosed with cancer at the time of enrollment were excluded from this group. Data were collected through direct interview with the participants and by seeking patient's hospital record as well as previous medical reports. Patient's claims were followed as an alternative source of information when his/her previous medical reports were not available. These data included age, previous and current occupation, smoking, drinking, residence and first relative family history of brain cancer.

Blood samples: The 5 mL of blood were taken from normal healthy controls and patients before the initiation

Table 1: Primers used for genes amplification

Gene	Primer	Sequence	Annealing temp. (°C) (sec)	Fragment size (pb)
IL1	Forward	GGGGGCTTCACTATGTTGCCCACACTG-3	58 for 30	304
	Reverse	GGGCATGGATTTTTACATATGAGCCTTCCA-3		
IL6	Forward	ATGCCAAGTGCTGAGTCACTA-3'	55 for 30	300
	Reverse	TCGAGGGCAGAATGAGCCTC		
IL8	Forward	CCATCATGATAGCATCTGT -3'	54 for 30	190
	Reverse	CCACAATTTGGTGAATTATTAA-3'		
IL17	Forward	CCCCCAAATGAGGTCATAGAAGAGAATC-3	57 for 30	470
	Reverse	AACAAGTAAGAATGAAAAGAGGACATGGT		
TGF	Forward	CGGACACCCAGTGATGGG	52 for 30	530
	Reverse	CCTCCTGGCGGCCAAGCGC-		

of chemotherapy or radiation therapy. The 2 mls were kept in an EDTA tube (for the SNPs study) at -20°C for further analysis. The remaining 3 mls were collected in an uncoated tube and serum was obtained by centrifugation then stored at -80°C until used for estimation of serum levels of IL-1, IL-6, IL-8, IL17 and TGF.

DNA extraction and genotyping: DNA was extracted from each blood sample using the gSYNCTM DNA Mini Kit with Whole Blood Protocol (Geneaid/Korea), according to the manufacturer's protocol. The primers used were in Table 1.

Template DNA (10 µL) from each sample and primers (5 µL of each) were added to the PCR master-mix (Bioneer/Korea) (50 µL). The mixture was then put in a shaker and spinner for 10 cycles for mixing. After mixing, the master-mix tubes were transferred to the thermocycler (MyGenie 32 thermal block/Bioneer/Korea). The PCR protocol involoved an iniatial denaturation of 94°C for 5 min followed by 35 cycle of denaturation at 94°C for 30 secm anneling then enlogation at 72°C for 30 sec.

Measurement of dna concentration of purified PCR product: The DNA concentration of the purified PCR products was measured using a Nanodrop/UVS-99 (ACTGene/USA). All products had a concentration of >100 ng mL^{-1} and DNA was sequenced.

DNA sequencing: Polymerase chain reaction products were sent to macogen Company/Korea for DNA sequencing. The obtained sequences were aligned using "ClustalW" software with a normal sequence from GenBan for IL1, 6, 17 and TGF and examined for the presence of polymorphisms.

Estimation of serum levels of IL-4: An Enzyme-Linked Immunosorbent Assay (ELISA) kit from (Maptech, USA) was used to estimate serum levels of IL1, 6, 8, 17 and TGF in each sample according to the manufacturer's instructions.

Statistical analysis: The Statistical Package for the Social Sciences (SPSS, Version 14) was used for statistical analysis. Risk association between the genotype and bladder cancer susceptibility and the distribution of different alleles between patients and controls were estimated by the calculation of adjusted odds ratio and 95% confidence intervals using binary logistic regression. Chi-square analysis was used for testing the deviation from Hardy-Weinberg equilibrium and serum levels of IL1, 6, 8, 17 and TGF. All $p<0.05$ were considered statistically significant.

RESULTS

Table 2 shows the characteristics and risk factors in this study of bladder cancer patients and healthy controls. For Interlukine-1(rs 16944) the CT genotype was significantly increased in patients compared to control frequency ($p = 0.005$, OR = 4.263, 95 CI = 1.566-11.603) While for Interlukine-6(rs1800795) the genotypes distribution frequency and their allele revealed no significant differences between patients and controls.

It has been shown that single nucleotide polymorphisms in the IL-8 gene are not associated with the incidence of bladder cancer, although, Interlukine-8 (rs 4073) and the genotype AT genotype show significant difference ($p = 0.03$), the frequency distribution was decrease in patient compared to control (OR = 3.773, 95% CI = 1.141-12.478) m bladder patients and controle show no deviarion from HWE.

Interlukine-17(rs 763780) the CT genotype (0R = 5.250, 95% CI = 1.402-19.661, $p = 0.014$) and T allele (0R = 4.20,95% CI = 1.546-11.409) frequencies significantly increase in patients compared to control ($p = 0.002$).

TGF beta (rs 1800496) genotype and allele frequency of this gene show no significant variation between bladder patients and controls (Table 3).

Systemic serum concentration: Serum level of cytokines show significant differences between bladder cancer patients and control, for IL6 and IL8 the levels in serum in patients (11.74 and 72.477 pg mL^{-1}, respectively) was higher than its level in control (9.977 and 53.68 pg mL^{-1}, respectively) while for IL17 the serum level revealed significant decrease in mean value in patients (10.713 pg mL^{-1}) than control (15.84 pg mL^{-1}). At the

Table 2: No significant variation between bladder patients and controls

Variables	Cases N = 60	Control N = 28	p-values	OR (95% CI)
rs16944 (IL-1B)				
Genotype				
GG	19 (31.67%)	18 (64.28%)	0.017	1.0
GA	36 (60%)	8 (28.58%)	0.005	4.263 (1.566-11.603)
AA	5 (8.33%)	2 (7.14%)	0.338	2.368 (0.407-13.794)
Alleles				
G	74 (61.67%)	44 (78.57%)	0.014	1.0
T	46 (38.33%)	12 (21.43%)		2.361 (1.131-4.928)
rs1800795(IL-6)				
Genotype				
GG	42 (70%)	22 (78.58%)	0.682	1.0
GC	14 (23.33)	5(17.85%)	0.512	1.467 (0.467-4.604)
CC	4 (6.67%)	1 (3.57%)	0.520	2.095 (0.221-19.903)
Alleles				
G	98 (81.67%)	49 (87.5%)	0.228	1.0
C	22 (18.33)	7 (12.5%)		1.571 (0.628-3.932)
rs4073(IL-8)				
TT	32 (53.33%)	22 (78.57%)	0.044	1.0
AT	21 (35%)	5 (17.86%)	0.030	3.773 (1.141-12.478)
AA	7 (11.67%)	1 (3.57%)	0.143	5.031 (0.579-43.745)
Alleles				
T	85 (70.83%)	50 (89.29%)	0.005	1.0
A	35 (29.17%)	6 (10.71%)		3.431 (1.349-8.730)
rs763780				
Genotypes				
CC	32 (53.33%)	24 (85.71%)	0.022	1.0
CT	21 (35%)	3 (10.71%)	0.014	5.250 (1.402-19.661)
TT	7 (11.67%)	1 (3.57%)	0.133	5.250 (0.605-45.573)
Alleles				
C	85 (70.83%)	51 (91.07%)	0.002	1.0
T	35 (29.17%)	5 (8.93%)		4.20 (1.546-11.409)
Rs1800469 (TGFB1)				
Genotypes				
AA	12 (20%)*	7 (25%)	0.549	1.0
AG	43 (71.67%)	17 (60.71%)	0.484	1.475 (0.497-4.381)
GG	5 (8.33%)	4 (14.29%)	0.701	0.729 (0.146-3.654)

Table 3: Systemic serum concentration

	Mean±SD		
Parameters	Control	BC patients	p-values
IL1	6.425±3.6	7.264±3.918	0.702
IL6	9.977±2.143	11.74±3.444	0.026
IL8	53.68±18.811	72.477±24.1	0.001
IL17	15.848±8.970	10.713±6.22	0.001
TGF	25.01±13.744	27.722±13.391	0.398

same time no significant differences was observed for IL1 and TGF in patients when comparing to control (Table 2).

DISCUSSION

Bladder cancer remains to be a challenging disease due to the high rate of recurrence and the accompanied high medical costs. Using genetic markers for determining risk may help to identify high risk population for early screening, diagnosis and therapy which may improve clinical outcome. In this study, we explored the role of polymorphisms in genes related to inflammation in bladder cancer. We found that a polymorphism in the promoter of the IL-1 and TGF gene is associated with a significantly increased risk of bladder cancer. The result pointed out that the genotype CT and allele T of IL1ß gene was associated with increase risk of bladder cancer. However, it is conceivable to know that IL1ß gene polymorphism might play a role in the process of bladder cancer initiation and progression. The initiation and progression of bladder cancer are an inflammatory process and thus IL-1 considered as one of the most important pro-inflammatory cytokine which control inflammation (Jiali *et al*., 2013). This genetic abnormality has been associate with increase in IL1ß level which suggests a mechanism by which genetic polymorphism act to modulate IL 1 protein production and this may influence the pathogenesis of bladder cancer. This finding has also been previously confirmed by Landvik, etc. who found an association between a specific polymorphism in the promoter region of IL 1 ß with increased in IL 1ß mRNA level and thus increased risk of lung cancer.

Our preliminary study showed that there was no relationship between IL-6, IL-8 and IL-17 with the susceptibility to bladder cancer in Iraqi patients. SNP in the promoter region of the transforming growth factor beta1 gene doesn't change the nature or level of the TGF-ß1 protein, these results are in agreement with Cardiolo, etc. and Huang *et al*. (2013) presented analyses

of the TGF-ß1 promoter where in the rs1800469 promoter was found to interfere with the identification of functional transcription factors which influenced the gene expression levels of TGF-ß1 (Kim *et al*., 1996).

Studies have reported an association between bladder cancer susceptibility and various inflammatory factors that either modulate the immune response or serve as a surrogate for immune dysfunction (e.g., polymorphisms in immune genes). TGF is one of the key players in an immune response and dysregulation in the production of this cytokine may strongly influence the immune system with subsequent decreased or increased susceptibility to bladder cancer (Burger *et al*., 2013). The results from our current study clearly indicate a estimation role of SNPs in the TGF gene against bladder cancer. These results are in agreement with a previously published study (Benson, 2004).

CONCLUSION

While the precise mechanism of this estimation is not fully understood, it is thought that SNP rs 1800469 results an increase TGF production (Stefanovic *et al*., 1994). In the present study, we also noted an increase in serum levels TFG among control versus bladder cancer patients Therefore, it is reasonable to believe that these SNPs increase production of TGF, thereby influencing bladder cancer susceptibility in the patients.

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