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Association of IL-4 and IL-1 Ra Gene Polymorphisms with the Risk of Bladder Cancer

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ABSTRACT: The aim of this study is to evaluate the association of interleukin-4 (IL-4) gene intron 3 and interleukin-1 receptor antagonist (IL-1Ra) gene intron 2 variable number tandem repeat (VNTR) polymorphisms with bladder cancer (BC) susceptibility in Turkish population. A total of 75 BC patients and 126 healthy controls were included in this case-control study. Genotyping for the interested polymorphisms were analyzed through polymerase chain reaction (PCR). The strength of association between both IL-4 and IL-1Ra gene VNTRs and BC susceptibility was estimated utilizing odds ratio (OR) with corresponding 95% confidence interval (CI). In the study, no statistically significant differences were determined in the allele distributions for either in IL-4 gene intron 3 VNTR (OR= 1.33; CI 0.704-2,41, p=0.390) or in IL-1Ra gene intron 2 VNTR polymorphisms (OR= 0.890; CI 0.569-1.394, p=0.346) between BC patients and control groups. The genotype distributions of IL-4 gene were estimated for RP1/RP2 (OR= 1.55; CI 0.11-7.74, p = 0.590) and RP1/RP1 (OR= 2.08; CI 0.48-9.06, p = 0.320), found no difference between BC and control groups. The genotype distributions of IL-1Ra gene were estimated for 2L (OR= 1.401; CI 0.753-2.610, p = 0.287) and 22 (OR=0.908; CI 0.252-3.276, p = 0.883) and found no difference between BC and control groups. This study suggest that there were no statistically significant differences determined either in genotype or allele distributions between BC patients and control groups for both IL-4 intron 3 VNTR and IL-1Ra intron 2 VNTR polymorphisms in Turkish population and therefore there was no association of these variants with BC risk in this population.

Keywords: Bladder cancer, VNTR, Polymorphism, IL-4, IL-1Ra

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INTRODUCTION

Bladder cancer (BC), as a common type of cancer in humans, occurs more often in men than women (approximately 3:1) and its recurrence rate in some populations is observed as 90%. As a reason for the disproportion between genders, it has been drawn attention that androgen /androgen receptor (AR) signals might have a significance on BC progression, and AR is a key factor regulating the progression of many tumors, primarily BC (Tao *et al*. 2016).

Chronic inflammation might be a risk factor for some type of cancers. Cytokine molecules, as being important inflammatory mediators, take part in inflammatory response and provide the protection of body from pathogens or external inducers. Various studies have come up with the important evidences explaining the relationship between polymorphisms of genes encoding cytokines or their receptor and susceptibility to cancer in humans (Tsai *et al.* 2005; Duan *et al*. 2014).

Interleukin-4 (IL-4), a cytokine is produced by T cells and induces activation of B cells and lymphocytes, thus providing inhibition of angiogenesis, as well as activation of granulocytes and eosinophil, and surveillance and clearance of tumor cells (Swain *et al*. 1990; Olver *et al*. 2007; Izol *et al.* 2021). IL-4 gene resides on the 5q31.1 chromosome in a cytokine gene cluster (Duan *et al*. 2014). Especially a VNTR sequence with 70-bp length on its intron 3 might be important. Because many studies have revealed that there could be important relations between IL-4 intron 3 VNTR polymorphism and various human diseases (Makhlouf *et al*. 2014; Kok *et al*. 2017; Elghoroury *et al*. 2018). Moreover, in a meta-analysis study, it has been suggested that there could be an association of this polymorphism even with cancer risk (Duan *et al*. 2014).

The interleukin-1 (IL-1) family, encoded by a 10.9 Mb gene region located to the 2q14 region, plays an important role in some mechanisms such as inflammation, growth and repair (Vamvakopoulos *et al*. 2002). Inflammation has a crucial role in a tumor microenvironment, since inflammatory cells produce cytokine molecules and other factors inducing the tumor progression. As a central tool for immunity and inflammation, IL-1 family has cytokines with 11 members and receptors with 10 members (Lewis *et al*. 2006; Garlanda *et al*. 2013; Dinarello 2018). IL-1Rα is one of the receptor antagonists among three (IL-1Rα, IL-36Rα, IL-38) (Garlanda *et al*. 2013). The second intron of the Interleukin-1 Receptor Antagonist (IL-1Ra) gene carries a functional VNTR with 86 base pairs in length, and it has a role in altering the serum IL-1Ra protein level, thus affecting immune response and cancer risk (Tarlow *et al*. 1993). IL-1Ra does not cause signal transduction due to the difference in its three-dimensional structure, but it blocks the effects of agonists (IL-1α and IL-1β) and thus suppressing inflammation (Tao *et al*. 2016; Kok *et al*. 2017). Six alleles are identified for intron 2 VNTR of the *IL1-Ra* (*IL1-RN*) gene; allele 1 (410 bp, 4 repeats); allele 2 (240 bp, 2 repeats); allele 3 (500 bp, 5 repeats); allele 4 (325 bp, 3 repeats); allele 5 (595 bp, 6 repeats) and allele 6 (154 bp, 1 repeat) (Hallegua *et al*. 2002). They are also categorized as long allele (L, 3-6 repeats) and short allele (2, 2 repeats); hence LL, 2L and 22 are used for named the genotypes (Cauci *et al*. 2010; Cai *et al*. 2014).

To date, some different population studies have been conducted to research the correlation of *IL-4* intron 3 VNTR and *IL-1Ra* intron 2 VNTR polymorphisms with risk of human cancers (Tsai *et al*. 2005; Bhayal *et al*. 2015). In our study, we aimed to research whether the polymorphisms of *IL-4* gene intron 3 and *IL-1Ra* gene intron 2 VNTR are associated with susceptibility to bladder cancer in Turkish population.

MATERIALS AND METHODS

Study Groups

75 BC patients aged 36 to 81 years (62.30 ± 9.21), who were admitted to the Urology Clinic of Nigde State Hospital and Luleburgaz State Hospital, were included in this study. The control group consisted of 126 individuals who were healthy and aged 37 to 97 years (61.15 ± 11.33), being not BC and being similar in demographic features such as age, sex, and smoking features which were regarded as important in BC patients (Table 1). The present study was a retrospective study and EDTA whole blood samples from all participants were used for extracting DNAs and genotyping gene variants (rs79071878 and rs2234663). The Power Calculator software was used to calculate power and effective sample size. This research was conducted following the principles of Helsinki Declaration. The informed consent forms were signed by the patient with BC and healthy individuals. Ethics committee approval was obtained from the Non-invasive Clinical Ethics Committee of Kocaeli University School of Medicine (Ethics no: KU GOKAEK 2016/93). MoNE and Governorship approvals were also obtained (2016/12.2).

Genotyping

Genomic DNA was extracted from the whole blood treated with EDTA using the QIAamp DNA Blood Mini Kit (Maryland, USA), following the manufacturer's protocol. Genotyping of the genes was determined by PCR amplification (Thermal Cycler- Kyratec, Supercycler) with specific primers. Primer pairs for the regions comprising the *IL-4* intron 3 and *IL-1Ra* intron 2 VNTRs polymorphism were as follows:

For the *IL-4* gene intron 3 VNTR polymorphism (rs79071878), primers were 5'-AGGCTGAAAGGGGGAAAGC-3' for forward, 5'-CTGTTCACCTCAACTGCTCC-3' for reverse. PCR steps were as follows: in the total volume of 25 µL, 4 minutes at 95 °C for the first denaturation, then 35 cycles at 95 °C 30 seconds for denaturation, after that 30 seconds at 55 °C for annealing, later 30 seconds at 72 °C for extension and finally 5 minutes at 72 °C for the last extension (Mout *et al*. 1991). The PCR products and 100 bp DNA ladder were run in 1.5% agarose gel for 55 minutes at 100 volts and the band lengths were monitored and recorded. The RP1 allele was determined as 183 bp and the RP2 allele was as 253 bp in length.

For the *IL-1Ra* gene intron 2 VNTR polymorphism (rs2234663): 86 bp VNTR region was amplified by using 5'- CTCAGCAACACTCCTAT-3' primer for forward and 5'-TCCTGGTCTGCAGGTAA-3' primer for reverse (Bid *et al*. 2006). The PCR reaction conditions were arranged as follows: 4 minutes at 95 °C for the first denaturation, then 35 cycles at 95 °C 30 seconds for denaturation, 30 seconds at 58 °C for annealing, 30 seconds at 72 °C for elongation and 5 minutes at 72 °C for the last extension. The amplification products after PCR were visualized with a UV device by running electrophoresis on 1.5% agarose gel with ethidium bromide. The alleles were determined as follows: 1 (410 bp, 4 repeats of the 86 bp), 2 (240 bp, 2 repeats), 3 (500 bp, 5 repeats), 4 (325 bp, 3 repeats), 5 (595 bp, 6 repeats) (Cai *et al*. 2014). These alleles were also represented as L for 3-6 repeats and 2 for 2 repeats, and genotypes were determined as LL, 2L and 22 (Cauci *et al*. 2010). In the present study, allele 5 and allele 6 were not identified.

Statistical Analysis

Data was analyzed using SPSS software for windows version 20 (SPSS Inc. Chicago, IL). The Kolmogorov-Smirnov test was used to test for the conformity of the variables to the normal distribution. Student's t-test and chi-square tests were used for statistical analysis of demographic feature. The descriptive statistics related with age were presented with n (%) and mean ± standard

deviation. For measuring associations between the genotypes and BC, odds ratios (OR) and 95% confidence intervals (CI) were estimated from binary logistic regression analysis and adjusted after controlling for age and smoking habits. $P < 0.05$ value was considered to be statistically significant. Hardy-Weinberg Equilibrium (HWE) was conducting by comparing genotypic frequencies (observed and expected) using chi-square analysis.

RESULTS AND DISCUSSION

There were 75 BC cases including 7 females and 68 males who were aged 36-81, with an average age of 62.30 ± 9.21 years. The control group contained 126 healthy individuals, including 26 females and 100 males; they were aged 37-97 years, with an average age of 61.15 ± 11.33 years. The control and case groups were matched in age ($P > 0.005$ for both). The distributions of gender, smoking and nonsmoking status were significantly different between case and control groups ($P < 0.05$). The demographic characteristics of case and control groups were summarized in Table 1.

All genotypes of *IL-1 Ra* gene intron 2 VNTR conformed to HWE in controls ($P > 0.05$), suggesting the representativeness of the study sample. However, for *IL-4* gene intron 3 VNTR polymorphism, the results were detected to deviate from the HWE in controls ($P < 0.05$).

The genotype and allele distributions of patients and controls for *IL-4* gene intron 3 VNTR were showed in Table 2. The distribution of allelic frequency between patient and control groups was not statistically significant (OR= 1.33; CI 0.704-2.41, $P = 0.390$). Distribution of either RP1/RP2 or RP1/RP1 genotypes was not different between patient and control groups (OR= 1.55; CI 0.11-7.74, $P = 0.590$; OR= 2.08; CI 0.48-9.06, $P = 0.320$ respectively) (Table 2).

Allele 1 frequency of *IL-1 Ra* intron 2 VNTR was 64% in patient group and 67.5% in control group. Allele 2 frequency was 29.4% in patient group and 27.4% in control group, with no significant difference (OR= 0.886; CI 0.563-1.394, $P = 0.599$). The frequency of allele 3, which is less common, was 5.3% in patient group and 4% in control group, and the distribution between them was not statistically significant (OR= 0.706; CI 0.270-1.849, $P = 0.476$). The frequency of allele 4 was 1.3% in patient group, 1.1% in control group, and the distribution was not significantly different (OR= 0.847; CI 0.139-5.158 $P = 0.857$). Allele 5 and allele 6, which are rare, were detected in neither patient nor control groups (Table 3). The genotypes of *IL-1Ra* gene polymorphism were observed as 1/1, 1/2, 2/2, 1/3, 1/4 and 2/3 and the distributions of these genotypes were not statistically significant ($P > 0.05$) (Table 3).

Table 1. Distribution of demographic characteristics of BC patient and control groups

Parameters	Patients n=75 (%)	Controls n=126 (%)	*P* value	OR (95% CI)
Age (years) (Age range)	62.30 ± 9.21 (36-81)	61.15 ± 11.33 (37-94)	0.200	
Sex				
Male	68 (91)	100 (79)		
Female	7 (9)	26 (21)	0.036*	2.52 (1.04-6.19)
Smoking status				
Smoker	42 (56)	37 (29)		
Non-smoker	33 (44)	89 (71)	0.0001*	0.327 (0.180-0.593)

*$P < 0.05$; significantly different from control group

Table 2. Genotype and allele distributions of *IL-4* gene intron 3 VNTR polymorphism patient and control groups

Gene			Crude values		Adjust values	
IL-4 intron 3 VNTR	Patients n= 75(%)	Control n=126(%)	*P* value	OR (95% CI)	*P* value	OR (95% CI)
Genotypes						
RP2/RP2	61 (81)	97 (77)		1		1
RP1/RP2	11 (15)	22 (17)	0.840	1.16 (0.252-5.40)	0.590	1.55 (0.11-7.74)
RP1/RP1	3 (4)	7 (6)	0.580	1.46 (0.36-5.89)	0.320	2.08 (0.48-9.06)
Alleles						
RP2	133 (90)	216 (87)		1		
RP1	17 (10)	36 (13)	0.390	1.33 (0.704-2.41)		

*$P < 0.05$; significantly different from control group. Adjusted with the age and smoking status

Table 3. Genotype and allele distributions of *IL-1Ra* gene intron 2 VNTR polymorphism patient and control groups

Gene			Crude values		Adjust values	
IL-1 Ra intron 2 VNTR	Patients n=75 (%)	Control n=126 (%)	*P* value	OR (95% CI)	*P* value	OR (95% CI)
Genotypes						
1/1	27 (36)	59 (47)		1		1
1/2	34 (45)	44 (35)	0.108	1.689 (0.892-3.197)	0.222	1.519 (0.777-3.408)
2/2	4 (5)	10 (8)	0.832	0.874 (0.251-3.038)	0.913	0.931 (0.254-3.408)
1/3	6 (8)	5 (4)	0.137	2.622 (0.636-9.34)	0.472	1.626 (0.432-6.119)
1/4	2 (3)	3 (2)	0.690	1.457 (0.230-9.23)	0.829	0.812 (0.123-5.349)
2/3	2 (3)	5 (4)	0.877	0.874 (0.159-4.79)	0.553	0.589 (0.102-3.392)
Alleles						
1	96 (64)	170 (68)		1		
2	44 (30)	69 (27)	0.599	0.886 (0.563-1.394)		
3	8 (5)	10 (4)	0.476	0.706 (0.270-1.849)		
4	2 (1)	3 (1)	0.857	0.847 (0.139-5.158)		
Genotypes						
LL	35 (47)	68 (54)		1		1
2L	36 (48)	48 (38)	0.214	1.457 (0.804-2.640)	0.287	1.401 (0.753-2.610)
22	4 (5)	10 (8)	0.688	0.777 (0.227-2.656)	0.883	0.908 (0.252-3.276)
Alleles						
L	106(71)	184 (73)				
2	44 (29)	68 (27)	0.346	0.890 (0.569-1.394)		

*$P < 0.05$; significantly different from control group. Adjusted with the age and smoking status

Additionally, in Table 3, all alleles and genotypes for this gene variant were also showed as L, 2, LL, 2L and 22. L and 2 allele frequencies were 71%, 29% in patient group and 73%, 27% in control group, with no significant difference seen between two groups (OR= 0.890; CI 0.569-1.394, $P = 0.346$) (Table 3). The frequencies of LL, 2L and 22 genotypes were 47%, 48% and 5% in patient group respectively, and 54%, 38% and 8% in control group respectively. The genotype distributions for 2L (OR= 1.401; CI 0.753-2.610, $P = 0.287$) and 22 (OR= 0.908; CI 0.252-3.276, $P = 0.883$) were not statistically significant in either of the groups (Table 3).

BC is much more common in men than women in the US and European countries (Mungan *et al*. 2000; Tao *et al*. 2016; Madeb and Messing 2004). In the case of Turkey, BC is the sixth most common malignant disease, with an expected 12.248 newly diagnosed cases in 2020, and 3.771 deaths (Sung *et al*. 2021). In bladder cancer, like other cancer cases, early detection and timely treatment is very important and increase survival chances, and with the help of cytogenetic and molecular analyses methods finding more effective biomarkers also play important roles in survival rates.

Chaotic changes emerged in any stage of cell mechanisms such as development, progression and growth are typical characteristic of cancer cells. From various studies, interleukins could have critical roles in cancer development by serving as communication for immune or non-immune cells and driving cells in chronic inflammation (Briukhovetska *et al*. 2021). A review study have extensively

discussed that polymorphisms into the genes encoding cytokines and their receptors could influence the levels of expressions and immune response (Bidwell *et al*. 1999). Chronic inflammation triggered either by polymorphisms of cytokines or inflammatory mediators leads to the development and progression of cancer. In the case of their relation with bladder cancer, some recent studies suggest that polymorphisms of the IL-4 intro 3 and IL-1Ra intron 2 VNTRs could be modulate the risk of developing this cancer (Bid et al. 2006; Ahirwar et al. 2009; Duan et al. 2014; Schneider et al. 2021).

In the present research, two VNTR polymorphisms (rs79071878 and rs2234663) in IL-4 and IL-1Ra genes were analyzed in 75 BC patients and 126 healthy controls, and no significant association was detected between either of these polymorphisms and susceptibility of BC in Turkish population (P > 0.05). Epidemiological studies have observed the positive relation of *IL-4* intron 3 VNTR (rs79071878) with some other cancers, such as oral and pharyngeal, gastric, or leukemia cancer (Yang *et al*. 2014; Bhayal *et al*. 2015: Ahmed *et al*. 2016). There are several case-control studies that have pointed to association of *IL-4* gene variants with bladder cancer in different populations. One study observed that *IL-4* C-590T gene variant might be related with the risk of BC in Chinese population (Chu *et al*. 2012). Another study reported that *IL-4* gene intron 3 VNTR polymorphism significantly increased BC risk in Taiwanese population, suggesting it as a potential genetic marker for screening BC (Tsai *et al*. 2005). Further, positive association of *IL-4* gene intron 3 VNTR with late stage bladder cancer was reported in a northern Indian population (Ahirwar *et al*. 2008). The significant association of IL-4 gene intron 3 VNTR polymorphism with BC susceptibility was reported in Turkish population as well (Bozdogan *et al*. 2015). In contrast, the present study revealed no association between *IL-4* VNTR polymorphism and BC risk for Turkish population ($P > 0.05$). That could be caused by the small sample size of the study and by the population heterogeneity for the gene variants, even in the same country.

IL-1Ra is an important antagonist exerting anti-inflammatory activities. IL-1Ra deficiency and its significant relation to chronic inflammatory conditions with evidences in mouse models and human practices were discussed in a detailed review paper (Guo *et al*. 2015). In some expression analysis from tissue samples, results showed that loss of IL-1Ra expression could be related to increased bladder cancer progression (Worst *et al*. 2014; John *et al*. 2020). Genetic alterations and polymorphisms in the *IL-1Ra* gene might also be important in BC development. On the other hand, positive relations of IL-1Ra gene polymorphism with various cancers such as cervical, ovarian, gastric cancer have already been reported (Mustea *et al*. 2003; Sehouli *et al*. 2003; Oliveira *et al*. 2012). In the case of its relation with bladder cancer, there are few population-based studies. From a case-control study, *IL-1Ra* intron 2 VNTR polymorphism (rs2234663) has been shown to play a role in increasing the risk of bladder cancer in Indian population (Bid *et al*. 2006). Another case-control study also suggested positive association between allele distributions of the *IL-1Ra* VNTR and BC susceptibility in Turkish population, finding alleles with 4 and 5 repeats as statistically significant (Bozdogan *et al*. 2015). In contrast, in the present study, even being same country, no significant differences were detected for *IL-1Ra* intron 2 VNTR polymorphism in either genotype and allele distributions between case and control groups (P> 0.05), and again, we suggest that sample size and population heterogeneity for this gene variant could cause that result.

CONCLUSION

In the present study, the polymorphic features of the alleles and genotypes of IL-4 intron 3 VNTR and IL-1Ra intron 2 VNTR in the bladder cancer patients in Turkish population were investigated and detected that there were no statistically significant differences between BC patients

and control groups. There are not enough publications whether effect of *IL-4* gene intron 3 and *IL-1Ra* gene intron 2 VNTR polymorphisms may vary by cancer type and by ethnicity. To draw a more precise conclusion to check the impact of these polymorphisms on BC susceptibility further studies should be carried out with larger sample size from different populations or subgroups.

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Conflict of Interest

The authors declare that they have no conflict of interest. All applicable international, national, and/ or institutional guidelines for non-invasive clinical studies were followed.

Author's Contributions

The authors declare that they have contributed equally to the article.

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