RESEARCH ARTICLE

Interleukin-12 and Interleukin-6 Gene Polymorphisms and Risk of Bladder Cancer in the Iranian Population

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Abstract

Interleukin-12 (IL-12) as an antitumor and interleukin-6 (IL-6) as an inflammatory cytokine, are immunomodulatory products that play important roles in responses in cancers and inflammation. We tested the association between two polymorphisms of IL-12(1188A>C; rs3212227) and IL-6 (-174 C>G) and the risk of bladder cancer in 261 patients and 251 healthy individuals. We also investigated the possible association of these SNPs in patients with high-risk jobs and smoking habits with the incidence of bladder cancer. The genotype distributions of IL-6 (-174 C/G) genotype were similar between the cases and the control groups; however, among patients with smoking habits, the association between IL-6 gene polymorphism and incidence of bladder cancer was significant. After a control adjustment for age and sex, the following results were recorded: CC genotype (OR= 2.11, 95%CI=1.56-2.87, p=0.007), GC genotype (OR=2.18, 95%CI=1.16-4.12, p=0.014) and GC+ CC (OR=2.6, 95%CI=1.43-4.47, p=0.011). A significant risk of bladder cancer was observed for the heterozygous genotype (AC) of IL-12 (OR=1.47, 95%CI=1.01-2.14, p=0.045) in all cases, and among smokers (AC) (OR=3.13, 95%CI=1.82-5.37, p=0.00014), combined AC+CC (OR=3.05, 95%CI=1.8-5.18, p=0.000015). Moreover among high risk job patients, there was more than a 3-fold increased risk of cancer in the carriers of IL-12 beta heterozygous (OR=3.7, 95%CI=2.04-6.57, p=0.000056) and combined AC+CC(OR=3.29, 95%CI=1.58-5.86, p=0.00002) genotypes as compared with the AA genotype with low-risk jobs. As a conclusion, this study suggests that IL-12(3'UTR A>C) and IL-6 (-174 C>G) genotypes are significantly associated with an increased risk of bladder cancer in the Iranian population with smoking habits and/or performing high-risk jobs.

Keywords: Bladder cancer - IL-12 - IL-6 - polymorphisms - high risk jobs - smoking - Iran

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Introduction

Bladder cancer (BC) is the most common malignancy of the urinary tract. An estimated 72,570 adults (54,610 men and 17,960 women) will be diagnosed with bladder cancer in the United States annually. Among men, bladder cancer is the fourth most common cancer and the eighth most common cause of cancer death (Siegel et al., 2013). In Iran, bladder cancer is the fifth most common cancer, and among men it ranks fourth (Iranian Ministry of Health and Medical Education, 2012). Many epidemiological studies have suggested that bladder cancer is influenced by environmental factors, such as smoking, exposure to industrial chemicals, age, and several lifestyle factors like obesity, suffering from diabetes mellitus (DM), of which smoking is the primary risk factor (Letasiova et al., 2012; Qin et al., 2013; Yang et al., 2013; Otunctemur et al., 2014).

Although the immune system can recognize and eliminate cancer tumors at the cell and tissue level (Smyth et al., 2006), inflammation can cause tumorigenesis through damaging DNA and stimulating angiogenesis and cell proliferation (Rakoff-Nahoum, 2006).

Produced by many different types of lymphoid and nonlymphoid cells (Tahara, 1990), Interleukin-6 (IL6) is a pleiotropic inflammatory cytokine encoded by IL-6 gene localized at chromosome 7p21-14 that is important for immune response, cell survival, proliferation and apoptosis (Kishimoto, 2005; Tan et al., 2005; Schafer and Brugge, 2007). Elevated serum IL-6 has been shown to be associated with incidence or clinical outcome in several cancers (Bozcuk et al., 2004). Studies have shown that IL-6 and its major effector STAT3 play a central role in the epigenetic switch from non-transformed epithelia to cancer cells (Iliopoulos et al., 2009; Iliopoulos et al., 2010). Furthermore, high levels of IL-6 may favor a T-helper–2 (Th2) pattern of humoral immune response which leads to subsequent chronic inflammation and poor

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survival in patients with cancer (Grivennikov and Karin, 2008; Enewold et al., 2009).Until now, 7 pathogenic mutations (www.portal.biobase-international.com) and three functional polymorphisms, including 6331 T/C (rs10499563), 572 C/G (rs1800796) and 174 G>C (rs1800795), have been found in the IL-6 promoter region, associated with IL-6 transcription activity (Morgan L et al., 2006; Smith et al., 2008), and 174 G>C polymorphism was associated with cancer (Terry et al., 2000; Belluco et al., 2003).

Interleukin-12 (IL-12) is a heterodimeric cytokine with a molecular weight of 70 kDa composed of two disulfide-linked polypeptide chains consist of IL-12p35 (35 kDa) and IL-12p40 (40 kDa), each of which is expressed on different chromosomes (encoded by the IL-12A and IL-12B genes, respectively) and is mainly produced by activated macrophages and dendritic cells (DCs) (Wolf et al., 1991; Colombo and Trinchieri, 2002). The antitumor activity of IL-12 has been extensively reported in mouse models of cancer, where it has been shown to inhibit tumorigenesis and angiogenesis through the production of antiangiogenic factors (Noguchi et al., 1996; Sangro et al., 2005). Meanwhile clinical research has shown that there is a correlation between the IL-12 SNPs and levels of serum IL-12 with disease severity in cancer patients (Murakami et al., 2004). Based on HGMD, 14 pathogenic mutations have been found to date (www.portal.biobase-international.com), and many studies have focused mainly on 3'UTR, 1188A>C (rs3212227) in the IL-12B gene (Zhou et al., 2012). The 1188 A/C polymorphism has been shown to influenceIL-12 production or protein expression and was associated with Th1-mediated diseases (Lin and Chien, 2004). Moreover, results from the current meta-analysis indicate that 1188 A/C polymorphism was associated with a significantly increased overall risk of cancer (Zhou et al., 2012).

The aim of our study was to determine whether there was an association between IL-12 and IL-6 genes polymorphisms and susceptibility to bladder cancer in the Iranian population. To test this hypothesis, we investigated the risk of IL-12B (1188 A/C) and IL-6 (174G>C) polymorphisms with bladder cancer and the interaction of these SNPs with smoking habits and other lifestyle factors.

Materials and Methods

Study subjects

261 histological-confirmed incident bladder cancer specimens and 251 matched blood samples were recruited from Department of Urology, HasheminejadHospital, ImamKhomeini, and ZahedanUniversity of Medial Sciences summarized in Table 1. None of the patients had received chemotherapy or radiation before inclusion in the study. The criteria for the selection of patients were based on clinical proforma, pathological, and histopathological records. This study was approved by the Iran Ethical Committee of the Institute. Bladder tumor samples were reviewed by the study pathologist and classified according to the 1973 and 2004 WHO guidelines for bladder tumors (www.uroweb.org). Patients' data were collected through interview where demographic features, clinical details, and environmental exposure were recorded using a standard clinical proforma. For control group of patients who were admitted in hospital without any prior history of cancer, pre-cancerous lesions or acute inflammatory disease were selected. Cases and controls were matched by age, sex, and socio-economic status. Most of the subjects had completed their primary education. A signed consent form was obtained from each of the patients. The amount of exposure to dangerous workplace materials and chemicals, such as aromatic amines, polycyclic aromatic hydrocarbons and diesel, were regarded as occupational exposure. Subjects who were exposed to such material over 5 years were classified as the high risk cases. The smoking habits of patients were also studied among the subjects. Subjects who had smoked at least 100 cigarettes or chewed tobacco 100 times or more during their lifetimes were defined as smokers.

Methods

For extraction and purification of genomic DNA from formalin-fixed paraffin-embedded tissues, we used QIAamp DNA FFPE Tissue Kit (Cat# 56404) and QIAamp DNA midi kit (cat#51304) for blood samples obtained from controls. DNA samples were stored at -20°C until analysis.

PCR reaction

Amplification was carried out in a final volume of 50 μL reaction containing 100 ng genomic DNA, 200 mM each dNTP, 1mM $MgCl_2$, 10mM tris- HCl (pH 8.3), 50mM KCl, 0.1% Triton X-100, 1.5 units of Taq DNA polymerase (MBI fermentas, Canada), and 1mM of each of the primers that were performed by using the previously described protocols, IL-6 F: 5'-ACT TTT CCC CCT AGT TGT GTC TTTC-'3, R: 5'-AGA ATG AGC CTC AGA CAT CTC CAG T-'3 and for IL-12 F: 5'-GGC ATT CTC TTC CAG GTT CTG-'3, R: 5'-CCA TGG CAA CTT GAG AGC TG-'3 (Šery et al., 2003; Bergholdt et al., 2004). The PCR conditions were the same as Šery and Bergholdt's protocols.

The PCR products were digested with 5 units of Taq1 restriction enzyme (Fermentas, Canada) at 65C for 5 h for both polymorphisms. The digested products were analyzed by agarose gel electrophoresis on a 2% agarose gel containing CinnaGen DNA safe Stain (Cat. No.PR881603) and were visualized under UV light. For IL-6 the amplified fragment of 204 bp was cleaved into two fragments of 24 and 180 bp. The uncut product of 204 bp was identified as CC genotype; the GC genotype was revealed by two fragments of 204 and 180 bp, and the GG by a fragment of 180 bp (Fig. 1 and 2). Finally, the IL-12 β PCR products resulted in either two fragments of 173 and 70 bp (allele C) or a single fragment of 243 bp (allele A).

Statistical analysis

Statistical analysis was carried out with SPSS 11.5 and EPI info 3.2 software programs. Odd Ratios (ORs) were adjusted for confounding factors, such as age, smoking, and socio-economic status. Multivariate analysis, odds ratios, and 95% confidence interval (CI) were used to describe the strength of association.

Results

IL-6 (-174 C/G) and IL-12B (1188 A/C) genotypes were analyzed in patients with bladder cancer and healthy controls. Demographic variables are summarized in Table 1. Whereas 45.21% of patients were current smokers, cigarette smoking showed significant association with OR= 2.46, 95%CI= 1.66-3.65, p=0.000031 compared with healthy individuals.

Moreover, 37.93% of patients were performing high risk jobs (workers in the aluminum, dye, paint, rubber and textile industries and, in gases stations. High risk jobs status has shown significant association with increased risk of bladder cancer (OR=2.4, 95%Cl= 1.58-3.63, p=0.0000).

The frequencies of the different genotypes at IL-6 (-174 C/G) in the study population were as follows: 7CC, 34 CG and 59% GG for the case study group and 4.9 CC, 31.4 GC, and 63.7% GG for the controls, indicating that the genotypes distributions were similar between the cases and the control groups. Also, genomic analysis did not reveal a difference between bladder cancer patients and healthy controls in allelic frequency at the -174 position for the IL-6 gene promoter.

On the other hand, among patients with smoking habits, the association between IL-6 gene polymorphism and incidence of bladder cancer was significant. After adjustment for age and sex, the following results were recorded: CC genotype (OR= 2.11, 95%CI=1.56-2.87, p=0.007), GC genotype (OR=2.18, 95%CI=1.16-4.12, p=0.014) and GC+ CC (OR=2.6, 95%CI=1.43-4.47, p=0.011), (Table 2).

We found that smokers having C allele in the IL-6 (-174 C/G) position were more at risk of bladder cancer. Moreover, the bladder cancer patients having GC and GC+CC genotypes at C-174G and performing high risk jobs demonstrated an increased risk of developing bladder cancer, compared with carriers of the GG genotype who had low risk jobs, after adjustment for sex and age (OR=2.57, 95%CI=1.37-5.09, p=0.004) and (OR=2.59, 95%CI=1.36-4.98, p=0.002), respectively (Table 2). Thus, people with high risk jobs and having C allele in IL-6 (-174 C/G) position were 2-3 times more susceptible to bladder cancer. However, different clinical stages and grades did not show any association of IL-6 genotypes in patients with bladder cancer.

Distribution of IL-12 beta genotypes in cases and controls: The frequency of wild (AA) and homozygous mutant (CC) genotype was found more in controls (45% and 4.7% respectively), but that of the heterozygous genotype was higher (60.15%) in cases with bladder cancer. Significant risk of bladder cancer was observed for AC (OR=1.47, 95%CI=1.01-2.14, p=0.045) genotype of IL-12B.

The genomic analysis did not reveal differences in allelic frequencies of the IL-12B (A/C) gene between bladder cancer patients and healthy controls. On the other hand, the heterozygous (AC) (OR=3.13, 95%CI=1.82-5.37, p=0.00014) and combined AC+CC (OR=3.05, 95%CI=1.8-5.18, p=0.000015) genotypes of IL-12B among smoker patients showed significant association with an increased risk of bladder cancer (Table 2). Moreover, among high risk job patients there was more than 3-fold increased risk of cancer in the carriers of IL-12B heterozygous (OR=3.7, 95%CI=2.04-6.57, p=0.000056) and combined AC+CC (OR=3.29, 95%CI=1.58-5.86, p=0.00002), (Table 2) genotypes as compared with carriers of the AA genotype with low risk jobs after adjustment for age and sex. However, no association between IL-12 beta genotypes and clinical stages and grades of bladder cancer were found.

Table 1. Demographic Characteristic of Bladder Cancer Cases and Controls

Variable	Cases	Controls
Age (year)		
Mean (±DS)	57.42 (±12.59)	56.35 (±10.13)
Median Range	25-86	30-85
Sex		
Male	227 (86.97%)	221 (88.04%)
Female	34 (13.02%)	30 (11.95%)
Smoking		
Currently smokers	118 (45.21%)	63 (25.09%)
Non smokers	143 (54.78%)	188 (74.09%)
Job		
High risks	99 (37.93%)	51 (20.31%)
Low risks	162 (62.6%)	200 (76.69)
Alcohol		
Drinking	110 (42.14%)	98 (39.04%)
Non drinkers	151 (57.85%)	153 (60.95%)
Stage		
<T1 (superficial)	210 (80.45%)	
>T2 (invasive)	51 (19.54%)	

Table 2. Age and Sex Adjusted ORs (and Cornfield 95% CIs) for the Association of Cytokines Gene Polymorphisms among Smokers and High Risk Job Patients

genotype	Smoker Risk		High risk job Risk	
	P value	OR (95% CI)	P value	OR (95% CI)
Il-6				
CC	P=0.007	OR= 2.11	-	-
GC	P=0.014	OR=2.18	P=0.004	OR=2.57
GC+CC	p=0.011	OR=2.6	P=0.002	OR=2.59
AC	P=0.00014	OR=3.13	P=0.000056	OR=3.7
Il-12				
AC+CC	P=0.000015	OR=3.05	P=0.00002	OR=3.29

Discussion

The role of Single nucleotide polymorphisms (SNP) in altering expressions or functions of inflammation genes was shown; thus, SNPs could be associated with an altered risk of multiple cancers (Machado et al., 2003; Zhang and Wang, 2013). Today, the important role of pro-inflammatory cytokines during tumor development is increasingly gaining interest. IL-6 is a pleiotropic inflammatory cytokine, which is regarded as an important tumor-promoting factor in the development and progression of various types of human cancer, including bladder cancer (Waldner et al., 2012; Zhu et al., 2012; Chen et al., 2013). Moreover, increased IL-6 serum levels were reported to be associated with metastasis and poor prognosis of prostate, ovarian, and bladder cancers (George et al., 2005). The

174G>C functional polymorphisms in the IL-6 promoter region is associated with IL-6 transcription activity and levels of IL-6 expression (Lagmay et al., 2009). Therefore, we hypothesized that the polymorphism may be a predisposing factor for the development of bladder cancer.

Our findings indicate that the genotypes distributions of IL-6 (-174 C/G) were similar between the cases and the control groups. Also, genomic analysis did not reveal a difference between bladder cancer patients and healthy controls in allelic frequency at the -174 position for IL-6 gene promoter. This result is compatible with the four met-analysis of IL-6 -174G>C polymorphism and cancer risk (Xu et al., 2011; Liu et al., 2012; Yu et al., 2012; Zhang et al., 2012). However, this null association could be due a lack of consideration for the potential role of the environment; in other words, the gene-environment interaction role in cancer risk, as mentioned by Liu RY et al. (Liu et al., 2012). Epidemiologic studies have revealed significant associations between bladder cancer and environmental factors such as tobacco smoking and occupational exposures (Letasiova et al., 2012; Kobeissi et al., 2013). It has been shown that smoking increases the production of numerous pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-8 GM-CSF and decreases the levels of anti-inflammatory cytokines such as IL-10 (Hagiwara et al., 2001; Sopori, 2002; Arnson et al., 2010). Therefore, we have jointly considered tobacco smoking and high risk jobs as two well-known risk factors for bladder cancer in our study. Interestingly, results have shown strong associations between specific genotypes and incidence of cancer among smokers, CC genotype (p=0.007), in case of the GC genotype (p=0.014) and in GC+ CC (p=0.011).

Moreover, patients with high risk jobs and GC and GC+CC genotypes at C-174G have shown increased risk of developing bladder cancer as compared with carriers of the GG genotype and low risk jobs, after adjustment for sex and age (p=0.004) and (p=0.002) respectively. These findings show the possible role of gene-environment interaction at the -174 position for IL-6 gene and predisposing of a specific genotype at this region to bladder cancer in exposure to these risk factors.

IL-12 is an important antitumor cytokine that plays an important role in the development and progress of cancer (Sangro et al., 2005). Variation in the DNA sequence may lead to altered IL-12 production, and this can alter an individual's susceptibility to cancer. The IL12B 3'UTR A>C polymorphism is a functionally important SNP that alters Il-12 production and it has been a reported potential biomarker for risks of some cancers,such as cervical, colorectal, gastric, breast and several others(Zhou et al., 2012); thus, we aimed to investigate the association between this polymorphism and bladder cancer among the Iranian population.

Our findings indicates increased frequency of (AA) and (CC) homozygous among controls (45% and 4.7% respectively), but that of the heterozygous genotype was higher (60.15%) in cases with bladder cancer; thus, a significant risk of bladder cancer was observed for AC (p=0.045) genotype of IL-12B, which is compatible with the results of cervical cancer (Tamandani et al., 2009). Moreover, two meta-analyses of IL-12 gene functional polymorphisms by Chen H and Zhou L support our findings (Chen et al., 2012; Zhou et al., 2012). However, Jaiswal PK reported an inconstant result, discovering that A /C heterozygote genotype and C allele carrier demonstrated reduced risk of BC (Jaiswal et al., 2013); this inconstant report could be due to different ethnicity or sample size.

For the first time, we have also investigated the association of the IL12B 3'UTR A>C polymorphism in bladder cancer patients with tobacco smoking habits and high risk jobs as the main environmental risk factors of bladder cancer (Letasiova et al., 2012). Interestingly, (AC) and combined (AC+CC) genotypes of IL-12B showed significant association with an increased risk of bladder cancer in smokers.

Moreover, there was more than 3-fold increased risk of cancer in the carriers of IL-12B heterozygous (AC) and combined AC+CC, genotypes as compared with carriers of the AA genotype with low-risk jobs after adjustment for age and sex. These results may be due to the IL-12 gene interaction with the environment, and consequently, the functional polymorphisms' role in increasing susceptibility to bladder cancer among individuals with exposure to these risk factors.

In conclusion, this study suggests that IL-12(3'UTR A>C) and IL-6 (-174 C>G) genotype is significantly associated with an increased risk of bladder cancer in the Iranian population with smoking habits or working at high risk jobs..

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