Original Article
Association of pro-inflammatory cytokines gene polymorphisms with risk of bladder cancer in the han Chinese population

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Abstract: Epidemiologic studies have shown smoking increases the production of numerous pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-12 (II-12) and decreases the levels of anti-inflammatory cytokines such as Interleukin-10 (IL-10). Up to now, numberous studies of genetic epidemiology have assessed the association of pro-inflammation cytokines gene polymorphisms and risk of cancer in different populations, but conflicting results were obtained due to the heterogeneity of the genetic background among populations. Here, we recruited 248 bladder cancer patients, and 226 matched healthy controls from Zhegjiang Province to evaluate the influence of IL-6 rs1800795, IL-12 rs3212227 and TNF-α rs1800629 polymorphism on bladder cancer patients. Single nucleotide polymorphism locus was genotyped using PCR-RFLP. The genotypic and allelic frequency of TNF-α, IL-6 did not show significant difference between asthmatic patients and normal controls. However, people engaged high risk jobs with C allele in IL-6 rs1800795 position were 2-4 times more susceptible to fall ill bladder cancer. Whereas, the GA heterozygous genetype of TNF-α rs1800629 (OR=1.642, 95% CI=0.781-3.641, *P*=0.034) and combined GA+AA (OR=3.213, 95% CI=2.013-6.412, *P*=0.026) genotypes among smoker patients showed significant association with an increased susceptibility of bladder cancer. The AC heterozygous genetype of IL-12 rs3212227 (OR=3.312, 95% CI=1.282-5.731, *P*=0.011) and combined AC+CC (OR=3.502, 95% CI=1.781-5.814, *P*=0.045) genotypes among smoker patients showed significant association with an increased susceptibility of bladder cancer. Moreover, there was more than 2-fold increased risk of cancer in the carriers of IL-12 heterozygous (OR=3.712, 95% CI=2.401-6.752, *P*=0.001) and combined AC+CC (OR=3.923, 95% CI=1.852-5.681, *P*=0.001) genotypes. Specific IL-6 rs1800795, and TNF-α rs1800629 genotype are significantly associated with an increased risk of bladder cancer with smoking habits or working. Furthermore, we first identified IL-12 rs3212227 AC genotype is not associated with an increased risk of bladder cancer with smoking habits or working, but confer genetically susceptibility to bladder cancer in chinese population.

Keywords: IL-6, IL-12, TNF-α, polymorphism, bladder cancer

Introduction

Bladder cancer (BC) is any of several types of cancer arising from the epithelial lining of the urinary bladder. The most common type of bladder cancer recapitulates the normal histology of the urothelium and is known as transitional cell carcinoma or more properly urothelial cell carcinoma [1-3]. Five-year survival rates in the United States are around 77% [1]. BC is the 9th leading cause of cancer with 430,000 new cases and 165,000 deaths occurring in 2012 [4]. Among men, BC is the fourth most common cancer and the eighth most common cause of cancer death. In China, BC is the tenth most common cancer, accounting for 17,365 deaths in 2005, and mortality has steadily increased between 1991 and 2005 [5]. Many epidemiological researches have demonstrated that BC is affected by environmental factors, such as smoking, environmental exposure to chemical carcinogens, age, occupation and several lifestyle factors like obesity, suffering from diabetes mellitus and psyhiatry disorders, of which tobacco smoking is the primary risk factor [6]. However, only a few of the exposed individuals develop BC in their lifetime, indicating that genetic factor may also play a crucial role in the pathogenesis of BC. Therefore, a greater understanding on the molecular basis

Table 1. Demographic characteristic of study population

Characteristic	Case	Control	P-value
Male [n (%)]	158 (63.71)	142 (62.83)	0.342
Age (years)	46.48±10.25	46.58±12.25	0.765
Smoking [n (%)]			<0.001
Currently smokers	152 (61.29)	66 (29.20)	
Non smokers	96 (38.71)	160 (70.80)	
Job [n (%)]			<0.001
High risk	96 (38.71)	37 (16.23)	
Low risk	152 (61.29)	189 (83.77)	
Alcohol [n (%)]			<0.001
Drinking	110 (44.35)	58 (25.67)	
Non drinkers	138 (55.65)	168 (74.37)	
Stage [n (%)]			
<T1 (superficial)	198 (79.84)		
>T2 (invasive)	50 (20.16)		

underlying in BC susceptibility and prognosis is beneficial for prevention, treatment and prognosis.

Cancer is a hyperproliferative disorder involving sustaining proliferative signaling, escaping growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism, and evading immune destruction. Recently, clinical and epidemiologic studies have demonstrated pro-inflammation can play an important role in tumorigenesis, development, invasion and metastasis [7]. Pro-inflammation, which orchestrates the tumor microenvironment, is involved in tumor initiation, promotion, and progression [8]. In BC, evidences from multiple studies have shown that pro-inflammation cytokines, such as tumor necrosis factor alpha (TNF-α) [9], interleukin 6 (IL-6) [10, 11] and Interleukin-12 (IL-12) [12], are likely to have an important role.

IL-6 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. Elevated serum IL-6 has been shown to be associated with incidence or clinical outcome in several cancers [13]. Until now, 7 pathogenic mutations and three functional polymorphisms, including rs10499563 (T/C), rs1800796 (C/G) and rs1800795 (G/C), have been found, rs1800795 G>C polymorphism in the in the

IL-6 promoter region was associated with cancer [14]. Meanwhile clinical research has suggested that there is a correlation between the IL-12 SNPs and levels of serum IL-12 with disease severity in cancer patients. Many studies have focused mainly on 3'UTR, -1188 A/C (rs3212227) in the IL-12 gene [15]. Furthermore, several polymorphisms in the promoter region of TNF-α have been also associated with different TNF-α expression levels. Of these, the TNF-α -308 G/A (also referred to as rs1800629) is the best studied. It involves the substitution of a guanine (G) by an adenine (A) and is associated with an increase in TNF-α expression levels [16, 17]. Up to now, numberous studies of genetic epidemiology have assessed the association of pro-inflammation cytokines gene polymorphisms and risk of cancer in different populations, but conflicting results were obtained due to the heterogeneity of the genetic background among populations. Furthermore, this supports the need for replication studies among all ethnic groups.

Taken together the association of pro-inflammation cytokines and cancer, the aim of the present study was to analysis the influence gene polymorphisms of IL-6, IL-12 and TNF-α on the susceptibility to bladder cancer in Chinese population.

Patients and methods

Ethics statement

The Medical Ethics Committee of The First Hospital of Jiaxing Hospital approved this study. Written informed consents conforming to the tenets of the Declaration of Helsinki were obtained from each participant prior to the study.

Participants

A total of 248 histological-confirmed incident bladder cancer specimens, and 226 matched healthy controls were recruited from Department of Urology, The First Hospital of Jiaxing, between January 2013 to January 2015. All subjects are Han Chinese. None of the patients had received chemotherapy or radiation before inclusion in the study. The criteria for the selec-

tion of patients were based on clinical patho-logical, and histopathological records. Those patients who had previous cancer, previous radiotherapy or chemotherapy, and metasta-sized cancer from other or unknown origins were excluded. Control subjects were geneti-cally unrelated individuals and those with any personal or family history of bladder cancer or other serious disease were intentionally exclud-ed. **Table 1** summarized the baseline clinical characteristics of the patients and control groups.

Genotyping

Genome DNA from whole blood cells of each sample was extracted by using Blood Genomic DNA Miniprep Kit (Axygen, USA) according to the manufacturer's instructions. DNA samples were stored at -20°C until analysis. Genotyping for the IL-6 -174 G/C, IL-12 -1188 A/C, TNF-α -308 G/A polymorphisms in genomic DNA were performed using the PCR and restriction frag-ment length polymorphism (RFLP). The genom-ic region encompassing polymorphisms were amplified using the following primers: IL-6 F: 5'-ACTTTTCCCCCTAGTTGTGTCTTTC-3', R: 5'-AGAATGAGCCTCAGACATCTCCAGT-3', IL-12 F: 5'-GGCATTCTCTTCCAGGTTCTG-3', R: 5'-CCAT-GGCAACTTGAGAGCTG-3' and TNF-α F: 5'-AG-GCAATAGGTTTTGAGGGCCAT-3', R: 5'-TCCCTG-CTCCGATTCCG-3'. Polymerase chain reaction products were generated in a 10 µL reaction volume containing 50 ng of genomic DNA, 1×PCR buffer, 2 mmol/L MgCl$_2$, 0.2 mmol/L of each dNTP, 1 µmol/L of each primer, and 0.25 U of Taq DNA polymerase (Invitrogen Corpo-ration, Carlsbad, CA). Cycling conditions con-sisted of an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of denatur-ation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds and a final elongation step at 72°C for 1 minute. Polymerase chain reaction products were digested with 2 U of *NcoI* restric-tion enzyme at 37°C, according to the manu-facturer's instructions (New England BioLabs, Ipswich, MA). 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. Andthe IL-12 PCR products resulted in either two frag-

ments of 173 and 70 bp (allele C) or a single fragment of 243 bp (allele A). Finally, the -308G allele contains an *NcoI* restriction site not pres-ent in the -308A allele; thus, in the presence of the -308G allele, the PCR product (107 bp) is cut into 2 fragments of 80 and 27 bp in length.

Statistic analysis

Data were statistically described in terms of mean ± standard deviation (SD), or frequencies (number of cases) and percentages as requir-ed depending on their distribution. The Hardy-Weinberg equilibrium (HWE) was assessed for each variation to identify the deviation. The dif-ferences of the genotypes and alleles of detect-ed genes between patients and normal con-trols were evaluated by using Pearson Chi-square test. Exact test was used instead when the expected frequency is less than 5. The odds ratio (OR) and 95% confidence intervals (95% CI) were calculated. Unpaired Student's t test or Mann-Whitney tests were used for two-group comparisons. Statistical analysis of data was performed using the SPSS software package 18.0 (SPSS Inc. USA). *P*-value less than 0.05 was considered statistically significant.

Results

In this study, 248 asthmatic (108 males and 140 females) and 226 controls (112 males and 114 females) were screened for IL-6 rs1800795, IL-12 rs3212227 and TNF-α rs1800629 polymorphisms using PCR-RFLP methods. The mean age of asthmatic patients was 46.48 years, and mean age of matched controls was 46.58 years. There were no signifi-cant differences between two groups with regarded to gender and age distribution. **Table 1** showed the general characteristics of the studied subjects. Whereas 61.29% of patients were current smokers, cigarette smoking sh-owed significant association with increased risk of bladder cancer compared with healthy individuals (OR=2.47, 95% CI=1.66-3.65, *P*= 0.000031). Moreover, 38.71% of patients were performing high risk jobs (workers in the alumi-num, dye, paint, rubbe and textile industries and, in gases stations). High risk jobs status has shown significant association with increa-sed risk of bladder cancer (OR=2.8, 95% CI= 1.56-3.73, *P*=0.0000). The incidence of alco-hol drinker was also higher in bladder cancer patients (44.35% Vs 25.67%). These epidemiol-

Table 2. Genotype and allele frequency of IL-6 rs1800795, IL-12 rs3212227 and TNF-α rs1800629 and Pearson's chi-square test in bladder cancer patients and normal controls

Genotype/Allele	Patients (n=248)	Controls (n=226)	P-value	OR (95% CI)
IL-6 rs1800795	HWE *P=0.25	HWE P=0.21		
GG	147	146	0.620	0.905 (0.626-1.320)
GC	83	69	0.077	0.822 (0.681-1.484)
CC	18	11	0.122	4.560 (0.556-37.412)
G	377	361	0.314	0.841 (0.498-1.192)
C	119	91		
IL-12 rs3212227	HWE *P=0.28	HWE P=0.75		
AA	106	81	0.0521	0.805 (0.526-1.220)
AC	127	140	0.025	1.749 (1.104-2.417)
CC	15	5	0.0519	0.972 (0.653-1.521)
A	339	302	0.275	0.571 (0.328-0.981)
C	157	150		
TNF-α rs1800629	HWE *P=0.48	HWE P=0.56		
GG	172	169	0.559	0.869 (0.542-1.392)
GA	68	51	0.921	1.258 (0.787-2.010)
AA	8	6	0.350	3.358 (0.347-32.543)
G	412	389	0.356	0.813 (0.524-1.267)
A	84	63		

*Chi-square test for deviation from the Hardy-Weinberg equilibrium (a value of P<0.001 was regarded as a deviation from the HWE).

Table 3. Age and sex adjusted ORs (and Cornfield 95% CIs) for the association of proinflammatory cytokines gene polymorphisms among smokers and high risk job patients

Genotype	Smoker Risk (152)		High risk job Risk (96)	
	P value	OR (95% CI)	P value	OR (95% CI)
IL-6 rs1800795				
CC	0.006	2.012 (1.782-2.561)		
GC	0.041	2.812 (1.612-4.210)	0.031	2.754 (1.731-5.902)
GC+CC	0.012	2.162 (1.452-4.841)	0.028	2.951 (1.631-4.899)
IL-12 rs3212227				
AC	0.011	3.312 (1.282-5.731)	0.001	3.712 (2.401-6.752)
AC+CC	0.045	3.502 (1.781-5.814)	0.001	3.923 (1.852-5.681)
TNF-α rs1800629				
GA	0.034	1.642 (0.781-3.621)	0.067	0.784 (0.427-0.989)
GA+AA	0.026	3.213 (2.013-6.412)	0.219	1.214 (0.781-1.971)

ogy results suggested that smoking, occupation and drinking are the primary risk factor for BC and were consistent with previous studies [18].

Firstly, the frequencies of genotypes and alleles of IL-6 rs1800795, IL-12 rs3212227 and TNF-α rs1800629 were detected on patients and control groups. HWE of rs1800795, rs3212227 and rs1800629 in patients and controls were

listed in **Table 2**, and the results showed allelic distribution of detected SNP were not deviated from HWE in both case and control populations. IL-6 rs1800795 in the study population were as follows: 7.4% CC, 33.5% CG and 59.1% GG for the case study group and 4.9% CC, 30.4% GC, and 64.7% 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. Similarly, the genotypic and allelic frequency of rs1800629 did not show significant difference between asthmatic patients and normal controls. Then, Genotype and allele frequency of rs1800629 were detected in metabolic syndrome patients and normal control in **Table 2**.

The genotypic and allelic frequency of rs-1800629 between cases and health controls did not show significant difference.

Whereas, the frequency of wild (AA) and homozygous mutant (CC) genotype IL-12 rs3212227 genotypes in cases and controls was found more in controls (43% and 5.7% 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=1.74, 95% CI=1.10-2.41, *P*=0.025) genotype of IL-12. The genomic analysis did not reveal differences in allelic frequencies of the IL-12 (A/C) gene between bladder cancer patients and healthy controls.

Considering bladder cancer may be originated in the environmental and genetic factors interaction, we further investigate the association of proinflammatory cytokines gene polymorphisms among smokers and high risk job patients with age and sex adjusted in **Table 3**. 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=2.012, 95% CI=1.782-2.561, *P*=0.006), GC genotype (OR=2.812, 95% CI=1.612-4.841, *P*=0.041) and GC+CC (OR=2.162, 95% CI=1.452-4.481, *P*=0.012). We found that smokers with C allele in the rs1800795 position were higher risk of bladder cancer. Moreover, the bladder cancer patients with GC and GC+CC genotypes at rs1800795 position and performing high risk jobs suggested an increased risk of developing bladder cancer, compared with carriers of the GG genotype who had low risk jobs (OR=2.754, 95% CI=1.731-5.092, *P*=0.031) and (OR=2.951, 95% CI=1.631-4.899, *P*=0.028), respectively (**Table 2**). Therefore, people engaged high risk jobs with C allele in IL-6 rs1800795 position were 2-4 times more susceptible to fall ill bladder cancer. However, there were not significantly association between IL-6 genotypes and different clinical stages and grades bladder cancer patients.

On the other hand, the AC heterozygous genotype of IL-12 rs3212227 (OR=3.312, 95% CI=1.282-5.731, *P*=0.011) and combined AC+CC (OR=3.502, 95% CI=1.781-5.814, *P*=0.045) genotypes among smoker patients showed significant association with an increased suscep-

tibility of bladder cancer. Moreover, there was more than 2-fold increased risk of cancer in the carriers of IL-12 heterozygous (OR=3.712, 95% CI=2.401-6.752, *P*=0.001) and combined AC+CC (OR=3.923, 95% CI=1.852-5.681, *P*=0.001) genotypes as compared with AA genotype occupied low risk jobs among high risk job patients. However, associations also not were found between IL-12 genotypes and clinical stages and grades of bladder cancer. Whereas, the GA heterozygous geneotype of TNF-α rs1800629 (OR=1.642, 95% CI=0.781-3.641, *P*=0.034) and combined GA+AA (OR=3.213, 95% CI=2.013-6.412, *P*=0.026) genotypes among smoker patients showed significant association with an increased susceptibility of bladder cancer. However, the similar results were not shown in the high risk job with bladder cancer.

Discussion

The gene single nucleotide polymorphisms (SNP) have been thought to alter expressions or influence certain genes; thus, SNPs could be associated with an altered risk of multiple cancers [19-25]. Up to now, the important role of pro-inflammatory cytokines during tumor development and prognosis are 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 [26]. Furthermore, elevated IL-6 serum levels were reported to be associated with metastasis and poor prognosis of prostate and may favor a T-helper-2 (Th2) pattern of humoral immune response which leads to subsequent chronic inflammation and poor [27-36]. The -174 G/C functional polymorphisms in the IL-6 promoter region was correlated with IL-6 transcription activity and levels of IL-6 expression [37].

IL-12 is an important antitumor cytokine that plays important role in the development and progress of canc. Variation in the DNA sequence lead to altered IL-12 production, and this can alter individual's susceptibility to cancer. The IL-12 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 [38-

44]. TNF-α gene is located in the class III region of the human major histocompatibility complex (MHC) on chromosome 6p21 [45, 46]. Among the several single nucleotide polymorphisms (SNPs) identified in TNF-α, TNF-α rs1800629 is the most extensively studied. The A allele of this polymorphism can lead to high binding affinity of nuclear factors to the TNF promoter, resulting in a high level of transcription activity and secretion levels of TNF-α. So, it was suggested to have a significant functional effect [47]. Numerous studies have tried to determine whether the polymorphism of pro-inflammatory cytokines SNP influences susceptibility to bladder cancer, but no accordant result was obtained due to the heterogeneity of the genetic background among populations [48-50]. Whether genetic variations of the pro-inflammatory cytokines conferred susceptibility to bladder cancer in Chinese were puzzled. Therefore, we presume that the pro-inflammatory cytokines gene polymorphism may be a predicted factor for the tumorigenesis.

In the present study, our results suggested that the genotypes distributions of IL-6 rs1800795 and TNF-α rs1800629 were almost the similar in the cases and the control groups. Meanwhile, allelic analysis did not reveal discrepancy for the frequency at the -174 position, -308 A/G with IL-6 gene and TNF-α promoter respectively. This result is line with the four met-analysis of pro-inflammatory cytokines gene polymorphism and cancer risk [51-53]. Furthermore, our findings indicated increased frequency of IL-12 rs3212227 AA and CC homozygous geneotype among controls but that of the heterozygous AC genotype was higher in cases with bladder cancer; thus, a significant risk of bladder cancer was observed for AC genotype of IL-12 rs3212227, which is consistent with the results of Iranian Population [54]. Moreover, evidence from two meta-analyses of IL-12 gene functional polymorphisms by Chen H and Zhou L stand by our results [15, 55]. Jaiswal PK yet reported an inconstant result, discovering that AC genotype and C allele carrier demonstrated reduced risk of BC [56]; We speculated different ethnicity or sample size may lead to contradictory results.

Bladder Cancer may be as a result of gene-environment interaction. Epidemiologic studies have shown significant associations between bladder cancer and environmental factors such as tobacco smoking and occupational exposures [57]. 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 [58]. 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 revealed strong associations between specific genotypes and incidence of cancer among smokers rs1800795 CC genotype, in case of the GC genotype and in GC+CC. Moreover, patients with high risk jobs and GC and GC+CC genotypes at rs1800795 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. These findings suggested the possible role of gene-environment interaction at the rs1800795 position for IL-6 gene and predisposing of a specific genotype at this region to bladder cancer in exposure to these risk factors.

For the first time, we have also investigated the association of the IL-12 rs3212227 polymorphism in bladder cancer patients with tobacco smoking habits and high risk jobs. Special genotypes of IL-12B showed significant association with an increased risk of bladder cancer in smokers and high risk job. Whereas, the GA heterozygous geneotype of TNF-α rs1800629 and combined GA+AA genotypes among smoker patients showed significant association with an increased susceptibility of bladder cancer. However, the similar results were not shown in the high risk job with bladder cancer. TNF-α has many effects relevant to the pathogenesis of asthma, including neutrophil release, epithelial cell barrier permeability, macrophage activation, recruitment of inflammatory infiltrates, effectiveness of the local and systemic inflammatory response, and amplification of the effects of other proinflammatory cytokines [59].

These results may be due to the pro-inflammatory cytokines 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.

Conclusion

In summary, though no any relationship between IL-6, and TNF-α genotypes or alleles and bladder cancer suscepitibility was revealed, specific IL-6 rs1800795, and TNF-α rs1800629 genotype are significantly associated with an increased risk of bladder cancer in the chinese population with smoking habits or working. Furthermore, we first identified IL-12 rs3212-227 AC geneotype is not associated with an increased risk of bladder cancer with smoking habits or working, but confer genetically susceptibility to bladder cancer in chinese population.

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Disclosure of conflict of interest

None.

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