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Running Title: IL-17 Genetic Variations in Bladder Cancer

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IL17A and IL17F Genetic Variations in Iranian Patients with Urothelial Bladder Cancer: A Case-Control Study

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Abstract

Background: Interleukin 17 (IL17) is a pro-inflammatory cytokine with pivotal modulatory effects on antitumor immune responses and has been reported to play a particularly important role in the occurrence and development of bladder cancer. We aimed to investigate the possible influence of IL17 genetic variations on loci rs22775913 and rs763780 with genetic susceptibility to bladder cancer in southern Iran.

Method: In this case-control study, we enrolled 180 patients with urothelial bladder cancer (mean age 64 years) and 180 age/gender matched healthy controls without any family history of cancer and autoimmune disorders. Genomic DNA was extracted from peripheral whole blood, and genotyping was performed using PCR-RFLP method.

Results: Genotype distributions in both the patients and controls were in agreement with Hardy-Weinberg equilibrium. The frequency of GG, GA, and AA genotypes for IL17A were 87 (48.3%), 72 (40%), and 21 (11.7%) among patients, and 92 (51.1%), 75 (41.6%), and 13 (7.3%) in the controls. The frequency of AA, AG, and GG genotypes for IL17F in both the patients/controls were 160 (88.9%)/ 158 (87.8%), 16 (8.9%)/ 21 (11.7%), and 4 (2.2%)/ 1 (0.5%), respectively. Statistical analysis revealed no significant differences between the two groups regarding the frequency of genotypes and alleles (P>0.05).

Conclusion: Our data collectively suggested that genetic variations on loci rs22775913 and rs763780 of IL17 genes were not associated with bladder cancer susceptibility in the Iranian population. Our finding has to be confirmed in different ethnic groups.

Keyword: Immune system, Bladder cancer, IL-17 , Genetic variation

Introduction

The urothelial bladder cancer (UBC) is the seventh prevalent type of cancer worldwide and the fifth common cancer in Iran.[1] It is more common in men than in women due to the high prevalence of environmental toxin exposure, including smoking and industrial substances.[1] Hereditary UBC is rare, but genetic factors have been associated with this type of malignancy. An antitumor activity and tumor progression were detected following the release of modulatory cytokines in adaptive immune system.[2] Th17 is a distinct subtype of $CD4^+$ cells that plays an important role in innate and adaptive immune system by releasing Interleukin 17 (IL17) cytokine. IL17A and IL17F are the two most important subtypes of IL17 family that function through inducing multiple pro-inflammatory mediators, such as chemokines, metalloproteinase, and cytokines.[3] Th17 cells are potentially enriched in the tumor tissues of patients with bladder cancer, and imbalance between Th17 and Treg cells was reported to be related to its development or progression.[4] As a pro-inflammatory cytokine, IL17 is believed to be among the most potent antitumor cytokine.[5, 6] However, there are several reports suggesting the tumor promoting activity of this cytokine.[6,8] A large number of polymorphisms were reported in IL17, among which rs2275913 and rs763780, located in IL17A and IL17F genes, respectively, and the single nucleotide genetic variations were associated with several malignancies, especially gastric and bladder cancers.[9,11] An updated meta-analysis showed the existence of a significant association between rs763780 polymorphism and cancer susceptibility in Caucasian populations[12]. Another meta-analysis revealed that rs2273913 conferred a high risk of non-gastrointestinal cancer among Asian populations. [13] In bladder cancer, IL-17A and IL-17F genetic variations were found to be associated with tumor invasion, tumor staging, tumor development, and smoking status.[10] Additionally, 17A and IL-17F genetic variations had combined effects with H. pylori infection or tobacco smoking on gastric cancer risk.[9,11] Furthermore, AG and AA genotypes of IL-17A were reported to increase the susceptibility to colorectal cancer by 2-3 times.[14] These genetic variants may provide valuable evidence as to the occurrence of cancer or cancer risk prognosis. Since rs2275913 and rs763780 affect the expression and production of IL-17A and IL-F cytokines at protein level, the objective of the present study was to investigate the association between IL17A and IL17F genetic variations on rs2275913 and rs763780 loci with UBC in a population from the south of Iran.

Subjects and Methods

The cases were selected from among patients who referred to hospitals affiliated with Shiraz University of Medical Sciences (from 2014 to 2016) according to clinical examinations and laboratory assessment. The inclusion criterion was primary, non-recurrent UBC based on the results of cystoscopy and pathological evaluation. We enrolled 180 patients with UBC (153 males and 27 females); aged 45-85 years (mean age 64 years). None of the patients had been treated with chemotherapy and radiotherapy before sample collection, and samples were taken prior to surgery. The control group comprised 180 age/gender matched healthy adults aged 40 to 85 years (153 males and 27 females) with no acute or chronic diseases, such as autoimmune disease, diabetes mellitus, thyroid disease, hypertension, hyperlipidemia, ischemic heart disease, cerebrovascular accident, renal disease, pulmonary disorders. This study was approved by the local Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1392.4611). The participants were informed about the objectives of this study, and safety and security measures were obtained before their informed consents.

On the day before the surgery, 4 mL of whole blood was collected from the patients' peripheral veins. Genomic DNA was extracted through the use of salting out.[15] IL17A and IL17F genetic variations were determined via Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) reactions. Both loci were amplified with specific primers and under the same conditions (Table 1). IL-17A -197 G/A genotyping was performed by XagI restriction enzyme, and the products were then run on 3% agarose gels. As shown in figure 1, in AA genotype, the restriction enzyme cut the PCR products into two fragments, namely68 bp and 34 bp while for GG genotype, the enzyme was unable to digest the 102bp fragment. Therefore, the GA genotype consisted of three fragments: 102bp, 68 bp, and 34 bp. IL-F 7488A/G genotyping was conducted by NlaIII restriction enzyme. For AA genotype, the enzyme digested the DNA to two fragments (80 bp and 63 bp) whereas for GG genotype, it failed to digest 143bp. AG genotypes contained three fragments of 143bp, 80 bp, and 63 bp. (Figure1). Primer sequences, restriction enzymes, length of the digested fragments, and annealing temperature are shown in Table 1. The data was assembled and analyzed by SPSS software package (version 11.5; SPSS Inc, Chicago, IL, USA). Chi-square test was used to compare the genetic data between the studied groups, and *P*-value < 0.05 was considered to be statistically significant.

Results

In both patients and controls, the genotype frequencies of both IL-17A and IL-17F were in agreement with the Hardy-Weinberg equilibrium (P>0.05) when analyzed by Arlequin software package.[16] Table 2 depicts the frequencies of genotypes and alleles at of IL-17A and IL-17F loci in the patients and controls. As shown, the genotype frequencies of GG, GA, and AA in IL-17 A locus in the patients and controls were 87 (48.3%) vs. 92 (51.1%), 72 (40%) vs. 75 (41.6%) and 21 (11.7%) vs. 13 (7.3%). G and A allele frequencies were 246 (68.3%) vs. 259(71.9%) and 114 (31.7%) vs. 101(29.1%). However, there was no significant difference between patients and controls concerning the frequencies of genotypes (P=0.35) and alleles (P=0.32) at this locus. Regarding IL-17 F gene, the frequency of GG, GA, and AA genotypes were 160 (88.9%) vs. 158 (87.8%), 16 (8.9%) vs. 21(11.7%), and 4 (2.2%) vs. 1 (0.5%) in the patients and controls, respectively. In addition, at this locus, the frequency of G and A alleles were found to be 336 (93.3%) vs. 337 (93.6%) and 24 (6.7%) vs. 23 (6.4%) in the patients and controls. No significant differences were observed in the frequencies of genotypes (P=0.28) and alleles (P=1) between the two groups. Additionally, no significant association was found between IL17A and IL17F SNPs and the histological grade of UBC in the patients.

Discussion

Our study showed no significant differences between the patients and controls in terms of the genotype and allele frequencies on rs22775913 and rs763780, which is in line with a study on breast cancer patients of the same population in southern Iran.[17] There was no relationship between these polymorphisms and pathological grading, where grading is an important factor in UBC progression. rs22775913 and rs763780 were found to be associated with the changes in serum cytokine levels in cancer, in which high levels might be related to susceptibility to cancer.[18] In comparison to G allele, A allele at -197 position in IL-17 A gene (rs2275913) had a higher affinity for NFAT transcription factor, thereby possibly inducing a higher cytokine production.[19] Substitution of adenine with guanine in IL-17F 7488A/G (rs763780) changed the amino acid from histidine to arginine (His161Arg) in the protein sequence, which might have directly regulated the IL-17F expression.[20] IL-17 is an important pro-inflammatory cytokine mainly generated by $CD4^+$ memory T cells, which is implicated in both innate and acquired immunity.[3] IL-17A plays a dual role in the inflammatory process during tumorigenesis, either as protection from tumor or as promotion of tumor

progression. IL-17 can be generated by malignant cells, leading to angiogenesis and tumor growth.[6] In a previous study by our group, it was reported that IL-17 was higher in the early stages of UBC.[21] Zhou et al. showed that the degree of systemic inflammation pertained to UBC aggressiveness.[22] In this regard, altered patterns of IL-17 expression regarding genetic variation on IL-17 gene might induce the infiltration of inflammatory cells, thus contributing to the occurrence and development of bladder cancer.[23] As a pro-inflammatory cytokine, IL-17-targeted therapies are currently under development for the treatment of autoimmunity and inflammation, which could be employed in UBC treatment in the future.

Because rs2275913 and rs763780 influence the expression and production of IL-17A and IL-17F cytokines at protein level, two SNPs were investigated in our study. We observed no relationship between the SNPs of IL17A and IL17F genes on rs22775913 and rs763780, which is similar to the breast cancer and basal cell carcinoma of skin in the same population in southern Iran.[17,24] In contrast to the present study, Zhou et al. showed that rs2275913 of IL17A and rs763780 of IL17F SNPs were associated with UBC development, gender, and smoking status in a Chinese Han population.[10] Similarly, Si *et al*. indicated that inheriting AA genotype or at least one A allele at this locus (GA+AA genotypes) might render genetic susceptibility to laryngeal cancer in the Chinese population.[25] Additionally, Qinghai et al. reported that rs2275913 and rs3748067 polymorphisms of IL-17 gene were related to increased risk of gastric cancer among Chinese population.[9] Genetic background variation of participants as well as the differences in molecular pathology of the investigated cancers/ cancer subtypes could explain the observed differences between Iranian and Chinese populations. Furthermore, the discrepancies might be explained by the differences in the distribution of minor allele frequency (MAF) in both populations. In our study, the minor allele frequency (MAF) (G allele) was observed to be 0.35. Several studies have shown that MAF affects the statistical differences amongst the genetic studies on different populations.[26] UBC is a multifactorial, complex disease whose complex interrelation of immune system and tumor might not simply be interpreted by one immunological parameter even in the tumors of the same type and genetic and immunological condition. To better understand the polymorphisms affecting cancer susceptibility, more genetic and immunologic studies with larger sample populations are required.

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Conflict of Interest

None declared.

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Table 1. Primers, reaction conditions, and RFLP product size for IL-17A and IL-17 F genotyping

Locus	Primer sequence		Annealing temperature	Restriction endonuclease enzyme	Fragments length	Ref.
IL-17A G197A (rs2275913)	Forward	5'-AAC AAG TAA GAA TGA AAA GAG GAC ATG GT-3'	65 ºC	*Xag*I	AA: 102 bp AG: 102، 68 and 34 bp GG: 68 and 34 bp	11
	Reverse	5'-CCC CCA ATG AGG TCA TAG AAG AAT C-3'				
IL-17F A7488G (rs763780)	Forward	5'-ACC AAG GCT GCT CTG TTT CT-3'	65 ºC	*Nla*III	GG:143 bp GA: 143، 80 and 63 bp AA: 63 and 80 bp	11
	Reverse	5'-GGT AAG GAG TGG CAT TTC TA-3'				

RFLP : Restriction Fragment Length Polymorphism, IL-17: Interleukin-17

Table 2. Frequencies related to the genotypes and alleles of IL-17A G197A and IL-17F A7488G in the patients with bladder cancer and healthy controls

Locus, alleles, and genotypes			Group		*P* value
			Patients (n=180)	Controls (n=180)	
Interleukin-17A -197G/A (rs2275913)	Alleles	G	246(68.3%)	259(71.9%)	0.32
		A	114(31.7%)	101(29.1%)	
	Genotypes	GG	87 (48.3%)	92(51.1%)	0.35
		GA	72(40%)	75(41.6%)	
		AA	21(11.7%)	13(7.3%)	
Interleukin-17F 7488A/G (rs763780)	Alleles	A	336(93.3%)	337(93.6%)	1
		G	24(6.7%)	23(6.4%)	
	Genotypes	AA	160(88.9%)	158(87.8%)	0.28
		AG	16(8.9%)	21(11.7%)	
		GG	4 (2.2%)	1(0.5%)	

IL-17: Interleukin-17

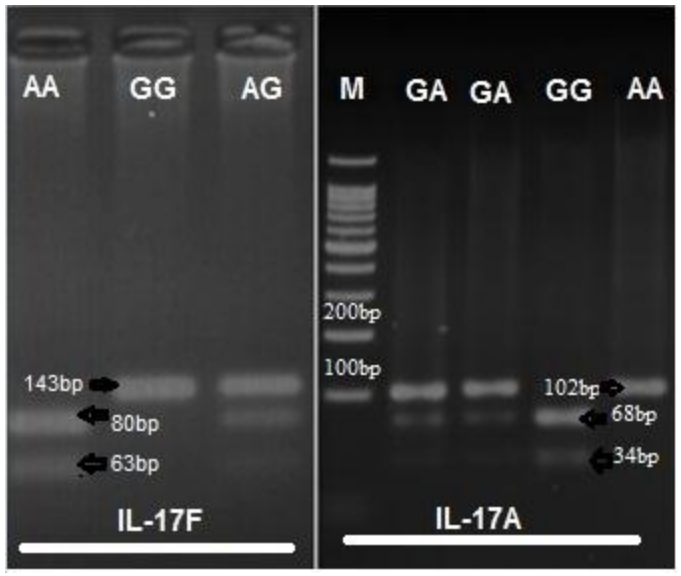

Figure 1. IL-17A rs2275913 (G197A) and IL-17F rs763780 (A7488G) genotyping in patients with bladder cancer by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). **Left side:** Genotyping of IL-17F: AA (80 and 63 bp), GG (143bp), and AG (143, 80 and 63bp). **Right side:** Genotyping of the IL-17A: GA(102, 68 and 34 bp), GG (68 and 34bp), and AA (102bp).

IL-17: Interleukin-17, M: Ladder Maker.