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RESEARCH ARTICLE

Allele A of -251A/T variant in interleukin-8 Promoter Associates with the Risk of Prostate Cancer in Iraqi Patients

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Abstract

***Background*:** Prostate cancer (PCa) is a disease of multifactor causes. Genetics is one of the most recognized predisposing factors for this disease.
Aim: This study aimed to investigate the association of the single nucleotide polymorphism (SNP) -251A/T in the promoter region of *interleukin 8* (*IL-8*) gene with incidence of PCa in Iraqi patients.
Subjects and Methods: A total of 60 histopathologically confirmed PCa patients and 40 apparently healthy control were recruited for this study. DNA was extracted from blood samples from these participants, and *IL-8* gene was amplified using specific primes. Restriction fragment length polymorphism (RFLP) was used for genotyping.
Results: Overall, there were no significant differences in genotype frequencies between patients and control neither for AT (OR=3.064, 95%CI=0.878-10.69) nor for AA (OR=2.61, 95%CI=0.348-19.553). However, allele A had higher frequency among patients (33.3%) compared to control (15%) (OR=2.833, 95%CI=1.377-58.3, *p*=0.05).
Conclusion: Allele A of the SNP -251A/T in the promoter region of *IL-8* gene may represent a risk factor for PCa in Iraqi patients.

Introduction

Prostate cancer is now considered as one of the most important medical problems facing the male population. It is the most common non-cutaneous neoplasm accounting for one quarter of all such cancers, and the second leading cause of death among men in the United States and many Western industrialized countries [1]. However its incidence and mortality on global scale vary widely [2]. This disparities in the incidence and aggressiveness of the disease among different races even those who live in the same geographical region and exposed to almost the same environmental factors highlighted the importance of genetic factors in this cancer.

Interleukin-8 is one of the key members of the human α-chemokine subfamily. It is mainly involved in the initiation and amplification of acute inflammatory reactions, as well as in chronic inflammatory process, since it attracts and activates neutrophils in the inflammatory region [3]. The -251A/T polymorphism has been found to be involved in the susceptibility of a range of cancer including gastric cancer [5], breast carcinoma [5], colorectal cancer [6], and oral cancer [7]. Regarding its role in PCa, there is not complete agreement about this SNP. McCarron and her coworkers showed that this SNP had significant association with PCa susceptibility in Caucasian patients, and the prevalence TT genotype, which is associated with low IL-8 production, was decreased significantly among those patients, but there were no significant association between the cytokine genotype and tumor stage, grade, or overall survival [8]. Similarly, Zabaleta *et al*. [9] found that Cucasian –American individuals carryning AT genotype of this SNP had a 3.5-fold risk of having aggressive PCa as compared with those individuals who do not carry that genotype at the same locus (OR=3.5; 95% CI= 1.13-10.88). However, when Wang *et al*. [10] conducted a meta-analysis involving 42 case-controls, they found that carriers of this SNP had reduced risk of PCa. In the light this

controversy, this study aimed to investigate the association of -251A/T in *IL-8* gene with the incidence of PCa among Iraqi patients.

Materials and methods

This study included 60 men with histopathologically confirmed PCa during the period from September 2011 to July 2012 from the Hospital of Radiation and Nuclear Medicine, and Al-Kadhumyia Teaching Hospital (Baghdad). Family unrelated, cancer free, 40 individuals from Al-Kadhumyia Teaching Hospital and College of Medicine/ Al-Nahrain University were selected to represent the control group. The mean ages of patients and control were 69.44±1.44 years and 66.48±1.23 years respectively. Informed consent from patients as well as control was taken which included age, previous and current occupation, smoking, drinking, residence, and first relative family history of prostate or breast cancer.

Blood samples

Three mL of venous blood was collected in EDTA tubes from each participant and kept at -20 ^{o}C until be used.

Estimation of serum level of prostate specific antigen (PSA) in control group

As some individuals with PCa have no obvious clinical signs, we conducted PSA test to confirm that control group have no PCa. The test was done using commercial kit (ACON laboratories, Inc./ USA) according to the manufacturer's instructions. Out of 40 serum samples from control group 3 gave serum PSA concentration which may indicates PCa according to the manufacturere's instruction, therefore three more samples from age-matched different men were collected, and PSA tests was performed and gave negative results.

DNA Extraction and genotyping

DNA was extracted from blood samples using ready kit (gSYNC^{TM} DNA Mini Kit Whole Blood Protocol/ Geneaid/ Korea) according to the manufacturer's instructions.

A nanodrop (UVS-99/ACTGene/USA) was used to estimate the concentration and purity of the extracted DNA (from blood and tissues) following the manual of the apparatus.

For PCR amplification of *IL-8* gene, we used the forward primer 5'-ACTTGTTCTAACACCTGCCACTCT-3', and reverse primer 5'-TAAAATACTGAAGCTCCACAATTTGG-3'. The PCR protocol was as the follows: an initial denaturation for 5 min 95 ºC, followed by 30 cycles of denaturation at 94 ºC for 30 sec , annealing at 63 ºC for 30 sec, extension at 72 ºC for 1min, followed by final extension at 72 ºC for 7 min. A ready 50 µL PCR mastermix (Bioneer/Korea) was used for amplification for both genes.

Template DNA (10 µL) from each sample and primers (5 µL from each) were added to each master-mix tube (50 µL PCR master-mix, Bioneer/Korea). The mixture then put in shaker and spinner for 10 cycles for better mixing. After mixing, the master-mix tubes were transferred to the thermocycler (MyGenie 32 thermal block/Bioneer/Korea) which is previously programmed with the above protocol according to gene to be amplified.

Restriction endonuclease digestion for *IL-8* PCR was done using *MfeI* endonuclease (New England Biolabs Inc./USA). A 1µg amount DNA from each *IL-8* PCR products mixed with a 5µL 10X NEB buffer (50mM NaCl, 10mM Tris-HCl, 10mM $MgCl_2$, 1mM dithiothreitol, pH 7.9), and 1µL *Mfe1* (10U). The reaction was adjusted to 50µL using sterile deionized H_2O. The solution was mixed by flicking followed by spinning in microcentrifuge at 5000 rpm for 30 sec, then incubated at 37 ^{o}C for 60 min. The A allele fulfills acorresponging restriction endonuclease site, while the T allele does not, with expected fragment size of 121bp for TT , 82bp, 39bp, and 121bp for AT, and 82bp and 39bp for AA. The resultants fragments were electrophoresed on a 3% agarose gel containing ethidium bromide (0.5 µg/mL). The bands were then visualized by UV-transilluminator.

Results

Restriction fragment length polymorphism

Restriction fragment length polymorphism of PCR products of *IL-8* gene revealed three genotypes; TT, TA, and AA ((figures 1 and 2), (figures 1 and 2), (figures 1 and 2), (figures 1 and 2). In PCa patients the TT, AT, and AA genotypes account for 28 (46.7%), 24 (40%), and 8 (13.3%) respectively, compared with 30(75%), 8(20%), and 2(5%) respectively, in control group, with no significant differences neither for AT (OR= 2.61, 95%CI=0.348-19.533, *p*=0.079) nor TT genotype (OR= 3.064, 95%CI=0.878-10.69, p=0.079).

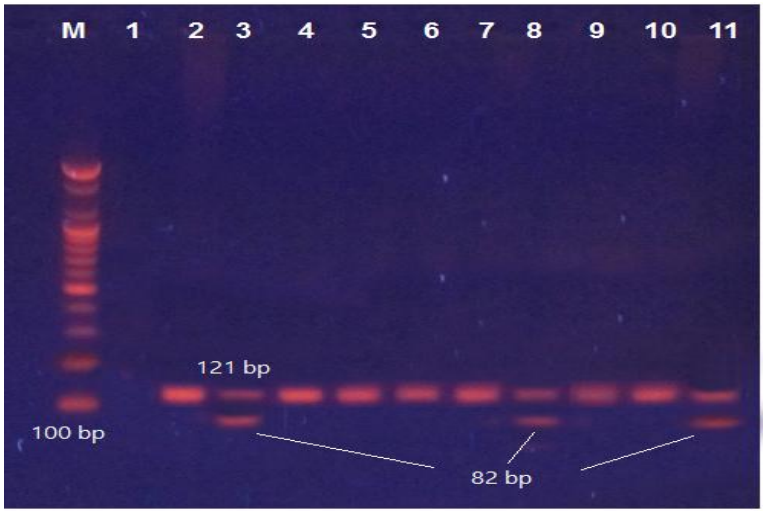

Figure (1): Various *interleukin*-8 -251T>Agenotype patterns in PCa patients observed after genotyping using RFLP-PCR. M: 1000bp DNA marker, lane 1: no product, lane 2,4,5,6, 7,9,10: wild type (CC), lane 3,7,11:heterozygous variant (AC)(bands of 39bp didn't appear).

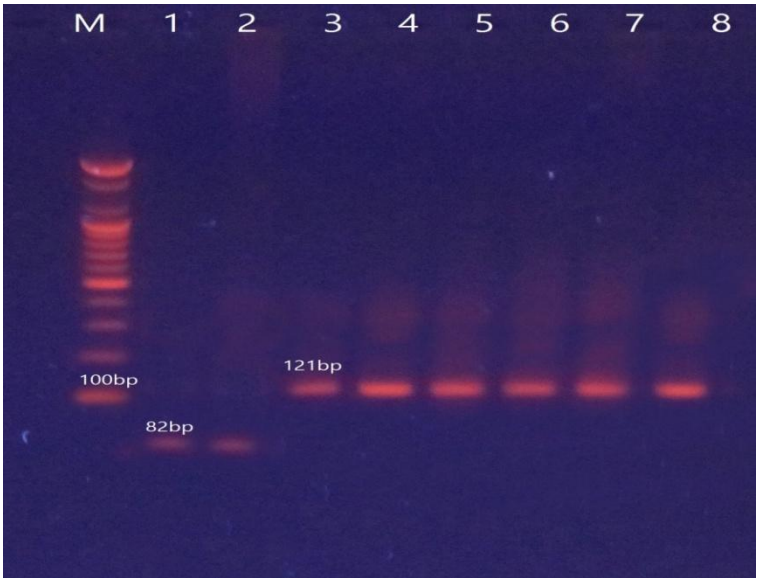

Figure (2): Various *interleukin-8* -251T>Agenotype patterns in PCa patients observed after genotyping using RFLP-PCR. M: 1000bp DNA marker, lane 3-8: wild type (CC), lane 1-2: homozygous variant (AA).

Using Chi-square for testing allele distribution, the result indicated that the SNP met Hardy-Weinberg equilibrium. Analysis of alleles frequencies revealed that T allele had 66.7% frequency among PCa patients and didn't differ from that of control group (85%), while, A allele had 33.3% frequency among PCa patients compared with 15% frequency among control with significant difference (p=0.05) (table 1).

Other risk factors

From the studied risk factors (other than polymorphism), only one (residency) has significant association with PCa (OR= 3.368, 95%CI=1.137- 9.978, p= 0.028). Since we intended to select control individual with age class that matches the PCa patients, age appeared to have insignificant association with PCa (OR= 1.038, 95%CI=0.977-1.104).

Only 12 patients (20%) have first relative with prostate or breast cancer compares with 4 (10%) control have these relatives. However, the association was insignificant (OR= 2.353, 95%CI=0.581-9.535). Mean BMI among

patients was 26.44 and does not differ considerably from that of control (24.71), and again, there is no significant association with PCa (OR= 1.108, 95%CI=0.989- 1.243) (table 1).

We have categorized the smoking status into three categories: never, ex-smokers and current smokers. Larger percentage of never smoked is among PCa patients (56.7 against 45%), whereas, 32.5% of control are ex-smokers compared to 30% of PCa patients, and 22.5% of control are current smokers compared to 13.3% of PCa patients. Nevertheless, there were no significant association with PCa (OR= 3.342, 95%CI= 0.766-14.57 and OR= 1.116, 95%CI= 0.247-5.048 for ex-smoker and current smoker respectively).

Table (1): Risk factors in patients with PCa and control

Variables	Cases N=55	Control N=38	*P*-value	OR(95%CI)
***IL-8* SNP**				
Genotypes				
TT	28 (46.7%)	30(75%)	0.17	1.0
AT	24 (40%)	8 (20%)	0.079	3.064 (0.878-10.69)
AA	8 (13.3%)	2 (5%)	0.35	2.61(0.348-19.553)
Alleles				
T	80(66.7%)	68(85%)	0.05	1.0
A	40(33.3%)	12(15%)	0.05	2.833(1.377-58.3)
Mean age in years (SD)	69.6 (9.76)	66.68 (8.29)	0.229	1.038 (0.977- 1.104)
Family history			0.231	
No	48 (80%)	36 (90%)		1.0
Yes	12 (20%)	4 (10%)		2.353(0.581-9.535)
Mean BMI (SD)	26.44 (4.27)	24.71 (5.02)	0.077	1.108 (0.989- 1.243)
Smoking				
Never	34 (56.7%)	18 (45%)	0.123	1.0
Ex-smoker	18 (30%)	13 (32.5%)	0.108	3.342 (0.766-14.57)
Current smoker	8(13.3%)	9 (22.5%)	0.886	1.116(0.247-5.048)
Residency			0.028	
Rural	29 (48.3.1%)	27 (67.5%)		1.0
Urban	31 (51.7%)	13 (32.5%)		3.368 (1.137- 9.978)

N: number, OR: odds ratio, CI: confidence interval, SD: standard deviation. BMI: body mass index, SNP: Single nucleotide polymorphism, TLR4: Toll-like receptor-4, IL-8: Interleukin-8

Discussion

In the year 2000, Hull *et al*. reported single A/T nucleotide polymorphism at position -251 from the transcription starting site in the proximal promoter region of *IL-8* gene, and found that 251A allele tended to be associated with increased IL-8 production [11]. Since then, researchers devoted their concern to elucidate the association of this SNP with different cancers. It is not the SNP itself which received such attention, but the high level of IL-8 resulting from A allele of the SNP is the pivotal point. Over expression of *IL-8* associates with many consequences considered in favor of initiation, progression, and metastasis of cancer.

For cancer initiation, IL-8 is a potent factor for activation and migration of neutrophils in response to infection, and, thereby, high serum levels of this cytokine can recruit unusual numbers of neutrophils which cause inflammation that may extend beyond normal limits at the site of injury, and predispose that organ to cancer. In the same concern, IL-8 can inhibit apoptosis through inducing anti-apoptotic factors such as Bclx1 and Bcl-2 and decreasing levels of apoptotic factor Bax [5].

Regarding cancer progression, over expression of IL-8 induced VEGF-independent angiogenic response. Moore *et al*. provided direct evidence for the role of IL-8 in tumor progression. They showed that neutralizing antibodies to IL-8 reduce the angiogenic activity of PC-3 cell line, and inhibit tumor growth after ectopic implantation in SCID mice [12]. This angiogenic effect of IL-8 was further demonstrated recently by Lattanziol et al. [13] in uveal melanoma.

High levels of IL-8 induce up-regulation of gene transcription and activity of metaloproteinase-2 (MMP-2), and collagenase IV which result in enhanced invasion of surrounding healthy tissue by tumor cells (metastasis). Experimentally, this ability of IL-8 was shown when forced expression of this cytokine by the transfection of poorly tumorigenic and poorly metatstatic human PCa cells makes them able to establish several cell lines [14].

Among the few documented risk factors for PCa is family history, which is confirmed by three meta-analysis studies [15,16]. These studies suggested that affected first degree relatives confer more than a two-fold increased risk of PCa.

Family history of BCa was included because many researchers have reported an association of this malignancy and PCa in the same family, especially when the affected female is sister or mother [17,18]. The biological mechanism behind this association may be related to the SNPs in *BRCA1* and *BRCA2* which can be a risk factor for PCa [18]. In the present study there was 20% of PCa patients who have first relative with PCa or BCa compared with 10% in control group. However the association appeared insignificant may be because small size sample.

Body mass index has been reported to be associated with many malignancies [19]. The most accepted hypothesized biological mechanism for the effect of BMI on the PCa is the serum level of free testosterone. The obese men were observed to have lower concentrations of free testosterone [20] which were observed to be associated with a decreased of non-aggressive well-differentiated PCa and with an increased risk of aggressive low-differentiated PCa [21]. Beside there is no significant association between BMI and PCa, mean BMI of PCa patients is 26.44. Individuals having this BMI are described as overweight but not obese. Therefore we cannot say that they have low serum level of free testosterone, and thus there may be no effect for BMI on the PCa.

Smoking is a leading preventable cause of many cancers such as lung cancers [22]. However, conclusive epidemiological evidences for the association of smoking with PCa are not yet available.

Even if we concede that smoking have a role in PCa initiation through whatever mechanism, then there must be a threshold, which takes certain period of time, beyond which smoking can participate in the induction of cancer. In the current study, it was difficult to determine the time at which ex-smokers or current smokers started and gave up smoking. However, the percentages of ex-smokers in patients and control are convergent (30% and 32.5% respectively), while the percentage of current smoker in control (22.5%) is higher than that of PCa patients (13.3%), and logistic regression test gave insignificant association.

The sole risk factor in the study (other than thate related to the SNP) which appeared to have significant association with PCa is the residency (OR=3.368, 95%CI=1.137- 9.978, p=0.028), which means that urban residents have 3.368 fold opportunity to get PCa compared with rural resident. This result implies a bias estimation, and we believe that the overall access to health care, and screening with PSA are reprehensible factors in this disparities. Such disparity has been also reported in many other parts of the world. Studies in the US and Australia have identified significant disparities in PCa screening between rural and urban residents [23,24]. Rural resident, generally, have lower socioeconomic status and educational level, combined with limited knowledge of PCa. Hence, even with the available access to health centers, rural residents frequently refused to take Prostate Specific Antigen (PSA) test or Digital Rectal Exam (DRE). This is a matter of fact which has been seen during sampling period of the present study. On the other hand, many urban residents, especially those with high levels of education, want to do PSA test even without reasonable suspension thinking that early detection of the disease can ease its cure.

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Ethical Approval: The necessary ethical approval from the Ethical Committee in Babylon Medical College was obtained .Moreover, all subjects involved in this work were informed and the agreement required for doing the experiments and publication this work was obtained from each one prior the collection of samples.

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