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ORIGINAL ARTICLE

Association of elevated interleukin-33 serum levels with tumorstages in patients with prostate cancer

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ABSTRACT. *Background*: Inflammation has a prominent role in cancer development and interleukin (IL)-33 has both inflammatory and anti-inflammatory properties. The aim of this study was to measure IL-33 quantities and genetic alterations in the rs1929992 SNP within IL-33 gene in patients with prostate cancer (PC). *Methods*: This investigation was conducted on blood specimens from 150 newly diagnosed PC patients and 150 healthy age-matched controls. Serum IL-33 measurements and genotyping were performed by ELISA and PCR-RFLP, respectively. *Results*: Elevated IL-33 quantities were detected in PC patients compared with controls ($P < 0.001$). The PC patients with Gleason scores 7-10 displayed greater IL-33 quantities than those who had Gleason scores 1-6 ($P < 0.001$). Significant differences were found between PC stages regarding the IL-33 serum levels ($P < 0.001$). The frequencies of the genotype GG and allele G in rs1929992 SNP were higher, whereas the frequencies of the genotype AA and allele A were lower in PC patients, as compared with controls ($P < 0.05$, 0.01, $P < 0.002$ and $P < 0.01$, respectively). The genotype GG and allele G of rs1929992 SNP were associated with a greater risk of cancer development (OR: 4.533; $P < 0.001$, and OR: 1.516; $P < 0.01$, respectively). The IL-33 levels were not significantly different between the subjects carrier genotypes AA, AG and GG, or alleles A and G in rs1929992 SNP, neither in patients nor in controls. *Conclusion*: Higher IL-33 quantities were found in patients with PC, especially in those with greater stages which raises the possiblity that IL-33 may contribute to PC progression. The rs1929992 SNP-related genotype GG and allele G were associated with an increased risk of cancer development.

Key words: prostate cancer, interleukin-33, gene polymorphism

INTRODUCTION

Prostate cancer (PC) is one of the most frequently occurring types of cancer in men and its incidence rates are quickly increased with rising age, beginning about age 50 years, with the greatest rates seen in individuals aged 70-80 years [1, 2]. Annually, 1.6 million men are diagnosed with PC and 366,000 men die due to this malignancy [3]. A number of risk factors such as old age, family history, ethnicity, obesity, diet, alcohol consumption, smoking, lifestyle, sexually transmitted disease, vasectomy, and environmental and occupational parameters were associated with susceptibility to PC [4].

Various inflammatory cells are infiltrated in both prostate-related benign and malignant tumors. In benign tissues, the occurrence of chronic inflammation is usually named as prostatitis [2]. In PC tissue biopsies, a complex mixture of cells such as fibroblasts, endothelial cells, epithelial cells, and infiltrated leukocytes (including mast cells, neutrophils, macrophages, dendritic cells, T-, and B lymphocytes) was observed that may communicate to maintain the tumor development [5, 6]. Inflammation and inappropriate immune responses play essential roles in the PC development [7]. The inflammatory-related mediators may help tumor development through inducing cell proliferation, promoting angiogenesis, inducing DNA damage, remodeling of the cytoskeleton, and enhancing extracellular matrix degradation [6, 7]. Within the immune cells, the effector type 1 helper T (Th1) cells display powerful antitumorigenic action through the recruitment of the natural killer (NK) cells, type 1 (M1) macrophages, and $CD8^+$ cytotoxic T lymphocytes

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(CTLs) [8, 9]. Conversely, type 2 (M2) macrophages, MDSCs, Th2 cells, and regulatory T (Treg) cells have pro-tumor activities mainly through the suppression of the antitumor arms of the immune system [9, 10].

A complex network of cytokines is also released by cells located within the tumor microenvironment that can affect the PC development through influencing the tumor cells and/or immune cell functions [11]. Therefore, cytokines are considered intercellular key mediators within the tumor microenvironment, among which interleukin-33 (IL-33) may play a prominent role [12]. IL-33 is constitutively expressed in many cells; however, its expression is also elicited in response to different inducers in the macrophages, fibroblasts, epithelial cells, endothelial cells, smooth muscle cells, and adipocytes [13].

IL-33 operates as a transcription element and as a regulatory protein. The complete form of IL-33 is transported into the nucleus upon expression via its N-terminus, where it interacts with chromatin and suppresses the transcription of the inflammatory element [13]. Further, IL-33 also interacts with the NF-κB to inhibit the transcription of the NF-κB-related pro-inflammatory genes [14]. In homeostasis situations, the IL-33 arrestment in the nucleus prevents its cytokine effects [13].

As a cytokine, IL-33 is released into the extracellular regions in reaction to cellular injury to act as an alarmin [15, 16]. In the inflammatory conditions, the complete form of IL-33 is degraded by the serine proteases released from the neutrophils to produce an active form that acts much more powerful than full-length IL-33 [16, 17]. IL-33 then binds to a hetero-dimeric receptor consisting of the specific subunit ST2 and the IL-1 receptor accessory protein (IL-1RAcP) via its C-terminal domain and recruits signaling elements such as MyD88, IRAK-1, IRAK-4, and TRAF6, leading to the stimulation of transcription factors including NF-κB, ERK, and AP-1 [13, 15].

As a cytokine, IL-33 promotes Th1 cell responses accompanied by increased expression of TNF-α, IFN-α, IL-6, IL-12p40, and IFN-γ [15]. IL-33 triggers and expands the NKT- and NK cells. Further, IL-33 and IL-12 trigger the IFN-γ secretion from the NKT- and NK cells in a synergistic manner [18]. On the other side, IL-33 has also powerful stimulatory effects on the expression of Th2 cell-related cytokines such as IL-4, IL-5, and IL-13, and IL-33-administrated mice exhibit elevated serum IgE levels [9]. IL-33 also has stimulatory impacts on the group 2 innate lymphoid cells (ILC2), mast cells, neutrophils, and eosinophils [19]. Moreover, IL-33 displays anti-inflammatory effects through inducing the Treg cells, regulatory B (Breg) cells, as well as M2 macrophage development [15, 18]. There are also reports concerning the pro- or antitumor properties of IL-33. Elevated IL-33 expression was indicated in various types of cancer such as gastric cancer, breast cancer, hepatocellular carcinoma, lung cancer, and head and neck cancer [20].

The human IL-33 gene has been mapped on the chromosome 9 (at 9p24.1) and contains eight exons [19]. The presence of the several single nucleotide polymorphisms (SNPs) was indicated in the IL-33 gene, of those the 9894 A/G polymorphism (rs1929992) is placed in the intron 3 region. The cytokine gene-related SNP may influence the cancer development through influencing the immune/inflammatory-related signaling pathways [21]. There are some investigations regarding the association of the rs1929992 SNP with several inflammatory disorders such as cedar pollinosis, ankylosing spondylitis, ischemic stroke, and Behcet's disease [9]. To our knowledge, no study was found about the association of serum IL-3 levels and genetic variability in IL-33 gene with PC and tumor stages. The present study aimed to determine the serum IL-33 quantities and the genetic variability at rs1929992 SNP in PC patients to explore possible associations.

MATERIAL AND METHODS

Subjects

In total, two groups of men including 150 men with PC and 150 healthy age-matched men were enrolled in this study. The enrollment of the PC patients was done in the Shiraz University of Medical Sciences-affiliated hospitals. The PC patients were newly diagnosed and included in the study before receiving chemotherapy, radiotherapy, or immunotherapy.

The expert Uro-oncologists verified the PC based on the pathological, para-clinical, and surgical evidence. Tumors were staged using TNM classification and graded according to the Gleason score described in the Sixth Edition of the American Joint Committee on Cancer (AJCC) [22]. The healthy men were selected among individuals who referred to the Shiraz University of Medical Sciences-affiliated hospitals and health centers for routine checkup.

The control men were in health, without previous history of prostatic diseases and malignancy. Cigarette smoking, medication, infectious diseases, asthma, allergy and atopic illnesses, any suspected immunological disorders, surgery and great trauma within the past 6 months were considered as the exclusion criteria. The present investigation was performed under the approval of the ethics committee of Kerman University of Medical Sciences and the Shiraz Institute for Cancer Research. All men were also enrolled after the completion an informed written consent. A sample of the peripheral blood (5 ml) was collected from each participant and the serum- and DNA specimens were isolated and stored at $-70\ ^{\circ}C$ until analyzed.

Genomic DNA extraction and genotyping

A sample of the whole peripheral blood was collected in a pretreated EDTA tube; then, the genomic DNA was separated using a standard salting-out technique [23]. The quality and quantity of DNA were assessed using a spectrophotometer (Ependorf, Germany).

The PCR-RFLP method was applied to determine the genetic variability in rs1929992 SNP. The composition of the PCR reaction in a total 25 µl was: 1 µl of separated DNA, 2.5 µl of PCR buffer (10×), 0.3 µl of Taq DNA polymerase (5 U/mM), 1.5 µl of MgCl2 (stock concentration: 1.5 mM), 0.5 µl of dNTP (stock concentration: 10 mM)], 1 µl of each primer, and

remaining was topped with sterile distilled water. The amplification of the region cover rs1929992 SNP was done using forward primer (5'-GTCATCAT-CAACTTGGAACCTT-3') and reverse primer (5'-CTGTGGAGTGCTTTGCCTTT-3'). The PCR thermal schedule was planned as follows: an initial denaturation (at 94 °C for 5 minutes) and 30 cycles of denaturation (at 94 °C for 30 seconds), annealing (at 53 °C for 30 seconds), and extension (at 72 °C for 45 seconds). The PCR process was ended after a final extension at 72 °C for 5 minutes. The PCR product includes the rs1929992 SNP with a length of 529 bp *(figure 1)*. The genotyping in the rs1929992 SNP was performed by subjecting the PCR product to digestion, using a restriction enzyme SSP 1 (Fermentase, Finland) to determine the G-A changing. For this purpose, 0.75 unit/reaction of the SSP1 enzyme was added to the 5 µL of PCR and then incubated at 37 °C overnight. The enzyme has only one restriction site at rs1929992 SNP. Hence, the PCR product was cut into two 131 and 398 bp fragments following digestion. The genotype AA exhibits 2 DNA fragments of 131 and 398 bp, whereas the genotype GG shows an uncut single DNA fragment (529 bp), and the AG heterozygosity was specified with 3 DNA fragments (529, 398, and 131 bp). The digested PCR products were visualized using a Chemi-Doc model XRS (Bio-Rad, USA) after mixing with 2 µl KBC Power load (Kowsar Biotech Co, Iran) and electrophoresis on 3% agarose gel *(figure 1)*.

Quantitation of the IL-33 levels

Serum IL-33 quantities were detected using commercial human IL-33 ELISA kits (R & D, USA) according to the manufacturer's instruction.

Statistical analysis

The statistical tests including ANOVA, Student t, χ^2, and Binary logistic regression were used to compare the variables, and the *P*-values of <0.05 were considered as the significance level. Analysis of the data was done using SPSS (version 21, Chicago, IL, USA).

RESULTS

Demographic characteristics of participants

The participants' mean age was 65.07 ±7.78 years in PC patients and 63.94 ±8.88 years in the control group ($P = 0.24$). According to the cancer stages, the patients were sorted to 4 subgroups, so that 16, 61, 49, and 24 patients were placed in stages I, II, III, or IV, respectively. According to the Gleason grading system, PC patients were again categorized into two subgroups, so that 71 and 79 PC patients were in Gleason score 1-6 or Gleason score 7-10, respectively.

Serum IL-33 quantities in PC patients and controls

The PC patients expressed significantly higher IL-33 quantities than the healthy men (80.80 ± 41.89 Pg/mL *versus* 21.91± 6.85 Pg/mL, $P < 0.0001$). The IL-33 quantities in patients with PC concerning the cancer stages are displayed in *table 1*. The patients with cancer stages I, II, III, and IV had significantly higher IL-33 concentrations than those detected in the control group ($P < 0.001$, $P < 0.001$, $P < 0.0001$, and $P < 0.0001$, respectively) *(table 1)*. Significantly raised IL-33 levels were detected in greater tumor stages, so that a significant difference was found between PC stages concerning the IL-33 quantities *(table 2)*.
The patients with Gleason scores of 1-6 and those with Gleason scores of 7-10 displayed higher IL-33 quantities as compared with the control men ($P < 0.001$). The PC patients with Gleason scores of 7-10 showed significantly higher IL-33 amounts than patients with Gleason scores of 1-6 ($P < 0.02$) *(table 3)*.

The rs1929992 SNP-related genetic variabilities in the PC and control groups

The frequencies of the rs1929992 SNP-related genotypes GG, AG, and AA were 22.7%, 70.7%, and 6.7%

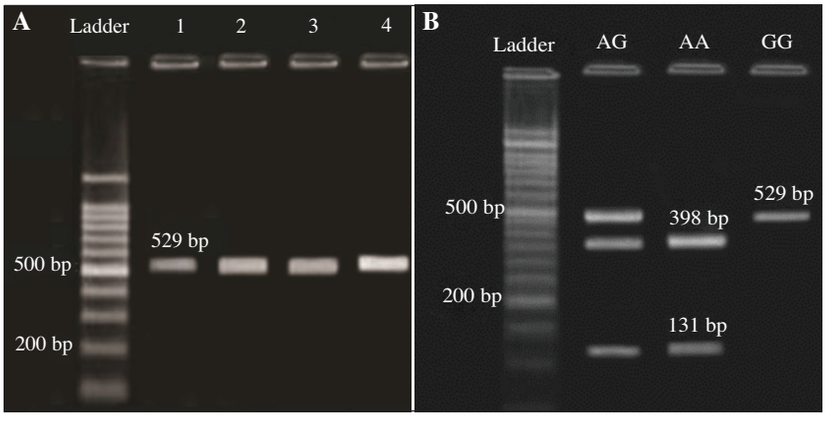

Figure 1
A) The PCR product includes the IL-33 rs1929992 with a size of 529 bp. The ladder pattern is observed in the first column. PCR products are observed in columns 2, 3, and 4. **B)** The patterns of genetic variations at rs1929992 SNP after enzymatic cutting of PCR products in PCR-RFLP. The first column indicates a ladder pattern. Columns 2, 3, and 4 indicate the AG, AA, and GG genotypes, respectively.

Table 1
Circulating amounts of IL-33 in patients with PC according to cancer stages.

Groups	Tumor stages	No.	IL-33 levels[a] Mean ± SD	IL-33 levels[a] Median (min-max)	*P*-values
PCa patients	I	16	40.00 ± 6.90	40.06 (28.78-54.28)	0.001*
	II	61	65.32 ± 18.61	59.24 (40.78-109.44)	0.001**
	III	49	85.47 ± 29.80	79.54 (45.66-158.67)	0.0001***
	IV	24	137.87 ± 60.45	125.58(48.12-270.12)	0.0001****
	Total	150	80.80 ± 41.89	70.31 (28.78-270.12)	
Healthy group	–	150	21.91 ± 6.85	18.80 (13.60-38.75)	0.0001[b]

*, **, *** and **** indicate the differences of the IL-33 levels in patients possess cancer stages I, II, III and IV with controls.
[a] The IL-33 levels displayed as Pg/mL.
[b] Indicate the difference of the IL-33 levels between healthy controls and whole patients with PC.

in the PC patients and 14.0%, 67.3%, 18.7% in the healthy men, respectively *(table 4)*. A significant difference was found between the PC patients and control group concerning the frequencies of the rs1929992 SNP-related genotypes ($P < 0.003$). The PC patients were found to have a significantly higher frequency of the genotype GG but a lower frequency of the genotype AA at rs1929992 SNP compared to the control group ($P < 0.05$ and $P < 0.002$, respectively). Similarly, the PC patients had a significantly higher rate of allele G but lower frequency of the allele A at rs1929992 SNP compared to the control group ($P < 0.01$).

Considering the genotype AA as a reference revealed that both genotypes AG and GG are associated with a greater risk for the development of PC [OR: 2.939 (95% CI: 1.358-6.358), $P < 0.006$; and OR: 4.533 (95% CI: 1.836-11.195), $P < 0.001$, respectively]. Considering the allele A as a reference also revealed that the allele G is associated with a greater risk for the development of PC [OR: 3.213 (95% CI: 1.500-6.883), $P < 0.003$] *(table 4)*.

The IL-33 levels concerning the genetic variability at rs1929992 SNP

The IL-33 levels were not significantly different between participants who possess genotypes GG, AG and AA, or between participants who possess alleles G and A at rs1929992 SNP, neither in PC- nor in control group *(table 5)*. The PC patients who carried the genotypes GG, AG, and AA, or alleles A and G at rs1929992 SNP had greater amounts of IL-33 concentrations compared with healthy men who carried same genotypes and alleles *(table 6)*.

DISCUSSION

In this study, elevated IL-33 levels were detected in patients with PC and its amounts had positive association with the progression of tumor stages. The PC patients with Gleason scores 7-10 exhibited higher amounts of IL-33 than those who had Gleason scores of 1-6. These findings indicate a positive association between overexpression of IL-33 and

Table 2
The statistical comparison of IL-33 levels between PC patients who were in different stages.

Stages	I	II	III	IV
I	–	0.001*	0.0001	0.0001
II	0.001	–	0.001	0.0001
III	0.0001	0.001	–	0.001
IV	0.0001	0.0001	0.001	–

*The decimal numbers indicate the *P*-values.

Table 3
Circulating IL-33 quantities in PC patients based on cancer Gleason score.

Groups	Gleason score	No.	IL-33 levels Mean ± SD	IL-33 levels Median (min-max)	*P*-values
PC patients	1-6	71	72.80 ± 34.68	16.49 (8.65-33.01)	0.02*
	7-10	79	89.05 ± 47.07	20.47 (10.50-34.70)	0.001**
	Total	150	80.80 ± 41.89	70.31 (28.78-270.12)	0.0001***
Healthy group	–	150	21.91 ± 6.85	18.80 (13.60-38.75)	0.0001[a]

Symbols *, **, and *** indicate the *P*-values concerning the differences of IL-33 amounts between cancer Gleason score 1-6 and cancer Gleason score 7-10; between cancer Gleason score 1-6 and control group; and between cancer Gleason score 7-10 and control group, respectively.
[a] Represent the difference of the IL-33 quantities between healthy group and whole patients with PC.

Table 4
The distribution of the IL-33 rs1929992 SNP-related genotypes and alleles in PC patients and healthy controls.

IL-33 rs1929992 SNP		PC patients No. (%)	Controls No. (%)	*P*-values	Odds ratio (OR)	(95% confidence interval of OR)	*P*-values
Genotypes	AA	10 (6.7)	28 (18.7)	0.003	1	Reference	–
	AG	106 (70.7)	101 (67.3)		2.939	1.358-6.358	0.006
	GG	34 (22.7)	21 (14.0)		4.533	1.836-11.195	0.001
Alleles	A	126 (42.0)	157 (52.3)	0.01	1	Reference	–
	G	174 (58.0)	143 (47.7)		3.213	1.500-6.883	0.003

The genotype GG and allele G was more frequent, whereas AA genotype and A allele was less frequent in cancer patients when compared with healthy men ($P < 0.05$, 0.01, $P < 0.002$ and $P < 0.01$, respectively). The genotypes GG and AG, and allele G were associated with a higher risk for the development of PC.

development of PC. Therefore, IL-33 may play an outstanding function in the development and progression of PC. In accordance with our findings, overexpression of IL-33 was reported in some malignancies such as breast, colorectal and gastric cancers, hepatocellular carcinoma, and hepatobiliary cancers [13].
Within the tumor microenvironment, IL-33 may be released from the cancerous cells, fibroblast, macrophages, and damaged cells which can display pro- and antitumor properties [24]. The antitumor activities of IL-33 may be exerted through the stimulation of the Th1 cells, CTLs, NKT cells, and γδ T cells [24]. Depending on the tumor, IL-33 may exert pro-tumor activity through the inducing T regulatory cells, stimulating M2 macrophages (tumor associated macrophages), triggering the cancer-associated fibroblasts (CAF), promoting MDSC activity, inducing Th2 cells, generating immature DCs, inducing angiogenesis, and eliciting mast cells that suppress the antitumor arms of the immune system such as NK

Table 5
IL-33 amounts in PC- and control groups based on the genetic variability in IL-33 rs1929992 SNP.

Groups	Genotype/allele	IL-33 levels Mean ± SD	IL-33 levels Median (min-max)	*P*-value
PC patients	AA	73.20 ± 36.28	66.49 (32.41-141.98)	0.48
	AG	83.66 ± 45.24	71.84 (28.78-270.12)	
	GG	74.21 ± 31.34	65.02 (31.59-161.90)	
	A	82.74 ± 44.47	71.45 (28.78-270.12)	0.81
	G	81.36 ± 42.35	70.31 (28.78-270.12)	
Healthy men	AA	21.30 ± 6.12	18.60 (14.80-35.09)	0.81
	AG	21.96 ± 6.73	19.00 (13.60-38.75)	
	GG	22.61 ± 8.52	17.00 (14.80-36.56)	
	A	21.79 ± 6.56	19.00 (13.60-38.75)	0.75
	G	22.08 ± 7.05	18.90 (13.60-38.75)	
Total men	AA	33.93± 28.81	20.73 (14.80-141.98)	0.02
	AG	54.20 ± 45.17	39.25 (13.60-270.12)	
	GG	54.20 ± 35.58	46.75 (14.80-161.90)	
	A	50.71 ± 43.43	35.09 (13.60-270.12)	0.39
	G	54.20 ± 43.21	40.78 (13.60-270.12)	

[a]The IL-33 levels displayed as Pg/mL. In all men (PC patients plus controls) the IL-33 levels in persons with AA genotype were lower than in individuals with genotypes GG or AG at rs1929992 SNP ($P < 0.006$ and $P < 0.01$, respectively).

Table 6
IL-33 quantities in IL-33 in PC and healthy controls based on the genetic variability in IL-33 rs1929992 SNP.

IL-33 rs1929992 SNP		Healthy group	PC patients	*P*-value
Genotypes	AA	21.30 ± 6.12	73.20 ± 36.28	0.003
	AG	21.96 ± 6.73	83.66 ± 45.24	0.0001
	GG	22.61 ± 8.52	74.21 ± 31.34	0.0001
Alleles	A	21.79 ± 6.56	82.74 ± 44.47	0.001
	G	22.08 ± 7.05	81.36 ± 42.35	0.001

The serum IL-33 quantities in PC patients with genotypes AA, AG, and GG or alleles A and G, were greater than controls with same genotypes and alleles at rs1929992.

cells, NKT cells, and CTLs [24-28]. It has been indicated that the ST2-deficient mice with breast cancer display lower metastasis, slower tumor growth, higher serum IL-17, IFN-γ and TNF-α levels, higher NK cell- and CD8+ CTL-mediated cytotoxicity, more intratumoral aggregation of the activated NK cells and CD8+ CTL cells, higher numbers of CD4+ and CD8+ T cells in the local lymph nodes and spleens, lower frequencies of splenic M2 macrophages, and lower serum IL-4 levels, compared with wild-type mice [29]. In ST2-defective mice, M1 macrophage-derived IL-12 enhances the DCs maturation and reinforces the Th1 cell response that activates the NK cells, NKT cells, and CTLs [29].

The results presented here also showed an association between PC and SNP rs1929992. The frequencies of the genotype GG and allele G in the rs1929992 SNP were higher, whereas the frequencies of the genotype AA and allele A were lesser in the PC group compared with the controls. Moreover, the rs1929992 SNP-related genotype GG and allele G were related to a greater risk for the development of PC. Therefore, the genotype GG and allele G of the rs1929992 SNP could be considered the risk factors for PC.

In our previous study, no significant association was observed between the rs1929992 SNP-related genotypes and alleles with breast cancer development [30]. Therefore, it seems that rs1929992 SNP may have different roles in various types of malignant diseases. We did not find more studies concerning the association of the rs1929992 SNP with malignant diseases. However, the association of the rs1929992 SNP with susceptibility to a number of inflammatory disorders including ischemic stroke [31], ankylosing spondylitis [32], coronary heart disease [33], and systemic lupus erythematosus [34, 35] was indicated.

How the rs1929992 SNP may increase the incidence of PC is not known. Our results indicate that the IL-33 levels did not significantly differ between individuals having different genotypes and alleles in rs1929992 SNP, neither in PC patients nor in the control group. Further studies need to clarify the mechanisms by which genetic variation at rs1929992 may affect the potential for the PC disease.

The rs1929992 SNP is located in the intron 3 region of the IL-33 gene and may regulate its expression. Previously, it was reported that the rs1929992 SNP may interfere with the IL-33 gene expression via affecting the activity of the promoter and enhancer regions, transcription and post-transcription process. The presence of the allele A may increase the expression of IL-33 by enhancing the binding of IL-33-inducing transcription factors. By contrast, allele G may reduce the IL-33 expression by inhibiting the binding of the transcription factors to the regulatory regions situated in the IL-33 gene [31].

In conclusion, higher serum IL-33 levels were found in patients with PC, especially in those with greater tumor stages which represents that this cytokine may contribute to PC progression. The rs1929992 SNP-related genotype GG and allele G were more prevalent in PC patients and were associated with a higher risk for the development of PC. However, the IL-33 levels were not influenced by genetic variability in rs1929992 SNP. The clinical values of the IL-33 and its related rs1929992 SNP in the PC diagnosis, progression, and prognosis need more consideration in future studies.

Disclosure. Authors declare that there is no conflict of interest.

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