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Serum concentration of interleukin-35 and its association with tumor stages and FOXP3 gene polymorphism in patients with prostate cancer

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ABSTRACT

IL-35 is an immunosuppressive cytokine that is largely synthesized by regulatory T (Treg) cells and may inhibit antitumor immune responses. This investigation aimed to determine the serum IL-35 concentrations and a single nucleotide polymorphism (SNP) in position of rs3761548, within the promoter region of *FOXP3 gene*, in patients with prostate cancer (PC). The blood specimens were obtained from 150 PC patients prior to using radiation therapy, chemo- or immunotherapy and 150 age-matched healthy men as a control group. The serum IL-35 concentrations and the pattern of genetic variation at position of rs3761548 were assessed using ELISA and polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP), respectively. The mean serum IL-35 concentrations were significantly higher in PC patients when compared with healthy control group (20.01 ± 7.03 Pg/mL vs. 11.60 ± 2.49 Pg/mL, $P < 0.001$). The serum IL-35 concentrations raised with progression of PC stages so that there was a significant difference between PC stages concerning the IL-35 concentrations ($P < 0.001$). The mean serum IL-35 concentrations in patients with Gleason scores of 1–6 and Gleason scores 7–10 were significantly higher as compared with healthy controls ($P < 0.001$). Moreover, the serum IL-35 concentrations in patients with having Gleason scores of 7–10 were significantly higher as compared with patients with Gleason scores of 1–6 ($P < 0.001$). Evaluation of the genetic variations in position SNP rs3761548 revealed that the AA genotype and A allele were more prevalent whereas CC genotype and C allele were less prevalent in PC patients when compared with healthy men ($P < 0.01$, $P < 0.001$, $P < 0.002$ and $P < 0.001$, respectively). The AA genotype and A allele were associated with higher risk of PC incidence [OR: 2.42 (95% CI: 1.179–4.99); $P < 0.001$ and OR: 1.732 (95% CI: 1.244 – 2.413); $P < 0.001$, respectively]. The mean serum IL-35 concentrations were significantly higher in total subjects (PC patients + healthy individuals) with AA genotype and A allele than individuals with CC genotype and C allele at SNP rs3761548 ($P < 0.05$ and $P < 0.01$, respectively). Higher serum IL-35 concentrations observed in patients with PC that were increased with progressive tumor stages. These findings indicate that the IL-35 is possibly involve in tumor progression. Moreover, SNP rs3761548 may affect the susceptibility to PC and the serum IL-35 concentrations.

1. Introduction

Prostate cancer (PC) is the most common malignant disease among men worldwide, and the third cause of cancer-related deaths in men from developed countries with a peak of incidence at age ~70 years [1,2]. The most recognized risk factors associated with PC include ethnicity, age, obesity, cigarette smoking, alcohol consumption, and so on [2]. The epidemiological data reveal that the genetic background and some gene polymorphisms have been linked with PC development [3].

According to the immunosurveillance concept, the tumor-derived antigens are recognized by the leukocytes and induce immune

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responses that lead to elimination of the cancerous cells. Therefore, the impairments in the immune system or inappropriate immune response play a principal role in tumorigenesis and tumor progression [4,5]. Various infiltrated cells of the leukocytes including macrophages, dendritic cells (DCs) and lymphocytes were observed in the PC tissue biopsies [6].

The effector $CD4^+$ T cells are classified into diverse subsets, which may exert pro- or anti-tumor activities [4]. For example, the Th1 cell-related cytokines (such as IFN-γ, IL-2 and IL-12) display powerful anti-tumorigenic influences by inducing natural killer (NK) and $CD8^+$ cytotoxic T lymphocytes (CTLs)-mediated cytotoxicity [4,7]. Conversely, Treg cells suppress the immune responses against tumor cells, thus promoting cancer progression [8].

Regulatory T (Treg) cells comprise 5–10% of the peripheral $CD4^+$ T lymphocytes that play a major role in the maintaining of the immunologic tolerance, preventing autoimmunity, and controlling the immune responses [9]. The increased numbers of the circulating Treg cells and the increased infiltration of Treg cells into tumor tissues were associated with poor prognosis in malignant patients with lung, breast, ovarian, colorectal, esophageal, renal and gastric cancers [10,11]. The Treg cells also act as terminators of T cell immune response during PC progression and development and a considerable association was found between the high number of Treg cells and poor prognosis in these patients [12,13].

The inhibitory activities of Treg cells are exerted via several mechanisms, especially by secreting immunosuppressive cytokines such as TGF-β, IL-10 and IL-35 [8,14]. It has been indicated that in addition to IL-10 or TGF-β, IL-35 was also required for Treg cell-mediated suppression [14]. IL-35 is a dimeric cytokine comprised of two subunits, including Epstein-Barr Virus (EBV)-induced gene 3 (EBI3) and P35 proteins, and is synthesized principally by $FOXP3^+$ Treg cells [14]. In addition, IL-35 is produced by a subset of $CD4^+$ Treg cells, named iTr35 cells [15]. IL-35 signals via a unique heterodimer or homodimers receptor composed of IL-12Rb2 and gp130 chains [16]. IL-35 also expands the $FOXP3^+$ Treg cells and potentiates the immunosuppressive properties of these cells [9,16]. IL-35 plays a key role in the tumor development, progression, metastasis and prognosis [17]. The results of a study in patients with breast cancer indicated that the elevated IL-35 expression in tumor-infiltrating lymphocytes (TIL) was associated with poor prognosis through promoting tumor immune evasion [18]. Using mice with IL-35 deficient in their Treg cells, it has been observed that Treg cells were the main producers of IL-35 within the tumor microenvironment where they display potent immunosuppressive activity that leads to the tumor immune evasion [19]. In mice models of melanoma and colon adenocarcinoma, IL-35 neutralization using specific monoclonal antibody reduced the tumor growth, limited the metastasis, intensified the specific anti-tumor responses of T cells, increased the $CD8^+$ T:Treg ratio in TIL, enhanced the long-term T cell memory to the tumor, and decreased the expression of suppressive molecules (including PD-1, TIM-3, LAG-3) on TILs [19].

The generation, maintenance and suppressor function of Treg cells are regulated by a master transcription factor, named Forkhead Box P3 (FOXP3) [14]. The continued FOXP3 expression is essential for the maintenance of the Treg cell-mediated tolerance [20]. The *FOXP3* gene is placed on the X chromosome, within the Xp11.23 locus, and encodes for FOXP3 protein [21]. There are several single nucleotide polymorphisms (SNPs) within the promoter, intron and exon regions of *FOXP3* gene [22]. The SNPs in the regulatory regions, especially in the promoter area, may change the binding of transcription elements to their binding sites and lead to enhancement or reduction of the transcription process [20,23]. The SNPs in the promoter region of the FOXP3 gene may influence the FOXP3 production and/or activity, thereby resulting in an immune deviation in cancers [14,21,24]. The SNP − 3279C/A (rs3761548) is among the SNPs placed in the promoter area of the FOXP3 gene [14]. This study aimed to investigate the serum IL-35 concentrations and the genetic variations at position of SNP rs3761548 in PC patients to explore possible associations.

2. Material and methods

2.1. Subjects

150 men with PC (age 65.07 ± 7.78 years) were recruited among patients who referred to the hospitals affiliated to Shiraz University of Medical Sciences (Shiraz, Iran) from January 2016 to August 2017. The PC patients were enrolled in the study prior to using radiation therapy, chemo- or immuno-therapy. The presence of PC was approved by expert Uro-oncologists according to the para-clinical, pathological and surgical reports. The PC staging was done based on the principles from the Sixth Edition of the American Joint Committee on Cancer (AJCC) [25]. Moreover, 150 age-matched healthy men who visited the health centers and hospitals affiliated to Shiraz University of Medical Sciences were enrolled in the study. The healthy men were in good health, without acute or chronic sickness, and none of them had a previous history of malignancy. Indeed, each person with cigarette smoking, medication and disorders such as history of recurrent infections, asthma, allergy and atopic illnesses, or any suspicious immunological diseases were all excluded from the present study. The other exclusion criteria were surgery and major trauma within the past 6 months, prior to blood collection. This study was evaluated and approved by the Ethics Committee of Kerman University of Medical Sciences and designated as IR.KMU.REC.1395.226. Moreover, all individuals were recruited after giving informed written consent. A specimen of the peripheral blood (5ml) was obtained from all participants and the serum samples were separated and stored at −70 °C until analyzed.

2.2. Genomic DNA extraction

The salting out technique was used for DNA separation from the peripheral blood leukocytes, as previously described by Miller et al. [26]. The DNA concentration and its purity was determined using a spectrophotometry system (Ependorf, Germany) according to the optical density at 260 and 280 nm wavelengths. The extracted DNA specimens were stored at −20 °C until using.

2.3. Polymorphism genotyping

The genetic variations at SNP rs3761548 in FOXP3 gene was determined by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) technique. The PCR reaction in a total volume of 25 μl was made: 1 μl of prepared 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 of 10 mM)], 1 μl of each primer, and sterile double-distilled water. The forward primer (5′-TAACCAGACAGCGTAGAAGG-3′) and reverse primer (5′-CAATACAGAGCCCATCATCA-3′) were used to amplify the region surrounding the SNP rs3761548. The amplification program was designed as follows: an initial denaturation stage (at 94 °C for 5 min) and followed by thirty cycles of denaturation (at 94 °C for 30 s), annealing (at 60 °C for 30 s) and extension (at 72 °C for 45 s). A final extension stage was also done (at 72 °C for 5 min) to terminate the PCR.

The amplified PCR product includes the SNP rs3761548 with a length of 503 bp (Fig. 1). The restriction enzyme Pst 1 (Fermentase, Finland) was used to detect the C–A transition. Therefore, the PCR products (5 μl) were digested overnight (at 37 °C) in the presence of 0.75 unit/reaction of Pst I restriction endonuclease. This enzyme has solely a restriction site in this area; thus, the fragment is digested into two 184 and 319-bp fragments following digestion. The CC genotype exhibits two DNA fragments of 184 and 319 bp, whereas the AA genotype shows a single DNA fragment without any digestion (503 bp) and

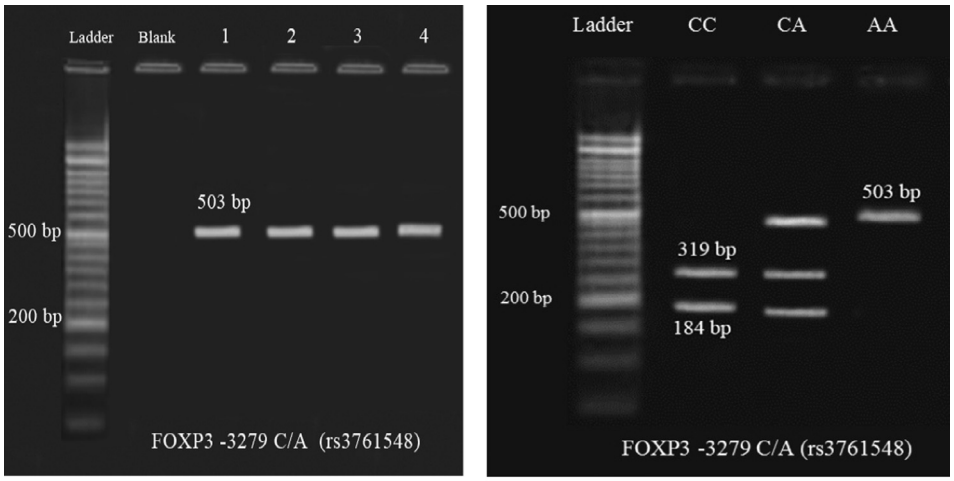

Fig. 1. (A) The amplified PCR product consist of SNP rs3761548 with a length of 503 bp. The first column shows a ladder pattern. The 2 column is blank. The 3, 4 and 5 columns show PCR product. (B) Determination of FOXP3 gene polymorphism at position of rs3761548 using PCR-RFLP method. The first column shows a ladder pattern. The 2, 3 and 4 columns represent CC, CA and AA genotypes, respectively.

the heterozygote CA genotype exhibits three DNA fragments (503, 319 and 184 bp). The enzyme-exposed products were electrophoresed on 3% agarose gel after adding 2 μl KBC Power load (Kowsar Biotech Co, Iran) and evaluated on a Chemi- Doc model XRS (Bio-Rad, USA) (Fig. 1).

2.4. Measurement of the IL-35 concentrations

The serum IL-35 concentrations were measured by commercial human IL-35 ELISA kits (Bioassay Technology, China) according to the manufacturer's instruction. The sensitivity of the assay was 0.047 Pg/mL.

2.5. Statistical analysis

Differences in variables were analyzed by using appropriate statistical tests, including ANOVA, *Student t* or $\chi 2$ and the P values of < 0.05 were regarded as statistically significant. Hardy–Weinberg equilibrium was estimated using genotype data. The Allele and genotype frequencies were calculated in participants by direct gene counting. The data were analyzed by a statistical SPSS software package (version 21, Chicago, IL, USA).

3. Results

3.1. General characteristics of the participants

The demographic characteristics and clinical data of the enrolled subjects are demonstrated in Table 1. The mean age of the PC patients was 65.07 ± 7.78 years and that of the control group was 63.94 ± 8.88 years (p = 0.24). The PC patients were distributed according to their tumor stages as follows: 16 patients were in stage I, 61 patients in stage II, 49 in stage III and 24 in stage IV. On the basis of the Gleason score, PC patients were again randomly classified into two groups including PC patients with Gleason score of 1–6 (n = 71) and those with Gleason score 7–10 (n = 79).

Table 1
Demographic and clinico-pathological characteristics for the enrolled prostate cancer patients.

Characteristic	Value	Number
Age (years)	65.07 ± 7.88	150
Tumor type	Acinar	86
	Adenocarcinoma	64
TNM stage	Stage I	16
	Stage II	61
	Stage III	49
	Stage IV	24
Tumor site	Left	26
	Right	19
	Multiple Lobes	61
	Unreported	44
Gleason score	1–6	71
	7–10	79

3.2. Serum IL-35 concentrations in PC patients and controls

The mean serum IL-35 concentrations in PC patients was significantly higher than those of the healthy control group (20.01 ± 7.03 Pg/mL vs. 11.60 ± 2.49 Pg/mL, p < 0.001) (Table 2). The serum IL-35 concentrations were 13.60 ± 6.31 Pg/mL in PC patients with stage I, 16.51 ± 4.88 Pg/mL in patients with stage II, 21.68 ± 5.13 Pg/mL in those with stage III, and 29.50 ± 4.78 Pg/mL in patients with stage IV. The serum IL-35 concentrations in patients with tumor stages I, II, III and IV were higher than those of the healthy control group (P = 0.08, P < 0.001, P < 0.001 and P < 0.001, respectively) (Table 2). The serum IL-35 concentrations increased with progression of tumor stages so that there were significant differences between PC stages concerning the serum IL-35 concentrations (P < 0.001) (Table 2 and Table 3).

Table 2
Serum levels of IL-35 in patients with PC according to their tumor stages.

Groups	Tumor stages	No.	IL-35 levels Mean ± SD	IL-35 levels Median (Min-Max)	P values
PC patients	I	16	13.60 ± 6.31	12.50 (8.65–34.70)	0.08*
	II	61	16.51 ± 4.88	15.82 (9.23–32.36)	0.001**
	III	49	21.68 ± 5.13	20.55 (13.40–32.42)	0.001***
	IV	24	29.50 ± 4.78	31.14 (14.06–34.66)	0.001****
	Total	150	20.01 ± 7.03	18.12 (8.65–34.70)	
Healthy group	-------	150	11.60 ± 2.49	11.57 (7.43 – 16.63)	†0.001

• The serum levels of cytokine expressed as Pg/mL. † Represent the difference of the serum levels of IL-35 between total patients with PC and healthy control group. *, **, *** and **** represent the differences of the serum levels of IL-35 between patients in stages I, II, III and IV with healthy control group.

Table 3
Statistical comparison of serum IL-35 levels N between patients with PC according to their stages.

Stages	I	II	III	IV
I	---------	P = 0.240	P < 0.001	P < 0.001
II	P = 0.240	---------	P < 0.001	P < 0.001
III	P < 0.001	P < 0.001	---------	P < 0.001
IV	P < 0.001	P < 0.001	P < 0.001	---------

The serum levels of IL-35 increased with advancing of tumor stages so that there was significant difference between cancer stages regarding the mean serum levels of IL-35.

Table 4
Serum levels of IL-35 in patients with PC according to their Gleason score (GS).

Groups	GS	No.	IL-35 levels Mean ± SD	IL-35 levels Median (Min-Max)	P values
PC patients	1–6	71	18.25 ± 6.71	16.49 (8.65–33.01)	0.003*
	7–10	79	21.83 ± 6.94	20.47 (10.50–34.70)	0.001**
	Total	150	20.01 ± 7.03	18.12 (8.65 – 34.70)	0.001***
Healthy group	-------	150	11.60 ± 2.49	11.57 (7.43–16.63)	0.001†

Symbols *, ** and *** represent the p values regarding the differences between patients having Gleason score 1–6 and patients having Gleason score 7–10, between patients having Gleason score 1–6 and control group and between patients having Gleason score 7–10 and control group, respectively.† Represent the difference of the serum levels of IL-35 between total patients with PC and healthy control group.

The serum IL-35 concentrations according to the PC Gleason score are shown in Table 4. The mean serum IL-35 concentrations in patients with Gleason scores of 1–6 and 7–10 were significantly higher in comparison with healthy individuals (P < 0.001). Moreover, the mean serum IL-35 concentrations in PC patients with Gleason scores of 7–10 were significantly higher as compared with patients who had Gleason scores of 1–6 (P < 0.001) (Table 4).

3.3. Genotype and allele frequencies of FOXP3 SNP rs3761548 in PC and healthy control groups

As shown in Table 5, the prevalence of CC, CA and AA genotypes in position of SNP rs3761548 in the FOXP3 gene were 21.3%, 48.7% and 30% in the healthy men and 8.7%, 48% and 43.3% in PC patients, respectively. There was a significant difference between healthy controls and PC patients as to the genotype distribution in SNP rs3761548 (P < 0.003). The AA genotype was more prevalent whereas CC genotype was less prevalent in PC patients when compared with healthy men (P < 0.01 and P < 0.002, respectively). When we used the CC genotype as a reference, both AC and AA genotypes were associated with a higher risk of PC development [OR: 2.42 (95% CI: 1.179–4.99) and OR: 3.55 (95% CI: 1.682–7.515), respectively]. Similarly, the prevalence of A allele was higher whereas the prevalence of C allele was lower in PC patients in comparison with the healthy group (P < 0.001). When we used the C allele as a reference, the A allele was associated with higher risk of PC development [OR: 1.732 (95% CI: 1.244–2.413)] (Table 5).

3.4. The serum IL-35 concentrations according to the genetic variations at SNP rs3761548

The association of the IL-35 concentrations with genetic diversities at SNP rs3761548 is shown in Table 6. In both PC patients and healthy control group, no significant differences were observed in the participants with CC, CA and AA genotypes or in individuals with C and A alleles at SNP rs3761548 with respect to the mean serum IL-35 concentrations. However, in all participants (PC patients plus healthy subjects) the IL-35 concentrations in persons with AA genotype and A allele were significantly higher than in individuals with CC genotype and C allele at SNP rs3761548 (P < 0.05 and P < 0.01, respectively) (Table 6).

The comparison of the serum IL-35 concentrations between the PC patients and healthy control group according to genetic diversities at SNP rs3761548 is demonstrated in Table 7. In PC patients with genotypes CC, CA, and AA or alleles A and C at rs3761548 in FOXP3 gene, the serum IL-35 concentrations were significantly higher than healthy subjects with the same genotypes and alleles (Table 7).

4. Discussion

IL-35 is an important anti-inflammatory cytokine which plays a considerable role in the immunoregulation process [14]. The results of the present study demonstrated that the serum IL-35 concentrations in PC patients were significantly higher than healthy individuals. These results suggest an association between elevated IL-35 concentrations and PC development. In accordance with our results, it has been recently indicated that the plasma amounts of IL-35 in PC patients were higher than those with non-malignant disorders of prostate [27]. Moreover, the plasma amounts of IL-35 in patients with advanced PC were higher than those with localized PC [27]. Elevated levels of IL-35 were also indicated in a number of other malignant diseases such as lung cancer [28], AML [29] and colorectal cancer [30]. It has been reported that IL-35 is produced by infiltrated intra-tumor Treg cells that reduce antitumor T cell-mediated immunity. Indeed, IL-35 increases the tumor growth through inducing the expression of inhibitory molecules such as PD1, TIM3, and LAG3, which leads to the impairment of the T cell functions in the tumor environment [19]. Moreover, IL-35 increases the myeloid-derived suppressor cell (MDSC) accumulation in the tumor and enhances the angiogenesis [31]. The MDSCs exhibit tumorigenic activities through several mechanisms such as production of IL-10 and TGF-β1 that inhibits the T cells and NK cells, production of cytokines that skew the immune reactions towards Treg cell type, secretion of angiogenic factors such as VEGF that promotes the tumor

Table 5
The frequencies of genotypes and alleles at SNP rs3761548 in FOXP3 gene in patients with prostate cancer and healthy control group.

FoxP3 – 3279C/A (rs3761548)		Patients No. (%)	Controls No. (%)	P value	Odds ratio (OR)	(95% Confidence interval of OR)	P value
Genotypes	CC	13 (8.7%)	32 (21.3%)	0.003	1	Reference	------
	AC	72 (48.0%)	73 (48.7%)		2.42	(1.179–4.99)	0.016
	AA	65 (43.3%)	45 (30.0%)		3.55	(1.682 – 7.515)	0.001
Alleles	C	98 (32.66%)	137 (45.66%)	0.001	1	Reference	-----
	A	202 (67.33%)	163 (54.33%)		1.732	(1.244 – 2.413)	0.001

The AA genotype and A allele was more prevalent, whereas CC genotype and C allele was less prevalent in PC patients when compared with healthy men (P < 0.01, 0.001, P < 0.002 and P < 0.001, respectively). Both AC and AA genotypes and A allele were associated with a higher risk of PC development.

Table 6
Serum levels of IL-35 in PC and healthy groups according to the genotypes of FOXP3 gene polymorphisms at rs3761548.

Groups	Genotype/Allele	IL-35 levels Mean ± SD	IL-35 levels Median (Min-Max)	P value
PC patients	CC	19.50 ± 7.87	16.36 (10.51–32.36)	0.15
	AC	18.91 ± 6.33	17.49 (8.65–33.01)	
	AA	21.36 ± 7.53	19.26 (10.34–34.70)	
	C	18.99 ± 6.49	17.28 (8.65–33.01)	0.29
	A	20.05 ± 7.00	18.28 (8.65–34.70)	
Healthy subjects	CC	12.22 ± 2.38	12.29 (7.82–15.63)	0.18
	AC	11.90 ± 2.34	11.56 (7.87–16.30)	
	AA	10.59 ± 2.69	9.73 (7.43–16.63)	
	C	12.02 ± 2.32	12.10 (7.82–16.30)	0.10
	A	11.37 ± 2.53	10.94 (7.43–16.63)	
Total participants	CC	15.38 ± 6.48	13.99 (7.82–32.36)	0.05
	CA	17.20 ± 6.37	15.66 (7.87–33.01)	
	AA	19.23 ± 8.09	17.38 (7.43–34.70)	
	C	16.81 ± 6.41	14.92 (7.82–33.01)	0.01
	A	18.12 ± 7.24	16.25 (7.43–34.70)	

• The serum levels of IL-35 expressed as Pg/mL.
In all participants (PC patients plus controls) the IL-35 levels in persons with AA genotype and A allele were higher than in individuals with CC genotype and C allele at SNP rs3761548 ($P < 0.05$ and $P < 0.01$, respectively).

Table 7
Serum levels of IL-35 in prostate cancer and healthy groups according to the genotypes of FOXP3 gene polymorphisms at rs3761548.

IL-35 (Mean ± SD)		Healthy group	PC patients	p-value
Genotypes	CC	12.22 ± 2.38	19.50 ± 7.87	0.017
	CA	11.90 ± 2.34	18.91 ± 6.33	0.001
	AA	10.59 ± 2.69	21.36 ± 7.53	0.001
Alleles	C	12.02 ± 2.32	18.99 ± 6.49	0.001
	A	11.37 ± 2.53	20.05 ± 7.00	0.001

In PC patients with genotypes CC, CA, and AA or alleles A and C, the serum IL-35 concentrations were higher than controls with same genotypes and alleles at rs3761548.

neovascularization, and production of matrix metalloproteinases [32]. IL-35 can also induce the differentiation of a subgroup of iTreg cells, named iTr35, which in turn inhibit the effector T-cell proliferation through the IL-35 production [33]. A subpopulation of $CD8^+$ $CTLA\text{-}4^+$ IL-35-secreting Treg cells was also demonstrated in some patients with PC that inhibits the immune response against tumor-associated antigens in an IL-35-dependent manner [34]. IL-35 may play an essential role in the PC development through contributing to T cell dysfunction and limiting anti-tumor immunity. Experimentally, in mouse models of melanoma and colon adenocarcinoma, it has been demonstrated that neutralization of IL-35 using monoclonal antibody significantly decreases the tumor growth and increases the anti-tumor immunity mechanisms [19]. Therefore, IL-35 targeting may be a favorable therapeutic potential for treatment of human cancers (including PC); this should be considered in future studies.

The results of this study also indicate that the serum IL-35 concentrations are increased with advancement of tumor stages. The serum IL-35 concentrations in PC patients with Gleason scores of 1–6 and in those with Gleason scores of 7–10 were significantly higher than the healthy control group. Moreover, the serum IL-35 concentrations in PC patients with Gleason scores of 7–10 were significantly higher in comparison with patients who had Gleason scores of 1–6. These findings indicate that the IL-35 may play prominent functions in the tumor growth and progression. In the same line with our results, elevated serum IL-35 concentrations or increased expression of IL-35 in tumor tissues, have been indicated in higher stages of breast cancer [19], colorectal cancer [30], non-small cell lung cancer [28], pancreatic ductal adenocarcinoma [35], and renal cell carcinoma [36]. Moreover, elevated serum IL-35 concentrations and overexpressed IL-35 in tumor biopsies were associated with more immunosuppression and regarded as an unfavorable prognostic factor for aforementioned malignant tumors. However, Zhou et al. [27] did not find a significant relationship between plasma levels of IL-35 and Gleason scores, which may be attributed to the small sample size in that investigation.

The reasons for the elevated levels of IL-35 in PC patients remain to be clarified in future investigations. In patients with colorectal cancer, the serum IL-35 concentrations were significantly associated with the number of peripheral Treg cells [30]. In addition to Treg cells, colorectal cancer cells also generate high amounts of IL-35, which recruit more Treg cells in the tumor microenvironment and facilitate tumor progression [30]. Furthermore, tumor infiltrating DCs also express a subunit of IL-35, EBI3 [37]. Collectively, Treg cells, tumor infiltrating DCs and cancer cells may be responsible for the elevated IL-35 levels in patients with malignant diseases such as PC.

The data from this study also demonstrated for the first time an association between SNP rs3761548 with PC. The PC patients had higher AA genotype and A allele in position SNP rs3761548 than healthy group, while the prevalence of CC genotype and C allele in the PC group was lower healthy men. Moreover, the existence of AA genotype and A allele in position rs3761548 was linked with a higher risk of PC development. Accordingly, the SNP rs3761548 may have an association with susceptibility to PC disease. There is no study on the association of the SNP rs3761548 with PC. However, in agreement with our results, a considerable relationship was also reported between the existence of AA genotype or A allele at rs3761548 in the FOXP3 gene and a number of malignancies such as breast cancer [24], colorectal cancer [38] non-small cell lung cancer [39] and thyroid cancer [15].

It has been also indicated that FOXP3 acts as an important tumor suppressor in breast cancer [40,41]. In the normal mammary cells, the wild type of FOXP3 is bound to *HER-2* and *SKP2* oncogene and represses them. In cancerous cells, however, the mutated FOXP3 does not exert suppressor function [40]. Similarly, in PC, FOXP3 is unable to repress an oncogene c-MYC. This leads to the prostatic hyperplasia and PC development [40,41]. In contrast to neoplastic cells, the normal prostate cells proliferate at a low rate and express low amounts of c-MYC [40]. Therefore, the presence of the AA genotype and A allele at rs3761548 in the *FOXP3* gene may reduce its expression and thus the oncogene c-MYC is activated. The presence of the AA genotype at SNP rs3761548 may cause defects in binding of some transcription elements to the promoter region of the FOXP3 gene, thus leading to defective gene transcription [20]. Further studies are required to clarify these speculations.

The results of the present study also indicated that in all participants (PC patients plus healthy subjects) the levels of IL-35 in persons with

AA genotype and A allele were significantly higher than the individuals with CC genotype and C allele at SNP rs3761548. Therefore, SNP rs3761548 may also probably contribute to the PC development through influencing the cytokine production such as IL-35. The SNP/rs3761548 is a functional polymorphism because it is placed in the promoter region of the FOXP3 gene [39]. The unusual expression of a gene may contribute to the development of diseases. Indeed, the SNP rs3761548 is placed in the core 'GGGCGG' sequence of the commonly accepted binding site for a transcription element that is called specificity protein 1 (Sp1) [42]. The SNP rs3761548 can influence the interaction of Sp1 with the promoter of *FOXP3* gene [42]. Thus, the presence of the genotypes AA, AC and CC or alleles A and C in rs3761548 may differentially affect the FOXP3 expression through changing the interaction of Sp1 with the *FOXP3* promoter. Accordingly, the Treg cell functions may differ in individuals carrying different genotypes and alleles at SNP rs3761548. Whether the SNP rs223818 directly exerts functional effects on the FOXP3 and IL-35 expression or whether it is linked with another operative SNP remains to be clarified. The SNP rs223818 may directly or indirectly alter the IL-35 expression and contribute to tumor development. It has been found that rs3761548 influences the alloimmune responses of the $CD4^+$ T cells during acute rejection after liver transplantation; however, no association was found between the frequency of Treg cells and the mentioned SNP [43].

In addition to the immunoregulatory effects, IL-35 may also directly influence the tumor cell growth. Our findings provide a powerful rationale for future studies to prove the exact role IL-35 in the PC progression and development. Future investigations are warranted to elucidate the effects of IL-35 on the tumor cell growth and apoptosis using different concentrations of IL-35 in different types of PC cells. We suggest more studies to evaluate the IL-35 effects on the expression of the cell proliferation-related genes (such as cyclin B, cyclin D and P27), the expression of anti-apoptotic molecules (such as Bcl-2 and survivin) and the expression of pro-apoptotic molecules (for example Fas, FasL, Bax and TRAIL). The effects of the IL-35 blockade using neutralizing monoclonal antibodies and the inhibition of the gene expression of IL-35 and/or its receptor using specific siRNA on the PC development also need to be considered in future studies.

In summary, higher serum IL-35 concentrations were observed in PC patients that are enhanced with progressive tumor stages. These findings indicate that the IL-35 is possibly involved in tumor progression. Moreover, our data represent that the SNP rs3761548 may affect the susceptibility to PC and the serum IL-35 concentration. The clinical value of the IL-35, as a biomarker for assessment of the PC progression and prognosis, needs to be considered in further studies.

5. Disclosures

There is no conflict of interest.

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