

Frequency of IL-10+CD19+ B cells in patients with prostate cancer compared to patients with benign prostatic hyperplasia

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Abstract

Background: The function of the immune system in prostate cancer (PC) might promote carcinogenesis. PC is a common cancer in men. Regulatory B cells (Bregs) are a new subtype of B cells that have suppressive roles in the immune system. Interleukin-10 (IL-10) is a dominant mediator of immune suppression released by Bregs.

Objective: The purpose of this research was to examine the frequency of CD19+IL10+ B cells and IL-10 mRNA expression in patients with PC compared to patients with benign prostatic hyperplasia (BPH).

Methods: Forty paraffin tissue samples from patients with PC and 32 paraffin tissue samples from patients with BPH were entered in this study. The immunohistochemistry staining was used to evaluate the pattern expression of CD19 and IL-10 markers. IL-10 mRNA expression in fresh tissue was determined by real time-polymerase chain reaction (RT-PCR).

Results: The frequency of CD19+IL-10+ B cells and IL-10 mRNA expression in PC patients were significantly higher than patients with BPH. Also, there was no meaningful relationship between the frequency of IL-10+CD19+ B cells and gleason scores in patients with PC.

Conclusions: Our findings suggested that frequency of IL-10+CD19+ B cells correlates with progressive stage of PC.

Keywords: Prostate cancer; benign prostatic hyperplasia; IL-10+CD19+ B cells.

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Introduction

Prostate cancer (PC) is the second most prevalent type of male cancer-related death all over the world. In the tu-

mor microenvironment (TME), the complex cellular interaction of immune cells is involved in progression of^{1,2}. Recently, multiple diagnostic approaches including typical cancer staging (TNM), histological characteristics of a prostate biopsy and prostate specific antigen (PSA) levels are considered to distinguish PC³. The immune system can promote cancer development⁴. Likewise, specific immune cells exclusively release mediators in TME, leading to immune system suppression⁵⁻⁷. There may be prognostic markers in identifying tumor promoting immune cells in patients with PCa⁴. Recently, the performance of regulatory B cells (Bregs) in immunosuppression has raised researchers' attention as a key novel cell in cancer development. In regard to the function, Bregs are highly sim-

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ilar to T regulatory cells (Tregs) and myeloid suppressive cells (MSC)⁸⁻¹⁰. Katz et al in 1974 recognized a different B cell subset, which delayed hypersensitivity on adoptive transfer¹¹. Bregs decrease has been shown to exacerbate the signs of disease in autoimmune diseases¹², chronic inflammatory diseases¹³, chronic inflammatory disease, or increase tumor growth¹⁴. CD19+CD24hiCD38hi¹⁵ and CD19+IL-10+16 were extensively considered as markers for Bregs in human. Bregs can arrest the T-cell-mediated inflammatory response mainly via secreting IL-10. In regulating human cGVHD, the secretion of IL-10 in B cells within the CD24hiCD27+ and CD27 high CD38 high markers is essential¹⁶. IL-10-producing B cells can transform effector T cells to IL-10-producing suppressive T cells for a longer period of time through interaction with CD4+CD25- T cells^{17,18}. It has been shown that CD19-/- significantly enhances the function of T-cells¹⁹. The high presence of CD5+CD19+IL-10+ Bregs in patients' peripheral blood indicated that Bregs play an important role in carcinogenesis growth and prognosis²⁰.

This research aimed at investigating the frequency of CD19+IL10+ B cells and IL-10 mRNA expression in PC patients compared to patients with benign prostatic hyperplasia (BPH) and their correlation with Gleason score.

Material and methods

Study population

The present study included all 72 patients diagnosed with biopsy obtained during prostatectomy at the Kashani hospital in Shahrekord. Before the study, none of the patients received radiotherapy, radiation or other medical interventions. Patients with concurrent malignancies, autoimmune diseases or immune defects were excluded from the study. The control group was selected from the benign prostatic hyperplasia (BPH) from the patients who were undergone open prostatectomy for PC screening. The mean age was 65.63 ± 14.42 . Data on age is expressed mean \pm SD and other data are reported as percentages.

Patients and tissue samples

The suitable Institutional Ethic Committees of the Shahrekord University of Medical Sciences approved the use of human samples for this study. All patients gave written informed consent. Forty two patients with PC underwent radical prostatectomy and 32 patients with BPH underwent open prostatectomy. Besides, 20 fresh tissue

samples of both PC and BPH were obtained for RNA extraction. Clinical information such as age, grade and stage were collected from medical files, and the names of participants remained confidential. The initial diagnosis of the probability of prostate cancer was checked using radiology photos, serum levels of prostate-specific antigen (PSA), and digital rectum exam (DRE) by a urologist. Then after obtaining samples by prostatectomy, the samples were transferred to the pathology lab and a definitive diagnosis was made about the health or malignancy of the tissues. Also, in case of malignancy, low grade and high grade samples were detected and reported by pathologist. The histological grade and stage of PC was classified based on the Gleason system (GS Intermediate: <7; GS Progressive: >7)^{21,22}. GS is a grading system that is determined by adding the most and second most common patterns ranging from 2 to 10²².

Immunohistochemistry staining

Radical prostatectomy samples were cut and parts of PC specimens were first embedded in 10% formalin buffered and then embedded in paraffin. The paraffin blocks were cut into 4 μ m thick pieces with microtomes. For immunohistochemical staining, 4- μ m serial sections were made and spread on poly-L-lysine-coated slides. Paraffin parts were dried and warmed in a 68°C oven overnight. The parts were then deparaffinized using three xylene modifications and rehydrated using a sequence of alcohols (100%, 100%, 80% and 70%). For antigen retrieval the parts were inundated in citrate buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) and subjected to autoclaving pressure and heat for 20 minutes to reveal concealed antigens for improved adhesion to antibodies in the next stages. In the next step protein blocks were added to the sections (ab94665, Abcam, UK) in order to block and hinder nonspecific background staining and were incubated for 2 hours. After washing the parts with distilled water, Anti-human CD19 mouse antibody (ab31947, ebioscience, UK) at a 1:200 dilution, anti-human IL-10 Rabbit antibody (ab34843, Abcam, UK) at a 1:400 dilution was applied. Afterwards, the parts were then put overnight in a suitable moisture chamber at 4 °C. The next day, after rinsing the slides in TBST buffer, 3% H₂O₂ in TBS for 15 min was applied to parts for preventing endogenous peroxidase activity. Later, Biotinylated goat anti-rabbit and mouse IgG (ab93697, Abcam, UK) which is also called secondary antibody was added and the sections were in-

cubated for 1 hour at room temperature in moist chamber. After the incubation time, the sections were incubated with Streptavidin Peroxidase Plus at room temperature for 10 minutes. Next, the chromogenic segment called 3-diaminobenzidine tetrahydrochloride (DAB) was incubated for 10 minutes (ab94665, Abcam, UK). After washing with the TBST buffer, parts were placed with Meyer's hematoxyline for 1 minute and washed with cold water and mounted. Human tonsil tissue was used as positive control for CD19 and IL-10. Sections without any primary antibody were considered as negative control. The numbers of CD19 and IL-10 cells were determined in 10 high-power fields by calculating positive cells in the entire tissue area. Outcomes were expressed as the mean value of all tested patients in any group.

RNA extraction and Real-time PCR (RT-PCR)

Twenty fresh tissue biopsies containing 10 PC and 10 BPH samples were taken from radical prostatectomy and open prostatectomy. Before analysis, specimens were held at -70 ° C. TRIzol ® Plus RNA reagent extracted the total RNA. The concentration of each RNA sample was calculated by Thermo Scientific TM NanoDrop 2000. Complementary DNAs (cDNA) were produced with reverse transcriptase (RT) using the First Strand cDNA Synthesis Kit (Fermentas Life Sciences, cat- K1622) with 2.5 µg RNA at a 20 µL reaction volume. The amplification of IL-10 cDNA in a Rotorgene 3000 (Corbett Life Science) was performed using the SYBR Green RT-PCR method. The primer sequences of IL-10 applicated in the RT-PCR include: IL-10 5`-TCAAGGCGCATGTGAACTC-3` (Forward), 5`-CGGCCTTGCTCTTGTTC-3` (Reverse) and GAPDH 5`-TTCACCACCATGGAGAAGGC-3` (Forward), 5`-CCCTTTTGGCTCCACCCT-3` (Reverse) as internal control (housekeeping genes) was used. Also, each sample was accomplished in duplicate. RT-PCR reactions were performed in a total volume of 20 µl containing of 1 µL diluted cDNA, 1 µL of each oligonucleotide primers, 7 µL nuclease free H2O and 10 µL

2X SYBR Green Master Mix. Negative control samples were constructed by deleting the diluted cDNA from the reaction mixture. RT-PCR was started with a 5-minute denaturation stage at 95 ° C and next in 35 amplification cycles that each cycle was included of 95°C denaturation at 10s, 60°C annealing at 20s, and 72°C extension at 30s. At the melting step, which is the last stage quality of examination was determined.

Statistical analysis

Analysis was carried out in duplicate and all data were shown as mean ± SD. The usual data distribution was validated by the normality check of Shapiro-Wilk. T test (To compare the two samples) or Tukey post hoc test (for multiple comparisons) evaluated quantitative data, comparing diseased groups with a different GraphPad Prism software version 8 control group (GraphPad Software, La Jolla, CA, USA). Pearson's parametric information association analysis was used to determine the relationship between variables. The relationship between gene expression levels and PC existence was evaluated using a multivariate logistic regression model. Where the P-value was less than 0.05, statistical significance was considered.

Results

Increased expression of IL-10 in PC (tumor tissues) compared with BPH

In our analysis, real-time PCR of 20 PC samples and 20 BPH samples assessed the mRNA expression of IL-10. The IL-10 mRNA was significantly higher in PC patients compared with BPH group (Fig. 1A; P < 0.0001).). If the mean of IL-10 expression in tumor tissue is 4.80 and the mean of IL-10 expression in non-tumoral tissue is 1.35 then the difference in the IL-10 expression in tumor tissue compared with non-tumoral tissue is 3.55-fold (4.08/1.35). The IL-10 gene expression in both intermediate and progressive groups of PC was more than that in the BPH group; however, there was no significant difference between intermediate and progressive groups (Fig. 1).

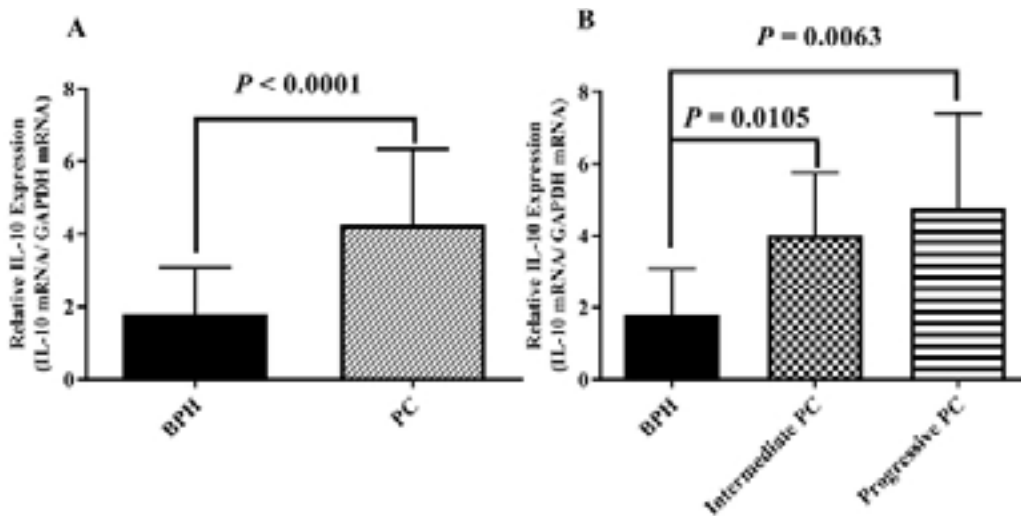


Figure 1. IL-10 mRNA level was evaluated in radical prostatectomy specimens. A) IL-10 gene expression in 20 PC samples and 20 BPH samples was shown. The real-time PCR results were standardized for human IL-10 versus GAPDH. B) In prostatectomy samples, the gene IL-10 was significantly over-expressed in intermediate and progressive PC patients rather than BPH. P-values less than 0.05 is statistically significant. PC: Prostate Cancer; BPH: benign prostatic hyperplasia.

The frequency of CD19+IL-10+ B cells was increased in prostatectomy tissue of PC

The prostatectomy tissues were immunostained with anti-CD19 and anti-IL-10 antibodies to detect Breg cells in samples (Fig.2). We observed that there is a significant increase in the frequency of CD19+IL-10+ B cells PC tissues than BPH tissues (Fig.3A; P =0.0014). Also, the number of CD19+IL-10+ B cells in intermediate and

progressive groups of PC was higher than the BPH group (Fig.3B; P = 0.0258 and 0.0225, respectively). The presence of PCs was positively correlated with the high frequency of IL-10 and CD19+IL-10+ B cells in multivariable logistic regression analysis. (P = 0.033, 0.014) (Table 2). Per 1 unite rise in frequency of CD19+IL-10+ B cells, the possibility of PC development was 1.34 times higher than the control group.

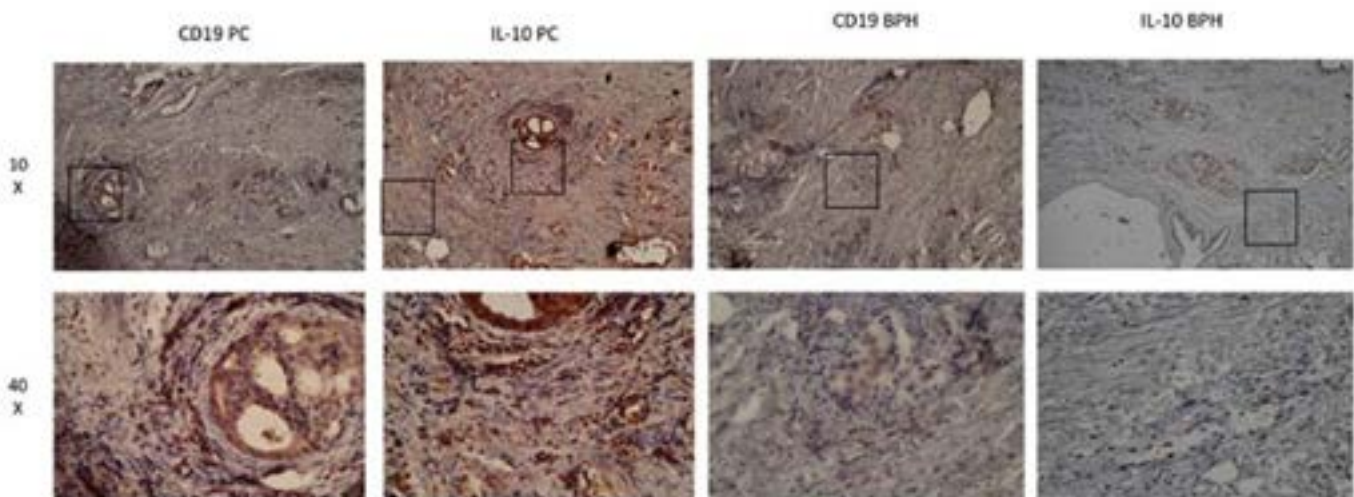


Figure 2. Assessment of prostatectomy tissues in immunohistochemistry for CD19 and IL-10 markers. Formalin-fixed and paraffin-embedded were stained with anti-CD19 and anti-IL-10 antibodies. Sequence sections of tissue were used to immunostaining CD19 and IL-10. CD19+IL-10+ B cells dominantly appear in the PC tissues.

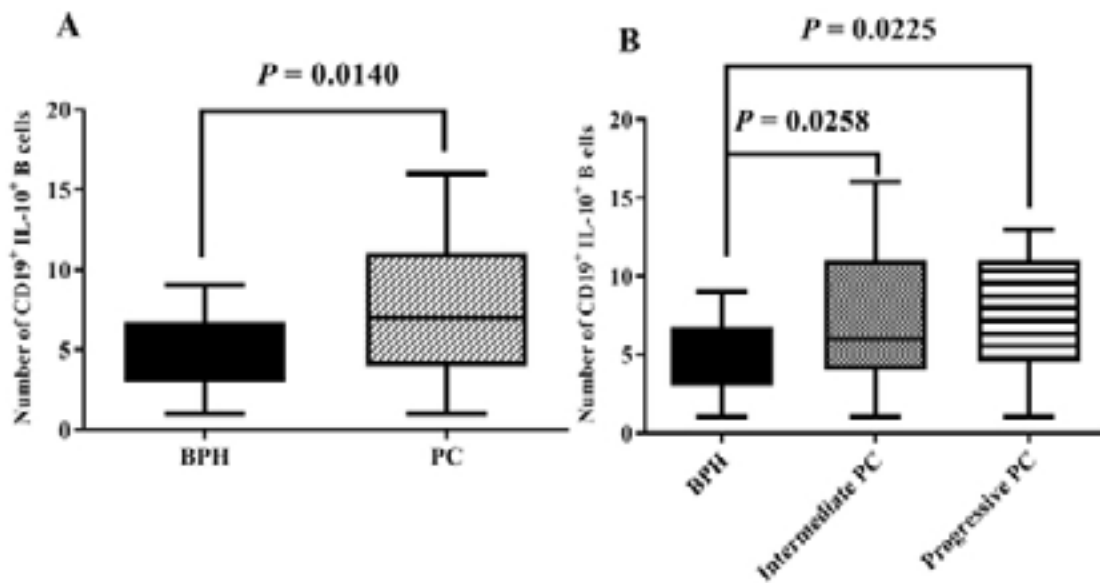


Figure 3. The frequency of CD19+IL-10+ B cells is shown. The number of CD19+IL-10+ B cells in the PC group was 1.5 higher than BPH group. P-values less than 0.05 is statistically significant. PC: Prostate Cancer; BPH: benign prostatic hyperplasia.

Table 1. Clinical and pathological characteristics

	All (n= 72)
FFPE samples PCa/BPH	72-40/32
Fresh samples PCa/BPH	20-10/10
Mean age \pm SD, years PCa/BPH	77.38/73.61 (p-value was not significant)
≤ 70 y	25/72
>70 y	47/72
Pathologic Gleason score	
<7	14/42
≥ 7	28 /42
Median PSA (ng/mL)	
<4 ng/ml	16/72
4-10 ng/ml	29/72
>4 ng/ml	27/72

PCa, prostate cancer; BPH, benign prostate hyperplasia; FFPE, Formalin-fixed paraffin embedded; PSA, prostate-specific antigen

Table 2. Coefficients of the logistic regression analysis for the existence of PC.

	Regression coefficient (β)	Standard error	P-value	95% C.I for Eep (B)	
				Lower	Upper
Breg cell	1.342922	0.1861459	0.033*	0.0231172	0.5665123
Frequency of IL-10	1.586061	0.2976891	0.014*	0.0933858	0.8291207
Frequency of CD19	1.086774	0.1608512	0.574	-0.2068765	0.3733041

* The presence of PC was significantly correlated with high level of IL-10 and Breg

Discussion

PC is one of the most generally diagnosed cancers among men all over the world, and mortality has been on the rise²³. The effects of immunotherapeutic strategies are dependent on interaction of tumor cells and host immune response²⁴. The lymphocytes that are infiltrated in tumor tissue have the potential to be used as the positive prognostic relation in cancer. An analysis of 50 high-grade PC transurethral prostate samples found that the rate of manually counted CD20+ B cells in the PC was significantly higher than in the control group²⁵. This investigation probably indicates that B cells may have an effective role in cancer development tumor genesis process. Another study showed that B cells can generate different cytokines such as lymphotoxin, which is able to activate signaling pathways in tumor cells of patients with castration-resistant prostate cancer (CRPC)²⁶. Shimabukuro-Vornhagen et al. suggested that a decrease in the number of B cells in colorectal cancer and an enhanced proportion of Bregs that contribute to metastasis may represent immune escape²⁷.

Several studies on various lymphoid malignancies in humans have shown that malignant B cells can act as Bregs that repress anti-tumor immune responses with their suppressive ligands^{28,29}. Overall, these studies on B cells can indicate that these cells have regulatory properties and have a direct effect on cancer growth³⁰. Our results

demonstrated that the frequency of CD19+IL-10+ B cells and IL-10 gene expression in PC patients were significantly higher than the patients with benign prostatic. In addition, the exponential rate of logistical regression showed that CD19+IL-10+ B cells and IL-10 cytokine could play a role in maintaining prostate cancer. Our findings suggest that the PC environment plays an important role in the induction of CD19+IL-10+ B cells differentiation or infiltration, which is confirmed by breast cancer cell studies that these cells can provoke Bregs expression in mice spleens³¹. According to available data, IL-10+CD19+ B cells accumulate in tumor areas and peri-tumor environment³². An immunohistochemical study on radical prostatectomy indicated the high frequency of B cell in malignant tissue against benign tissue³³. These findings suggest that B lymphocytes are critical in response to antitumor immunity and can differentiation to Bregs. PC microenvironment is more likely to be immunosuppressive³⁴. Low cytolytic activity of NK cells was detected in prostate tumor milieu³⁵. The high secretion of transforming growth factor beta (TGF- β) by prostate tissue inhibits NK cells and lymphocytes function and migration³⁶. Furthermore, the agglomeration of Tregs and Th17 lymphocytes down-regulates antitumor immunity in PC^{37,38}. A research by Zhou et al. found that factors released by lung cancer and infiltrated immune cells can play a significant role in Breg cell growth and the extent of inflammation³⁹. Our results presumably have

demonstrated that the expression of IL-10 has a relationship with the poor prognosis of cancer. IL-10 is an important mediator of immune suppression by Bregs⁴⁰. A recent study of tongue squamous cell carcinoma (TSCC) found that IL-10+CD19+ B cells played an important role in transforming CD4+ T cells into Tregs via IL-10 secretion and worse prognosis in patients with TSCC³². There are indications that Bregs can produce Tregs via cell contact and IL-10 secretion⁴⁰. Also, the study showed that Bregs suppressor is IL-10, CD80, and CD86 dependent, but not TGF- β in humans⁹, which was constant with our conclusions that IL-10 is critical cytokine in cancer. An analysis on the animal model of breast cancer, however, found that the conversion of CD4+ T cells to Tregs was associated with increased TGF- β levels rather than IL-10³¹. Perhaps IL-10+CD19+ B cells through IL-10 are capable of suppressing immune cells in tumor microenvironment and promote metastasis through interactions with Tregs. Our findings suggested that an enhanced frequency of IL-10+CD19+ B cells may promote tumor progression in patients with PC through the secretion of anti-inflammatory mediators. Altogether, the use of CD19+IL-10+ B cells in cancer treatment may be much welcomed in the future, especially in the field of immune response to tumors.

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Ethical approval

The ethical board of the University Of Medical Sciences Of Shahrekord authorized this research with the number: IR.Skums. REC.1397.63.

Conflict of interest

This manuscript was approved by all authors. No competing interests declared.

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