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Original Article

Upregulation of Cytotoxic T-Lymphocyte-Associated Protein 4 and Forkhead Box P3 Transcripts in Peripheral Blood of Patients with Bladder Cancer

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Abstract

Background: Regulatory T cells (Tregs) play a key role in the progression of tumors. These cells express forkhead box P3 (FOXP3) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4), which are the potential targets for cancer immunotherapy. The present study aimed to evaluate *FOXP3* and *CTLA4* transcripts in patients with bladder cancer (BC) compared with healthy individuals.

Methods: Transcripts of *CTLA4* and *FOXP3* genes in the peripheral blood mononuclear cells (PBMCs) of 50 patients with histologically confirmed BC and 50 healthy individuals were assessed at the Institute for Cancer Research, Shiraz University of Medical Sciences (Shiraz, Iran) during 2014-2016. RNA was extracted from PBMCs, then cDNA was synthesized and subjected to quantitative real-time PCR (qRT-PCR) using appropriate primers. Statistical analysis was performed using SPSS software (version 21.0).

Results: Significantly higher amounts of *CTLA4* and *FOXP3* gene transcripts were found in the peripheral blood of BC patients compared with healthy individuals. The expression of both genes was significantly higher in patients with non-invasive and grade I/II BC. The median of *CTLA4* and *FOXP3* transcript expressions was 3.74 and 5.39, respectively, in non-invasive BC patients, which was significant compared with the control group (P=0.0016 and P=0.009, respectively). The median of target gene mRNA expression in grade I/II BC patients was 2.9 for *CTLA4* and 6.61 for *FOXP3*, which was significant compared with the controls (P=0.013 and P=0.0037, respectively).

Conclusion: This study highlights the functional activity of Tregs in early stages of bladder cancer and showed the importance of CTLA4 and FOXP3, when it comes to screening BC.

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Keywords ● *CTLA4* ● *FOXP3* ● Urinary bladder neoplasms ● T-lymphocytes, Regulatory ● Immunotherapy

What's Known

- Regulatory T cells play a key role in lymph nodes and tissues of patients with different types of cancer such as bladder cancer for tumor progression.

What's New

- The significance of regulatory T cells in the peripheral blood of bladder cancer patients is demonstrated.
- Our findings provide valuable data, which contribute to the development of new strategies for the detection of bladder cancer.

Introduction

Bladder cancer (BC) is the most common urologic malignancy. It has a higher incidence in men and ranks among the top ten most prevalent cancer types worldwide.[1, 2] BC is mostly affected by various environmental factors (smoking, occupational exposure

to aromatic amines and industrial chemicals) and several lifestyle factors such as obesity.[3] Chronic inflammation plays a prominent role in developing BC and tumorigenesis through DNA damage, angiogenesis stimulation, and cell proliferation.[1, 4] Although T-cell proliferation provides inflammatory responses, it should be regulated and controlled to prevent damaging inflamed tissues and autoimmune diseases.[5, 6]

Different subsets of T cells including T helper (Th) 1, Th2, Th17, and regulatory T-cell (Treg) have differential effects on immune responses against tumor cells and tumor immune evasion. CD4+CD25+FOXP3+T cells, also known as regulatory T cells (Tregs), play inhibitory roles in the immune system.[7] In the context of suppressing immune responses of solid tumors, the presence of Tregs has been shown to be associated with negative prognosis in a variety of malignancies including lung and breast cancer and melanoma. In contrast, tumor-infiltrating Tregs may correlate with a favorable prognosis in certain tumors such as gastric and colorectal cancer by controlling inflammation.[8] The frequency of Tregs in peripheral blood was introduced as a new and important prognostic marker in BC.[9] High expression of forkhead box P3 (FOXP3) in Tregs was seen earlier in patients with BC, which indicates the presence of immune suppression.[10] Basic inhibitory mechanism of Tregs is applied by *FOXP3* to upregulate cytotoxic T-lymphocyte associated protein 4 (*CTLA4*) gene.[7] *CTLA4* is an inhibitory molecule expressed in many T cells and mostly in a large number of FOXP3$^+$Tregs.

Delayed diagnosis of BC is usually due to the late onset of unspecific symptoms. This in turn may increase the risk of recurrence and make it more difficult to find a desirable therapeutic method for patients with advanced-stage BC. Moreover, delayed diagnosis and the subsequent progression of cancer poses a huge challenge to healthcare systems and imposes a considerable financial burden on the community.[11-15] Although the cancer SEEK test has an acceptable sensitivity to detect solid tumors such as ovary, liver, stomach, pancreas, and esophagus cancer, it is not applicable to BC.[16] Therefore, it is important to identify other factors, which allow BC screening. The present study aimed to determine the association between *CTLA4* and *FOXP3* gene expression in the peripheral blood of patients with BC, their prognostic role, and applicability for early detection of BC.

Materials and Methods

In a cross-sectional study, 50 newly diagnosed BC patients from hospitals, affiliated to Shiraz University of Medical Sciences (Shiraz, Iran), were enrolled in the study from June 2014 to November 2016. The cancer diagnosis was confirmed via bladder biopsy during cystoscopy and histopathological examination. The type and stage of tumors were reported by an experienced pathologist. Patients with a positive history of other cancers, autoimmune diseases, and immune suppression were excluded from the study. The control group included 50 healthy age-matched individuals without any history of malignancy or autoimmune diseases. The sample size was determined in accordance with a previous study using Power SSC software.[17]

$n=2(z_{1-\alpha/2}+z_{1-\beta})^2\delta^2/d^2$

Where: n=Sample size, δ^2=Variance within the population, d=Effect size, α=Significance level at 0.05, and β=Power of 80%. The standard deviation (SD) was 6.

The study was approved by the Ethics Committee of Shiraz University of Medical Sciences (code of ethics: IR-SUMS. REC.1389.1798). All procedures were set up and performed at the Institute for Cancer Research (ICR), Shiraz University of Medical Sciences, Shiraz, Iran. Written informed consent was obtained from all the participants prior to enrollment.

Blood Samples, RNA Extraction, and Reverse Transcription

Peripheral venous blood samples were obtained from the participants. The BC patients did not receive any radiotherapy, chemotherapy, or immunotherapy prior to sampling. Total RNA of blood cells was extracted using lysis with ammonium chloride and by Trizol reagent (Invitrogen, USA), according to the manufacturer's instructions. The quality and quantity of RNA samples were measured using spectrophotometry at 260 and 280 nm. Contaminated DNA was removed from the RNA using DNase I treatment (Fermentas, Lithuania) before cDNA synthesis. The cDNA was synthesized from 5 µg of total RNA with the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Lithuania) using both oligo (dT) and random hexamer primers.

Quantitative Real-Time PCR (qRT-PCR)

Specific primers were designed to determine the expression of *CTLA4, FOXP3,* and *β-actin* using Primer-Blast online software.[18] The sequences of the designed primers are listed in table 1. The quantity and expression of target gene transcripts were determined using a Bio-Rad system (Chromo4 Real-time PCR Detector, Bio-Rad, USA) for quantitative RT-PCR. The

Table 1: Sequences of forward and reverse primers for the detection of *CTLA4* and *FOXP3* genes in the patients and control groups

Target Gene	Forward Primer Sequence	Reverse Primer Sequence
Beta-actin	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG
CTLA4	CCCTGTCTTCTGCAAAGCAATGCA	CAGCCTGCCGAAGCACTGTCA
FOXP3	CACCTGGAAGAACGCCATCC	CTCATCCACGGTCCACACAG

expression of the *β-actin* housekeeping gene was used to normalize the target gene expression level. Every PCR reaction was done in a final volume of 20 µL, that contained 0.5 µg of cDNA product, 150 nM of each specific primer, and 1× reaction mixture consisting of SYBR green PCR Master Mix (Applied Biosystems, USA). Thermal cycling for all genes started with an initial denaturation step at 95 °C for 10 minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds, annealing at 56 °C for 20 seconds, and extension at 60 °C for one minute. The qRT-PCR amplification products were verified using melting curve analysis and 1% agarose gel electrophoresis. The amplification efficiency of PCR reaction for all transcripts was determined by plotting a standard curve. The relative quantities of target gene transcripts were calculated using the $2^{-\Delta\Delta Ct}$ formula.[19]

Statistical Analysis

Statistical Package for Social Science (SPSS) version 21.0 was used for data analysis. Relative expression was plotted and assessed with Prism 6 software (Inc; USA). Total amounts of target gene transcripts in the peripheral blood were compared to the equivalent values from the control samples using the nonparametric Mann-Whitney U test.

Results

In total, 100 individuals were allocated into two groups; patients with BC (n=50) and healthy age-matched controls (n=50). The mean age of the patients and the controls was 66.5±10.9 and 66.3±7.8 years, respectively. There was no age difference between the two studied groups (P=0.999). The histopathological information of BC patients included TNM stage, histological grade, vascular invasion, and muscular invasion (table 2).

Transcripts Level of CTLA4 and FOXP3 in BC Patients

The median of *CTLA4* mRNA in BC patients and control groups was 4.04 and 1.00, respectively. A four-fold increase was observed for *CTLA4* gene expression in the peripheral venous blood samples of BC patients compared with the control individuals (P=0.0002). In the BC patient's group, the detected median of *FOXP3* mRNA expression was 4.83, while it was 1.00 in the control group. The level of *FOXP3* mRNA showed a 4.8-fold higher expression in BC patients than the control group (P=0.0075) (figure 1).

CTLA4 and FOXP3 Gene Expression and Tumor Stage

There was no correlation between BC stages and *CTLA4* gene expression, while *FOXP3* gene expression was decreased, when tumors had disseminated in subterranean tissues. The expression of *FOXP3* mRNA was 10.5-fold

Table 2: Baseline characteristics of the patients with bladder cancer

Variable		N (%)
TNM stage	Ta	14 (28%)
	I	11 (22%)
	II	13 (26%)
	III	6 (12%)
	IV	1 (2%)
	Undefined	5 (10%)
Histological grade	I	1 (2%)
	II	19 (38%)
	III	8 (16%)
	IV	17 (34%)
	Undefined	5 (10%)
Vascular invasion	Yes	20 (40%)
	No	25 (50%)
	Undefined	5 (10%)
Muscular invasion	Yes	20 (40%)
	No	25 (50%)
	Undefined	5 (10%)

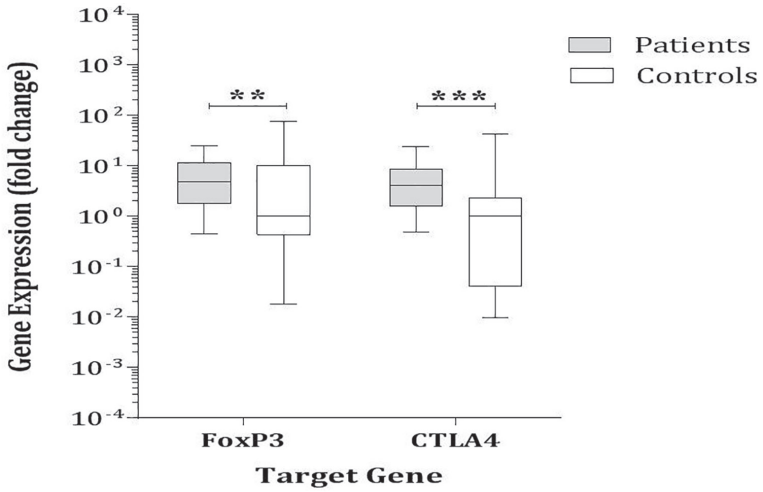

Figure 1: *CTLA4* and *FOXP3* genes transcript levels were detected in the peripheral blood of bladder cancer patients and control individuals. Both genes represent significantly higher expression in patients compared with controls. The graph shows whisker-box plot, and the data is compared with non-parametric Mann-Whitney test. **P<0.01; ***P<0.001

higher in Ta, while the transcript level of *FOXP3* was 2.6, 3.1, and 2.7-fold higher in patients with stage T1, T2, and T3 BC than the controls, respectively. Based on the Kruskal-Wallis test followed by Dunn's multiple comparison test, the difference in *FOXP3* mRNA expression between patients with stages Ta and T1 BC was statistically significant (P=0.026). The BC patients were divided into two sub-groups based on T stage and the patients with stage II (T2) or above were categorized as invasive BC. The median of *CTLA4* transcript expression was 3.74 and 6.43 in patients with non-invasive and invasive BC, respectively (P=0.0016 and P=0.024, respectively, compared to the control group) (figure 2A). The median of *FOXP3* mRNA expression in the peripheral blood samples of patients with non-invasive and invasive BC was 5.39 and 3.40, respectively. The difference in the *FOXP3* gene expression in patients with non-invasive BC was statistically significant (P=0.009) (figure 2B).

CTLA4 and FOXP3 Gene Expression and Tumor Grade

The median of target gene mRNA expression in grade I/II (low-grade) and grade III/IV (high-grade) BC patients were 2.9 and 7.39 for *CTLA4* and 6.61 and 2.73 for *FOXP3*, respectively. The expression of *CTLA4* and *FOXP3* genes were statistically correlated with the grade of bladder tumor cells, when compared to the control group. The results showed that the expressions of these genes, in particular *CTLA4*, were amplified by increasing the tumor grade (P=0.013 and P=0.0027) (figure 3). Furthermore, the muscular

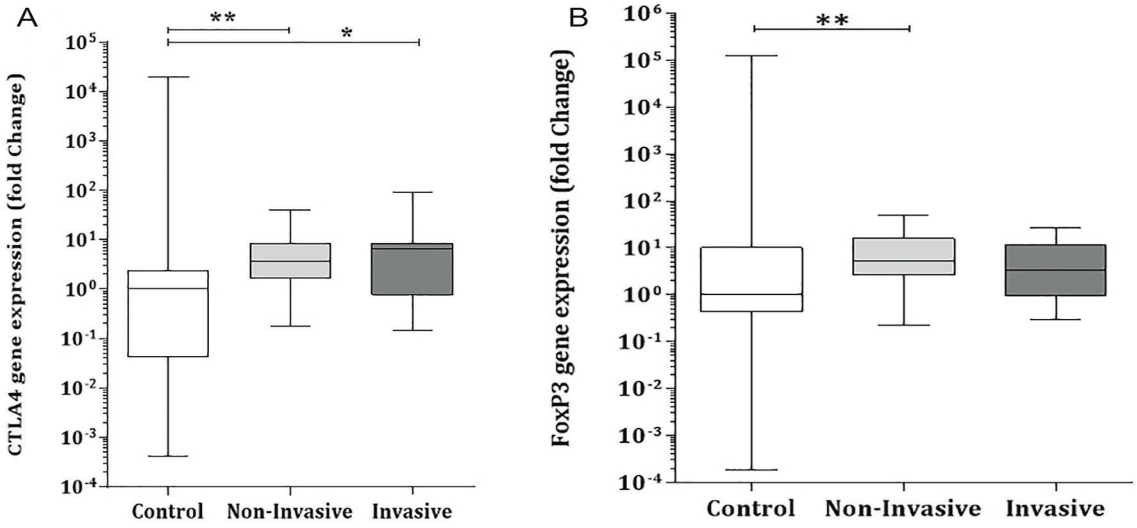

Figure 2: The correlation between tumor stage and target genes mRNA transcript level. (A) *CTLA4* gene showed significantly higher expression in both non-invasive and invasive BC patients compared with the control group. (B) *FOXP3* gene showed significantly higher expression in non-invasive BC patients compared with the control group. All data were compared with Kruskal-Wallis test followed by Dunn's multiple comparisons test. *P<0.05; ** P<0.01

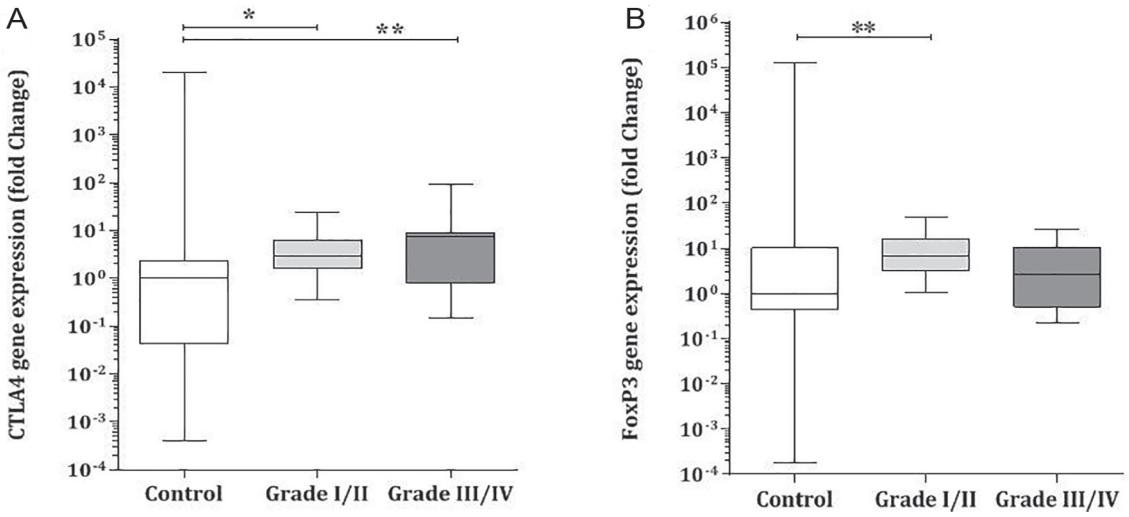

Figure 3: The correlation between tumor grade and target genes mRNA transcript level. The expressions of *CTLA4* (A) and *FOXP3* (B) genes were positively correlated with the various bladder cancer grades. Lines represent data range. All data were compared with Kruskal-Wallis test followed by Dunn's multiple comparisons test. *P<0.05; **P<0.01

and vascular invasion of tumor cells were positively correlated with the mRNA levels of both genes in BC patients.

Discussion

Evaluation of *CTLA4* and *FOXP3* gene expression in the peripheral blood of BC patients revealed significant overexpression of both genes. *CTLA4* and *FOXP3* were significantly expressed in the early-stage and low-grade BC patients, indicating the applicability of these markers to detect early-stage BC.

The hypothesis of downregulation of the immune system in malignancies has attracted much attention to *FOXP3*, a unique transcription factor of Tregs.[20, 21] High expression of this gene was detected in many malignancies such as gastric cancer.[22, 23] The overexpression of *FOXP3* and *CTLA4* in the peripheral blood of patients with breast cancer was previously reported by our cancer research center.[17] However, high expression of *FOXP3* in most malignancies have shown an opposite association with disease prognosis.[22] In line with our study, Zhang and colleagues evaluated *FOXP3* in BC patients and showed a direct correlation between tumor staging and metastasis with this gene.[24] They found an inverse correlation between *FOXP3* expression, patients' survival, and response to chemotherapy.[24] *FOXP3* can enhance hypoxia-inducible factor-1α (HIF-1α) in BC cells by influencing VEGF signaling and intratumoral immune responses.[25] Therefore, it is considered a poor prognostic factor for BC. Moreover, in patients with superficial BC, *FOXP3* expression is associated with lower levels of intratumoral CD8+T cells.[25] In a study by Winerdal and colleagues, *FOXP3* expression in urinary BC cells was associated with decreased long-term survival and thus, unfavorable prognosis. However, the presence of *FOXP3* positive tumor infiltrating lymphocytes was positively correlated with good prognosis, since *FOXP3* is also a T-cell activation marker.[26] In another study, an increase of Treg population in a tumor microenvironment of invasive BC was positively correlated with better prognosis due to the suppression of *MMP2* gene expression.[27] They showed that MMP2 protein and mRNA expression was downregulated in a dose-dependent Treg-mediated manner in BC cells and the surrounding macrophages. They concluded that *FOXP3* gene expression is positively correlated with better survival of patients with invasive BC.[27] Based on our results, we hypothesize that an increase of *FOXP3* expression in BC patients is indicative of its screening potential of this malignancy. However, further studies with a larger sample size are required to confirm our findings.

In our previous study, we showed the presence of a significantly higher level of *CTLA4* expression in the peripheral blood of breast cancer patients.[17] Previous reports showed downregulation of Tregs in BC with anti-CTLA4 treatment.[28-30] Based on this mechanism, ipilimumab is an approved drug with a good success rate in treating melanoma.[31] The results of the CheckMate-032, phase 1/2 trial, showed improved survival and tolerable side effects in 86 patients with metastatic BC, who received standard-of-care chemotherapy combined with a higher dosage of Yervoy (ipilimumab) together with Opdivo (nivolumab).[32, 33] Anti-CTLA4 therapy increases the production of IFN-γ by enhancing T-cell responses, which is

viewed as the main anti-tumor mechanism of this treatment pathway.[34] However, it has been shown that FOXP3+Tregs population did not decrease in the tumoral tissue of the bladder following anti-CTLA4 therapy.[35] However, it should be mentioned that anti-CTLA4 antibodies may act against Tregs through other mechanisms than antibody cell-mediated cytotoxicity.[36] Indeed, a major immune regulatory function of Treg was mediated by iTreg, which expresses a cytoplasmic variant of CTLA4, and escapes from antibody cell-mediated cytotoxicity through anti-CTLA4 antibodies.[36] Nevertheless, the impact of anti-CTLA4 antibodies has been confirmed in clinical trials.[33]

The main limitation of the study was the inability of patient follow-up to assess the relationship between the level of *FOXP3* and *CTLA4* genes and disease recurrence.

Conclusion

FOXP3 and *CTLA4* levels were significantly higher in patients with BC, especially in those with a low-grade and non-invasive type, indicating the importance of these molecules in BC screening. Although *FOXP3* and *CTLA4* gene expression levels may change in various types of cancers, the combination of a test based on these prognostic markers with other available tests may increase early detection of BC. Further studies with a larger sample size are required to confirm our findings.

Conflict of Interest: None declared.

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