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Increased CTLA-4 and FOXP3 Transcripts in Peripheral Blood Mononuclear Cells of Patients with Breast Cancer

Mansooreh Jaberipour · Mojtaba Habibagahi · Ahmad Hosseini · Saadat Rezai Habibabad · Abdolrasoul Talei · Abbas Ghaderi

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Abstract Generation of Regulatory T cells (Tregs) is known to play a major role in progression and modulation of the immune escape mechanisms in cancer. These cells express Forkhead/winged helix transcription factor (FOXP3) and also Cytotoxic T-lymphocyte antigen-4 (CTLA-4), as a negative regulatory molecule which, is a potential target for immunotherapy. We, therefore, evaluated FOXP3 and CTLA-4 transcripts in the peripheral blood mononuclear cells from 55 women with histologically-confirmed infiltrating ductal carcinoma of the breast. Blood samples from 40 healthy volunteer women without a history of malignancies or autoimmune disorders were also obtained as a control. The abundance of FOXP3 and CTLA-4 gene transcripts was determined by quantitative real-time PCR (qRT-PCR). Compared to healthy individuals, significantly higher amounts of these transcripts were found in the mononuclear cells from breast cancer patients. Also, a significant correlation was found between CTLA-4 and FOXP3 expressions in a group of patients. Among patients with early stage, nonmetastatic or low-grade disease, the relative expression of CTLA-4 was about 10-fold as much as in the control group. These patients also showed a significant increase, more than 10 fold, in mean relative FOXP3 expression. The results of this investigation point to functional activity of Treg cells in early stages of breast cancer, a finding which emphasizes the significance of Tregs as an imminent target for breast cancer immunotherapy.

Keywords Breast cancer · CTLA-4 and FOXP3

Abbreviations

CTLA-4	Cytotoxic T-lymphocyte antigen-4
GITR	Glucocorticoid-induced tumor necrosis factor receptor
TGF-β	Transforming growth factor- betta
FoxP3	Forkhead/winged helix transcription factor
qRT-PCR	quantitative real-time PCR

M. Jaberipour · A. Hosseini · A. Talei · A. Ghaderi
Cancer Gene Therapy Laboratory, Shiraz Institute for Cancer Research, Shiraz University of Medical Sciences, Shiraz, Iran

M. Habibagahi (✉) · S. R. Habibabad
Immunotherapy Laboratory, Department of Immunology, Shiraz University of Medical Sciences, P.O. Box 71345-3119, Shiraz, Iran
e-mail: agahim@sums.ac.ir

A. Talei
Department of Surgery, Shiraz University of Medical Sciences, Shiraz, Iran

A. Ghaderi
Department of Immunology, Shiraz University of Medical Sciences, Shiraz, Iran

Introduction

There is considerable evidence showing significant defects in the immune system of patients with cancer [1–3]. In fact, this concept has emerged that peripheral tolerance to tumors is maintained and could be enhanced by T cells with immunoregulatory functions [4]. Although the absolute number of peripheral blood lymphocytes decreases in many patients with breast cancer [5], the quantity of functionally suppressive CD4+CD25+ regulatory T cells (Tregs) is reportedly increased in the peripheral blood and tumor microenvironment [6]. T-regulatory cells are known to have a major influence on the immune response [7]. Under normal

conditions in healthy adult individuals, 5%−10% of CD4+ lymphocytes are CD4+CD25+ with regulatory functions [8, 9]. These cells express cytotoxic T-lymphocyte antigen-4 (CTLA-4) and glucocorticoid-induced tumor necrosis factor receptor (GITR), secrete IL-10 and transforming growth factor-β (TGF-β), and are specifically characterized by the expression of the most reliable markers of Tregs: forkhead/winged helix transcription factor (FoxP3) [10, 11].

A high prevalence of regulatory T cells with suppressive activity has been described in lung, ovary, liver, pancreas, breast and skin cancers either in the peripheral blood or around and within the tumor [12–15]. When the frequency of circulating Tregs was compared in patients with colorectal cancer and control group of patients with colonic inflammation, significantly more regulatory T cells were found in the former group. Therefore, this increase could not simply happen by chronic inflammation within the colon [16, 17]. Similarly, the frequency of Tregs with suppressor phenotype and function was significantly greater in patients with head and neck cancer than normal controls [18]. Bi Y et al. have studied the level of mRNA expression of CTLA-4 in normal and breast carcinoma tissues and showed statistically increased levels of the gene transcription in patients and that was correlated with disease progression [19]. Other studies show the elevated expression of FOXP3 mRNA in high and early stages of breast cancer tissues than in normal samples [20]. Similarly, association of Foxp3+ cell frequencies with more advanced diseases of breast cancer has been shown [21]. Foxp3 expression, which is an indicator of Treg activity, was identified as an independent prognostic factor and a marker of tumor progression and metastasis in breast carcinoma [22, 23].

Due to this evidence and the importance of CTLA-4 and Foxp3 genes in breast cancer disease, we used quantitative real time PCR to directly measure their mRNA transcripts in the peripheral blood cells of women with breast cancer and compared that with healthy controls. The results of this study show detectable overexpression of CTLA-4 and FOXP3 in patients in different grades of the disease.

Material and Methods

Patients and Healthy Controls

The participants in this study were 55 women with infiltrating ductal carcinoma of the breast, confirmed by histological studies. The patients were referred to our laboratory from the Breast Clinic of the Shiraz University of Medical Sciences in Shiraz, Iran during the year 2008. All the patients provided their informed consent to take part in this study. Peripheral venous blood samples (2 mL), with EDTA as an anticoagulant, were collected by venipuncture before any intervention. None of the patients had received chemotherapy, radiotherapy or immunotherapy before sampling. Blood samples from 40 healthy volunteer women without a history of malignancies or autoimmune disorders were also obtained as a control. The mean age of the patients and healthy control group were 51 years (ranged 25 to 81) and 45 years (ranged 23 to 68), respectively.

RNA Isolation and Reverse Transcription

Total RNA was prepared from blood cells after lysis with ammonium chloride and TRizol reagent treatment (Invitrogen, Paisley, UK) according to the manufacturer's instructions. The quantity and quality of the extracted RNA samples were estimated by spectrometry at 260 and 280 nm. RNA was treated with DNase I (Invitrogen-Gibco, Paisley, UK) before cDNA synthesis to avoid DNA contamination. cDNA was synthesized from 5 μg of total RNA, using the RevertAid First Strand cDNA Synthesis Kit (Fermentase, Vilnius, Lithuania).

Quantitative Real-Time RT-PCR

The abundance of CTLA-4 and FOXP3 gene transcripts was determined in triplicates by quantitative real-time PCR (qRT-PCR), using a Bio-Rad system (Chromo4 Real-time PCR Detector, Bio-Rad, Foster City, CA, USA) with Syber Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Expression of β-actin housekeeping gene was used as a reference for the level of target gene expression. Each PCR reaction was performed in a final volume of 25 μL and contained 0.5 μg of the cDNA product, 4.0 pmol of each primer, and 1× reaction mixture consisting of FastStart DNA polymerase, reaction buffer, dNTPs, and SYBR green I (Applied Biosystems). Table 1 shows the forward and reverse primers for β-actin, CTLA-4 and FOXP3 genes. Primers were designed by primer3 open source software (Sourceforge, USA).

Thermal cycling for all the genes was initiated with a denaturation step at 95°C for 10 min, followed by 30 cycles

Table 1 Forward and reverse primers of β-actin, CTLA4 and FOXP3 genes for real-time PCR amplification. Sequences were designed by Primer3 software (Sourceforge, USA)

Primer	Sequence
β-actin Forward	GGACTTCGAGCAAGAGATGG
β-actin Reverse	AGCACTGTGTTGGCGTACAG
CTLA4 Forward	CTTCAGTCACCTGGCTGTCA
CTLA4 Reverse	CTCAGCTGAACCTGGCTACC
FOXP3 Forward	CCACTTGCAGACACCATTTG
FOXP3 Reverse	CATGATCAGCCTCACACCAC

(denaturation at 95°C for15 s, annealing at 60°C for 30 s, and elongation at 60°C for 34 s when fluorescence appeared). The qRT-PCR amplification products were analyzed by melting curve analysis and by 1% agarose gel electrophoresis (data not shown).

Amplification Efficiency and Standard Curve Analysis

For each target gene, efficiency of the real-time PCR reaction was calculated from the slope of the standard curve. Standard curves were plotted by Ct values of serial dilutions of plasmids containing the genes of interest against the logarithm concentration of input template DNA. (Efficiency of the β-actin, CTLA-4 and FOXP3 PCR reactions was calculated as 95%, 96% and 98%, respectively; data not shown.) The relative amounts of CTLA-4 and FOXP3 transcripts were determined from the ΔCt and $2^{(-\Delta Ct)}$ formulas. Target-to-reference gene ratios were calculated with the Pfaffl method [24].

Statistical Analysis

The numbers of CTLA-4 and FOXP3 transcripts in the peripheral blood was compared to the corresponding values from control samples using nonparametric Mann-Whitney test using SPSS software v. 11.5 (SPSS, Chicago, IL, USA). Relative expression was plotted and analyzed using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). The relationships between different values were examined using Pearson's correlation coefficient and Spearman's rank correlation tests. For all the statistical analysis, $p<0.05$ was considered as significant.

Results

Clinical and Pathological Characteristics

Data on age, tumor histology, tumor size, clinical stage, histological grade, lymph node metastases, and presence of other organ metastases were obtained from the hospital records of the 55 patients. Clinical stage was determined with the tumor-node-metastasis classification. Figure 1 demonstrates the distribution of patients regarding different clinical criteria. The high-grade and metastatic breast cancer patients were a small group of patients and statistically they were not able to be compared with low-grade and non-metastatic patients.

CTLA-4 Gene Expression

Comparison of the findings shows significantly higher expression of CTLA-4 in patients than in healthy volunteers (P=0.0003) (Fig. 2). Among the patients in the early disease stages (stages I and II), the relative expression of CTLA-4 was about 16-fold as much as the control group (P=0.0006). Similarly, CTLA-4 expression in patients with nonmetastatic tumors and patients with low-grade tumors increased up to 12-fold (P=0.0007) and 10-fold (P=0.0021), respectively, compared to healthy controls (Fig. 2). There was no significant difference in levels of CTLA-4 gene expression among patients of different stages ($p>0.8$). No significant correlation was found between CTLA-4 expression, lymph node involvement and peritumoral vessel invasion. Also, right and left sided tumors showed similar levels of CTLA-4 expression.

FOXP3 Gene Expression

Expression of the FOXP3 gene transcript in different groups of breast cancer patients was not different ($p>0.99$). However, it was about 19-fold as much as healthy individuals (P=0.0001). Patients in the early-stage of the disease showed a significant increase in mean relative FOXP3 expression (P=0.0007). Increased expression of Foxp3 was found in nonmetastatic patients (P=0.0003) and those with a low-grade tumor burden (P=0.0002) (Fig. 3). No significant correlation was found between FOXP3 expression and lymph node involvement and peritumoral vessel invasion. Likewise, neither right-sided tumors nor left-sided tumors showed a significant correlation with FOXP3 expression.

Correlation of FOXP3 and CTLA-4 Gene Expression

There were significant positive correlations between Foxp3 and CTLA-4 gene expression in women with breast cancer. In fact, more Foxp3 transcripts were found in the peripheral blood cells of the patients with higher CTLA-4 expression ($r=0.47$, P=0.0003).

Discussion

Regulatory T cells can act as active suppressor of lymphocyte response to tumor antigens in malignant neoplasms and many recent studies have explored the cellular and molecular mechanisms of this suppression [25–27]. In this study, we evaluated CTLA-4 and FOXP3 transcripts, as acceptable indicators of Tregs, in the peripheral blood from women with breast cancer and found that these transcripts significantly increase even in early stages of breast cancer. In patients with leukemic cutaneous T-cell lymphoma, the expression of the same genes was evaluated with real-time PCR, and FOXP3 expression correlated with a poor prognosis of the disease [28]. In cervical cancer, Tregs

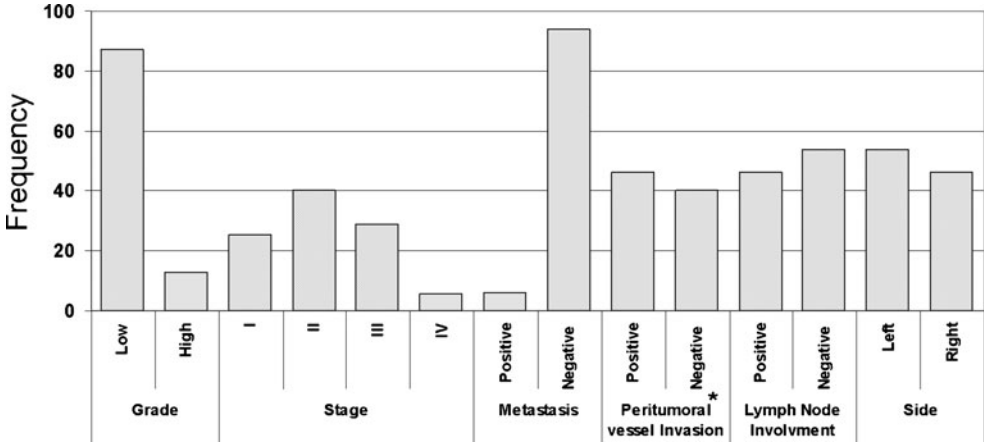

Fig. 1 Distribution of patients with breast cancer with regard to different clinical criteria. The majority of the studied patients showed non-metastatic and low grade tumor. * Corresponding data for 14 patients were not available

were frequently detectable in precancerous stages, indicating a possible role of these cells not only in the progression but also in the initial development of cancer [20, 29].

In a recent study by Ohara M et al. on 136 breast cancer patients, total RNA was extracted from frozen breast cancer tissues and normal tissues, and the expression of FOXP3 mRNA was evaluated. FOXP3 transcripts were significantly increased in cancer tissues, not only at late stages but also at the early stages of the disease [20]. Matsuura et al. also used quantitative real-time RT PCR and found significantly higher levels of Foxp3 transcripts in metastatic breast cancer patients than in those without it [30]. Besides, evidence shows a linear association between increasing levels of Foxp3 and advanced tumor stages in breast carcinomas [23]. It has also been suggested that FOXP3 expression can be used as a prognostic factor in early-stages of breast cancer [31]. Similarly, we found significantly higher FOXP3 gene expression (based on mRNA levels) in blood samples from patients with early-stages, nonmetastatic and low-grade breast cancer than healthy women. Therefore, based on pervious studies and our work, FOXP3 appears to merit further study as a potential target for immunotherapy in early-stages of breast cancer.

Transcripts of the CTLA-4 gene were also significantly increased in our low-grade and early stage breast cancer patients compared to healthy controls. Presence of CTLA-4 could result in sustained peripheral tolerance to tumors and may justify cancer immunotherapy approaches by CTLA-4 blockade using monoclonal antibodies [32, 33]. In a study by Tuve S et al., combination of local anti-CTLA-4 antibody with systemic Treg depletion in mouse model enhanced antitumor immune responses with no signs of autoimmunity [29].

Overall, the results of CTLA-4 and Foxp3 gene expression in the current study and those in similar investigations spot the

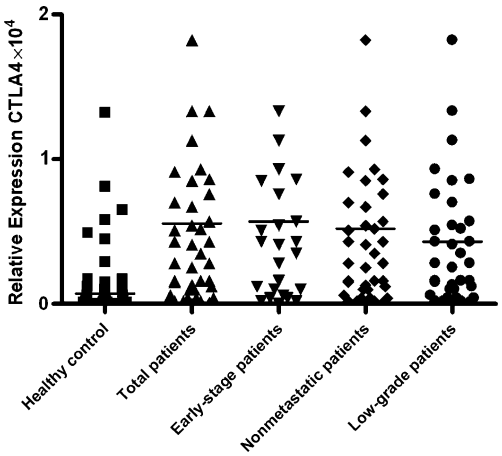

Fig. 2 Expression level of *CTLA-4* in the peripheral blood cells of breast cancer patients and normal controls. Presented data were calculated with the $2^{(-\Delta ct)}$ formula and analyzed with the nonparametric two-tailed Mann-Whitney test. Horizontal bars indicate median values. Significant differences were found in levels of *CTLA-4* expression among total, nonmetastatic, early-stage (I, II) and low-grade breast cancer patients compared to healthy controls (P=0.0003, 0.0007, 0.0006 and 0.0021, respectively)

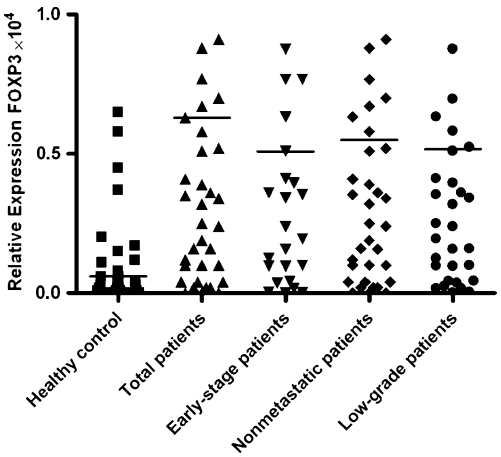

Fig. 3 Expression level of Foxp3 in the peripheral blood cells of breast cancer patients and normal controls. Presented data were calculated and analyzed as explained in Fig. 2. Horizontal bars indicate median values. Significant differences were found in the levels of Foxp3 expression among total, nonmetastatic, early-stage (I, II) and low-grade breast cancer patients compared to healthy controls (p=0.0001, 0.0003, 0.0007 and 0.0002, respectively)

importance of inhibitory mechanisms of regulatory T cells in the course of malignancies even from early stages. This inhibitory signal maintains self tolerance against tumors and provides opportunity for tumor cells to escape from immune effectors mechanisms.

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