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Concomitant Increase of OX40 and FOXP3 Transcripts in Peripheral Blood of Patients with Breast Cancer

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ABSTRACT

Background: Regulatory T cells (T-regs) have an important role in cancer by suppression of protective antitumor immune responses. Regulatory T cells express the forkhead/winged helix transcription factor (FOXP3) and OX40 molecules which have important regulatory roles in the immune system. **Objective:** To evaluate FOXP3 and OX40 transcripts in the peripheral blood mononuclear cells of women with breast cancer. **Methods:** Blood samples from 40 women with histologically-confirmed infiltrating ductal carcinoma of the breast and 40 healthy volunteer women without a history of malignancy or autoimmune disorders were collected. The abundance of FOXP3 and OX40 gene transcripts were determined by quantitative real-time PCR (qRT-PCR). **Results:** There was a significant positive correlation between FOXP3 and OX40 gene expression in women with breast cancer in a stage dependent manner. **Conclusion:** This finding emphasizes the importance of T-regs as predominant targets for breast cancer immunotherapy.

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Keywords: Breast Cancer, FOXP3, OX40

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INTRODUCTION

Regulatory T (T-reg) cells have a major role both in immune self-tolerance and also in tumor-related immune suppression (1), and in normal condition comprise about 5-10% of CD4+ lymphocytes (2,3). T-reg cells accumulate in both human and mouse tumors, as well as in secondary lymphoid organs. Two major groups of T-reg cells are known, natural (n)T-regs which mainly develop in thymus and induced T-regs which develop from the peripheral conventional CD4+ T cells (4-6).

T-reg cells express a variety of markers including fork head winged helix transcription factor (FOXP3) and OX40 (7). FOXP3 is now recognized as a reliable prognostic factor and also a marker of tumor progression. Several studies have demonstrated that the frequency of regulatory T cells with suppressive activity rises in lung, liver, pancreas, breast and skin cancers either in the peripheral blood or around and within the tumor (8-11). Other investigations indicate that the expression of FOXP3 mRNA increases in the tissue of breast in early and late stages of cancer (12). Similarly, relation between the levels of FOXP3+ cells with more advanced metastatic disease of breast cancer has been described (13). OX40, which also called CD134, is a new co-stimulatory molecule that belongs to the TNF-R superfamily (14), which transfers a potent co-stimulatory signal to effectors T cells (15). OX40 was initially identified as a T cell activation marker predominantly expressed by activated T cell. Recently investigations reported that OX40 is expressed on both natural and induced FOXP3+T-regs (7). Several studies show the effect of OX40 on CD25 FOXP3 T-regs which can influence T-regs activity (16). These studies have indicated that the ligation of OX40 can reduce suppressor activity of T-regs in autoimmune and allogeneic transplant mouse models.

Recently, OX40 is introduced as a new marker on FOXP3+T-regs that plays an important role in survival and proliferation of activated T cells. OX40 is a potent negative regulator not only for natural FOXP3+ T-regs but also for T-regs generated de novo from activated effector T cells, therefore OX40 is suggested as an important checkpoint in T-reg homeostasis (17). Due to this dual activity of OX40 on the immune system and the nature of interaction with FOXP3, this study aimed to find the level of gene expression of these two important markers in breast cancer using quantitative real time PCR to directly measure their mRNA transcripts in the peripheral blood cells of women with breast cancer compared with control group.

MATERIALS AND METHODS

Subjects. In this case series study, the participants were 40 women with diagnosed infiltrating ductal carcinoma of the breast which was confirmed by histological data. The sample size was determined based on statistical calculation and the prevalence of disease in our region. The blood samples were collected from patients in the surgery department of the Apadana and Emam Khomeini hospitals in Ahvaz, Iran, in 2010. All the patients provided their informed consent to take part in this study. Peripheral venous blood samples (2mL), 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 malignancy or autoimmune disorders were also obtained as control.

The mean age of patients and healthy control group were 51 years (ranged 25 to 81) and 45 years (ranged 23 to 68), respectively.

Clinical and Pathological Characteristics. All breast cancer patients were newly diagnosed cases and none of patients had received chemotherapy, radiotherapy or immunotherapy before sampling. Data on histological grade, clinical stage and the presence of organ metastasis were prepared from the hospital reports of the 40 patients. Table 1 shows the distribution of patients according to clinical criteria. The predominant cases were in low-grade, early stage (I, II) and negative metastasis group.

Table. 1 Distribution of patients with breast cancer regarding to different clinical criteria.

Characteristics	No.
Low grade	31
High grade	9
Stage I	7
Stage II	17
Stage III	14
Stage IV	2
Metastasis	3
Negative Metastasis	37
Total	40

RNA Extraction and Reverse Transcriptase Polymerase Chain Reaction. Fresh blood from patients and control groups were used for RNA extraction and total cellular RNA was isolated using RNX plus reagent according to the manufacturer's instructions after lysis with RBC lysis. To the resulting sediment 100 micro liters cold PBS and then 1 cc RNX plus reagent was added and the solution obtained was gently stirred to be completely transparent. 300 micro liters of chloroform was added to the solution and centrifuged 14,000 rpm, 4°C 20 minutes. After adding isopropanol and ethanol and centrifugation, supernatants were removed and were given time to dry completely, RNA quality was monitored by 1% agarose gel electrophoresis, and its concentration was determined by photo absorption at 260 nm and 280 nm. Then RNA was treated with DNase I before cDNA synthesis to avoid DNA contamination. Complementary DNA was synthesized from 5 µg of total RNA.

Quantitative Real-Time RT-PCR. The abundance of OX40 and FOXP3 gene transcripts was determined in triplicates by quantitative real-time PCR (qRT-PCR), using Applied Bio systems Step one™ and Step one Plus™ Real Time PCR systems. Expression of GAPDH housekeeping gene was used as a reference for the level of target gene expression. Each PCR reaction was performed in a final volume of 20 µL and contained 1 µg of the cDNA product, 1 µL of each primer, 50 nm, and 2X reaction mixtures consisting of Fast Start DNA polymerase, reaction buffer, dNTPs, and SYBR green I (Applied Bio systems). Table 2 shows the forward and reverse primers for GAPDH, FOXP3 and OX40 genes. Thermal cycling for all the genes was initiated with a denaturation step at 95°C for 10 min, followed by 40 cycles (95C for 15 s and 60C for 60 min).

Table 2. Forward and reverse primers of GAPDH, FOXP3 and OX40 genes for real-time PCR amplification.

	Primer	Sequence
GAPDH	Forward	5'-GCAAGAGCACAAGAGGAAGA-3'
	Reverse	5'-ACTGTGAGGAGGGGAGATTC-3'
FOXP3	Forward	5'-ACAGTCTCTGGAGCAGCAGC-3'
	Reverse	5'-CCACAGATGAAGCCTTGGTC-3'
OX40	Forward	5'-AACCAGGCCTGCAAGCCCT-3'
	Reverse	5'-GTCCCTGTCCTCACAGATT-3'

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 cDNA and PCR product containing the genes of interest amplicon against the logarithm concentration of input template DNA.

Statistical Analysis. The data for the number of FOXP3 and OX40 transcripts in the peripheral blood was compared to the corresponding values from control samples using SPSS software v.18 (SPSS, Chicago, IL, USA). Relative expression was plotted and analyzed using Rest 2008 V2.0.7. For all the statistical analysis, $p < 0.05$ was considered as significant.

RESULTS

Efficiency of the GAPDH, FOXP3 and OX40 was calculated as 99%, 92% and 95% respectively. Spearman's correlation analysis of FOXP3 expression by stage showed an increase in the expression of FOXP3 with stage of tumor (Figure 1). This increase positively correlated with the stage of tumor and OX40 expression (Figure 2).

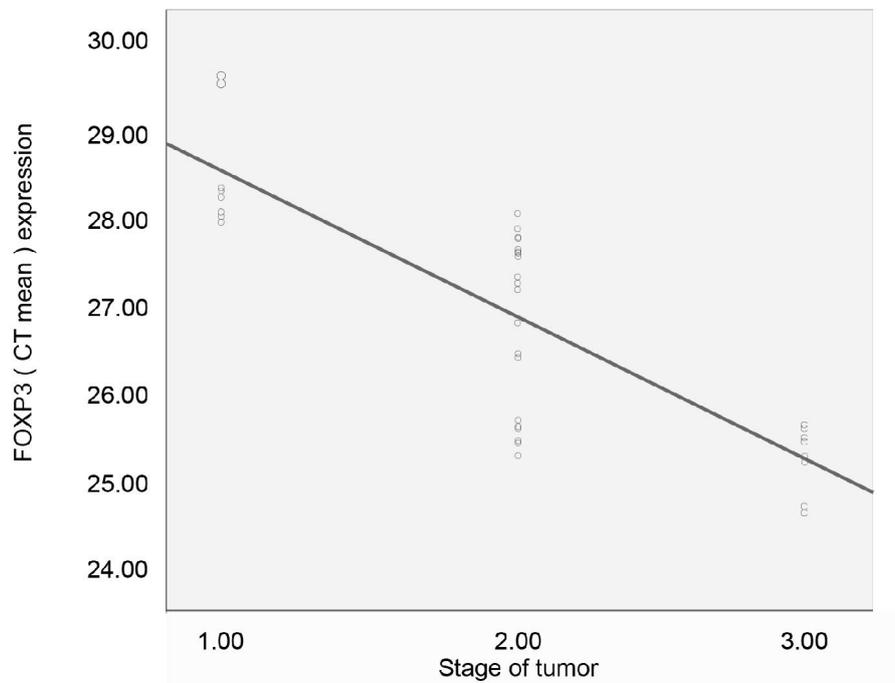


Figure 1. Spearman's correlation analysis of FOXP3 expression by stage, $R^2=0.73$, $p<0.01$.

Gene Expression. FOXP3 was up-regulated by 3.3 fold in cancer patients in comparison with control group by a mean factor of 3.303 (S.E. range = 0.805-13.157, $p<0.001$; Table 3 and Figure 1).

Table 3. Relative expression of FOXP3 and OX40 in peripheral blood cells of breast cancer patients generated by Rest RG 2008 Software.

Gene	Type	Reaction Efficiency	Expression	P(H1)Result
GAPDH	REF	0.99	1.000	
FOXP3	TRG	0.99	3.303	<0.001UP
OX40	TRG	0.95	2.536	<0.001UP

The level of FOXP3 expression in cancer patients was significantly different from that of control group. OX40 was also up-regulated in these patients in comparison with control group by a mean factor of 2.536 (S.E. range is 0.484-12.016, $p<0.001$; Table 3 and Figure 2).

OX40 and FOXP3 Transcripts in breast cancer

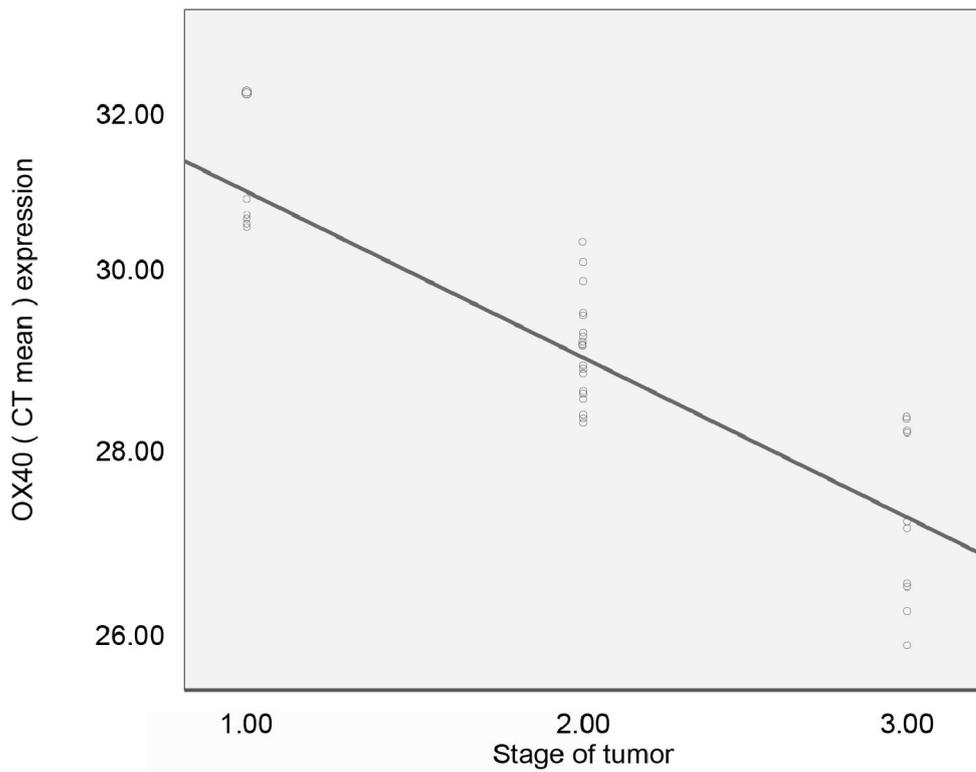


Figure 2. Spearman's correlation analysis of OX40 expression by stage, $R^2=0.82$, $p<0.01$.

Correlation of FOXP3 and OX40 Gene Expressions. There was a correlation between FOXP3 and OX40 gene expression in women with breast cancer (Figure 3).

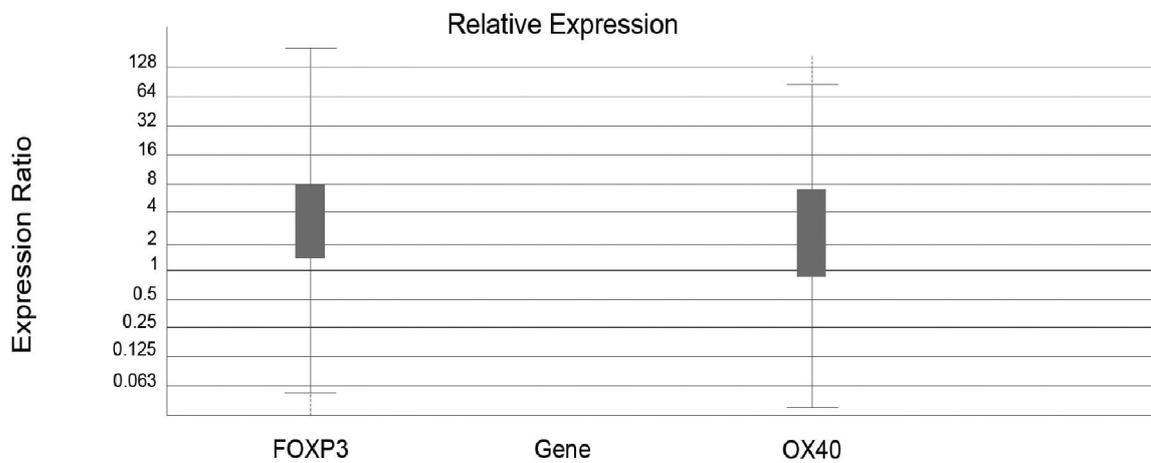


Figure 3. Relative expression of FOXP3 and OX40 in cancer patients generated by (Rest RG 2008 Software).

DISCUSSION

In this study, we evaluated FOXP3 and OX40 transcripts, as acceptable indicators of T-regs, in the peripheral blood from women with different stages of breast cancer and found that there was a correlated significant increase in these transcripts with the stage of cancer. There was also a significant increase in early stages of breast cancer.

It is now accepted that T-regs have an important role in suppression of tumor immune surveillance and are major factors which restrict the clinical success of cancer immunotherapy (18). In a recent study by Ohara et al., the expression of FOXP3 mRNA was evaluated on 136 breast cancer patients, total RNA was extracted from frozen breast cancer and normal tissues. FOXP3 transcripts were significantly increased in cancer tissues, not only at late stages but also at the early stages of the disease (19). Matsuura et al., using quantitative real-time RT PCR, also found significantly higher levels of FOXP3 transcripts in metastatic breast cancer patients compared with control cases (20). Furthermore, Merlo et al. found that FOXP3 expression was associated with overall and distant metastasis free survival but not with local relapse; therefore, these authors suggest that FOXP3 expression might be related to the metastatic potential of the tumor rather than to suppression of a specific immune response (21). It has also been suggested that FOXP3 expression can be used as a prognostic factor in early-stages of breast cancer (22). These findings come in agreement with our present findings in our patients.

On the other hand, OX40 can be expressed by both FOXP3+ T-regs and by activated effector T cells. Activated effector T cells, which express both CD25 and OX40, can be converted to FOXP3 T-regs and execute as potent suppressor cells (7).

Previous studies showed that OX40 expression on T-regs point to its possible dual functional role: one to mask OX40L expressed on DCs, rendering OX40L unavailable for effector T-cell costimulation; and the other is controlling T-regs homeostasis, because mice lacking OX40 were found to have reduced numbers of CD4+CD25+ T cells early in life (23). These new findings suggest that the impact of OX40 on the FOXP3+ T-regs is likely to be more complex than initially anticipated. Recently it was proposed that OX40 expression by T-reg cells was indispensable for suppression of OX40-dependent colitogenic T cell responses (24).

Our results show that there is a concomitant increase in the expression of OX40 molecules in the peripheral blood of breast cancer patients in a stage-related manner. This finding was unexpected because there are several reports that show the level of these molecules have inverse relation to each other, since OX40 has a negative regulatory effect on the FOXP3 expression in T-reg cells (25). In a study conducted by Sarff et al., a decrease in expression of OX40 on TCD4+ in both sentinel lymph node draining and peripheral blood cells among patients with primary melanoma has been found which may be due to inhibition of T cell function through regulatory T cell (26). The reason for this contrasting finding may be the differences in the end point, while our method assessed the OX40 gene expression; other studies used flow cytometry methods for measurement of surface of proteins. In addition to the differences in the type of cancer under investigation, it is known that expression of a gene does not guarantee its translation into a protein. These findings bring forward the notion that it is possible that translations of protein are inhibited by unknown tumor-suppressing factors. Prudently, we should have used flow cytometry to assess this discrepancy.

The remarkable increase in OX40 markers in our study may also be attributed to the types of T cells taken from the peripheral blood samples. The peripheral blood cells are known to be expressed by naive T-reg, CD4⁺ T and memory T cells (27).

It is clearly obvious that in situ measurement of these markers (i.e. on samples taken from local sites of the tumors), will provide a clearer picture on the relevance of these findings under clinical conditions (28).

In conclusions, new strategies that simultaneously stimulate effector T cells while inhibiting or depleting T-regs are needed to improve the outcome of cancer immunotherapy. In the current study, we demonstrated that the suppressive function of T-regs can be reversed through OX40 costimulation that is known to be able to durably enhance the function of effector T cells. These findings suggest that OX40 signaling could modulate the balance between T-regs and effector T cells, resulting in tipping the balance towards antitumor immunity.

Since a detailed understanding of how OX40 signaling turns off T-reg function may provide a useful approach for exploring of how suppression itself is manifested. Possible therapeutic implications of these findings will be promising. Moreover, it is possible that additional work on OX40 in T-regs may lead to the development of new strategies for augmenting or decreasing suppression in situations where such manipulations might afford therapeutic benefits such as decreasing tumor immune tolerance or increasing immune response to tumors.

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