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Research Article

Increased P-35, EBI3 Transcripts and Other Treg Markers in Peripheral Blood Mononuclear Cells of Breast Cancer Patients with Different clinical Stages

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Abstract

Purpose: Currently, cancer as a major problem around the world threatens human health and has a high incidence in developing countries. Many reports have indicated that patients suffering from cancer demonstrate decreased antitumor immune responses as well as a high prevalence of T regulatory population. It has been reported that $Foxp3^{+}T_{regs}$ exert suppression by cell contact-dependent mechanisms which are mediated by soluble factors such as immunosuppressive cytokines like IL-10, TGF-β and IL-35. Consequently there is a great need to identify prognostic and diagnostic biomarkers of regulatory T cells for vaccine and drug development.

Methods: In this study IL-10, TGF-β, IL-35 and Foxp3 mRNA gene expression has been measured in peripheral blood of 40 breast cancer patients and 40 normal age-matched women using quantitative real-time PCR (qRT-PCR) method with Master Mix reaction containing SYBER Green. GAPDH gene was used as housekeeping gene.

Results: Our data demonstrated a significant up-regulation of IL-10, TGF-β, P35, EBI3 and Foxp3 gene expression in patients' peripheral blood compared to normal healthy controls ($p<0.05$).

Conclusion: The data suggests that the immune system is suppressed in breast cancer patients, which may be due to elevated T_{reg} cells population. These results may be useful for diagnostic or therapeutic purposes. However it may require more investigations

Introduction

Breast cancer is by far the most common cancer in women worldwide, ranking second when both sexes are considered. It is estimated that about 1.38 million of women around the world suffered from breast cancer in 2008, which accounts for nearly a quarter (23%) of all cancers in women.[1,2] Iran with annually 8 per 100,000 affected women has the highest status of breast cancer incidence in the Middle East.[3] According to the most recent report from Iranian Ministry of Health and Medical Education in 2009, 525 new cases of all Iranian breast cancer (23.1%) have been founded in Khuzestan province.

The role of healthy immune system in controlling the progress of malignant disease is well established. This means that immune suppression along with cancer is the major cause of cancer progression.[4] Regulatory T cells (T_{reg} cells) are a subset of $CD4^{+}$ T cells that are necessary for monitoring auto inflammatory diseases.

They also prevent the antitumor immune responses, but the in vivo mechanisms of prevention have not been well recognized. Thus, the prominent aims of immunotherapy undoubtedly are the regulation of T_{reg} cell activity and *ex vivo* generation of T_{reg} cells. There known to be two main subsets of T_{reg} cells: naturally occurring, thymic-derived $CD4^{+}T_{reg}$cells and peripherally antigen-induced $CD4^{+}$ T cells deriving from $CD4^{+}CD25^{-}$ non-T_{reg} cells. Regulatory T cells comprise approximately 5-10% of $CD4^{+}$ T cells.[5-8] In spite of their limited number, T_{reg} cells have a key role in immune homeostasis. Natural Regulatory T cells as a small population help to keep the immune system in balance by a process called infectious tolerance. This is an in vivo process in which non-T_{reg} cells convert to regulatory and suppressor cells. Although the mechanism of infectious tolerance remains to be unclear, the immunomodulatory cytokines such as transforming growth factor-β (TGF-β) and interleukin 10 (IL-10) have both important roles in this process.[9]

Induced regulatory T cells (iT_R cells) can be induced both by peripheral conventional $CD4^{+}Foxp3^{-}$ T cells (T_{conv} cells) and by *in vitro* exposure to antigen. Two

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major classes of iT_R cells have recently been described based on the cytokines that stimulate their production: TGF-β-iT_R and IL-10-iT_R cells. TGF-β-iT_R cells are generated by T_{conv} cells activation in the presence of TGB-β. These cells mainly secrete TGF-β and also express Forkhead/winged helix transcription factor (Foxp3). IL-10-iT_Rcells are produced by activation of T_{conv} cells in the presence of IL-10. These cells do not express Foxp3 but are known by immense secretion of IL-10.[10,11]

Interleukin-35 (IL-35) as a novel inhibitory cytokine is a new member of IL-12 cytokine family (which contains IL-12, IL-23 and IL-27). IL-35 is a heterodimer cytokine which consist of EBI3 (Epstein-Barr virus-induced gene 3 protein) and IL-12A (IL-12 subunit p35).[8] IL-35 is a T_{reg} cell-specific cytokine that is essential for the maximum regulatory and suppressive function of T_{reg} cells under both *in vivo* and *in vitro* conditions.[12] IL-35 which is not expressed by effector or naive T cells, but considerably expressed in T_{reg} cells induces proliferation and activation of Treg cells as well as reducing Th17 cells activity.[13] Several studies have been performed on IL-35 effects on T_{reg} $CD4^+CD25^+Foxp3^+$ cells (isolated from spleen and lymph nodes of BALB/C mice) which demonstrate that induction of T_{reg} $CD4^+CD25^+Foxp3^+$ cell may be responsible for high level production of IL -10.[8] Many other observations suggest that T_{reg} cells have an important role in immune evasion mechanisms used by cancer.[14] Various tumors abolish antitumor immunity through a variety of mechanisms such as differentiating, developing and activating T_{reg} cells.[15] Tumor T_{reg} cells may facilitate local tumor growth, and also could be associated with systemic disease progression in peripheral blood or lymphoid organs.[15,16] It is believed that Treg cells (also defined as $CD4^+CD25^+Foxp3^+$ T cells) presence is inversely associated with the development of numerous human malignancies.[17] Thus, T_{reg} cells may be considered as the main barrier for cancer immunotherapy. Moreover detection of a well-defined population of T_{reg} cells is crucial for a favorable cell-based immunotherapy.

Due to the mentioned evidence and the importance of T_{reg} cells cytokines in breast cancer, we have determined T_{reg} cytokines and surface marker mRNA transcripts by applying quantitative real time PCR (qRT-PCR) to quantify mRNA transcripts in peripheral blood cells of women with different grades of breast cancer and normal healthy controls.

Materials and Methods

Subjects

Blood samples from 40 women with invasive ductal carcinoma of breast cancer were collected during 2010. The disease was histologically confirmed by the surgery department of Apadana and Emam Khomeini hospitals, Ahvaz, Iran. All the cases signed an informed consent of participation in the study. All breast cancer patients were newly diagnosed cases which did not receive chemotherapy, radiotherapy or immunotherapy before sampling. Hospital reports provided the required data of patients' histological grade, clinical stage and metastasis. Table 1 illustrates that all patients had been chosen according to clinical criteria. The majority of the patients belonged to the group with Low-grade, early stage (I, II) and no metastasis. Peripheral venous blood samples of 2mL each were collected in EDTA vials as an anticoagulant before any intervention. Blood samples from 40 healthy volunteer women without any history of malignancy or autoimmune disorders were also utilized as normal control. The mean age of patients and control group were 51years (range, 25-81) and 45 years (range, 23-68) respectively.

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

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 Isolation and Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted from blood cells using RBC lysis and RNX-Plus reagent (Cinnagen, Iran) according to the manufacturer's instructions. The resulting pellet was resuspended in 100 µL of cold PBS, followed by adding l ml RNX plus reagent. Subsequently it was gently mixed until the solution was completely transparent. Adding 300 µL of chloroform, solution was centrifuged at 14,000 rpm and 4°C for 20 min. 300 µL isopropanol and 700 µL ethanol were added in next step follow by centrifuging at 12,000 rpm for 30 min. Afterward supernatant was discarded, the pellet was dried completely, dissolved in 50 µl of DEPC treated water and located in 55-60 °C of water bath for 10 min.

RNA quality was assessed by 1% agarose gel electrophoresis, and the optical density of the extracted RNA samples was estimated at 260 nm and 280 nm by spectrophotometer (eppendorf-Biophotometer-Germany). First-strand cDNA synthesis was performed 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 FOXP3, IL10, TGF-β, EBI3 and P35 gene transcripts were assessed by a triplicate determination of qRT-PCR (Applied Biosystems Step one™ and Step one Plus™ Real Time PCR systems). Expression levels of GAPDH as a housekeeping gene was measured as a reference level of target gene

expression. Each PCR reaction was performed in a final volume of 20 µL including 1 µg of the cDNA product, 1 µL (50 nm) of each primer and 2 × reaction mixtures containing Fast Start DNA polymerase, reaction buffer, dNTPs, and SYBR green I (Applied Biosystems). Table 2 shows forward and reverse primer sequences used for qRT-PCR reactions. The thermal cycling conditions of all genes comprised a temperature profile of 95°C for 10 min for denaturation step, followed by 40 cycles (including 95°C for 15 s and 60˚C for 60 min). The qRT-PCR amplification products were analyzed by melting curve analysis and 1% agarose gel electrophoresis.

Table 2. Forward and reverse primers of genes for real-time PCR amplification

Primer		Sequence
GAPDH	Forward	GCAAGAGCACAAGAGGAAGA
GAPDH	Reverse	ACTGTGAGGAGGGGAGATTC
Foxp3	Forward	ACAGTCTCTGGAGCAGCAGC
Foxp3	Reverse	CCACAGATGAAGCCTTGGTC
IL-10	Forward	ACTTTAAGGGTTACCTGGGTTGC
IL-10	Reverse	TCACATGCGCCTTGATGTCTG
TGF-β	Forward	CCCAGCATCTGCAAAGCTC
TGF-β	Reverse	GTCAATGTACAGCTGCCGCA
EBI3	Forward	TGGCGGCTCAGGACCTCACA
EBI3	Reverse	GGGGCTTAGGGTGGCGAGGA
P35	Forward	GCAGCCTCCTCCTTGTGG
P35	Reverse	GGGAACATTCCTGGGTCTGG

Amplification Efficiency and Standard Curve Analysis

Real-time PCR reaction Efficiency for each target gene was estimated by the slope of the standard curve. Standard curves were designated by Ct values of cDNA serial dilutions. The efficiencies for GAPDH, Foxp3, IL10, TGF-β, EBI3 and P35 were 91%, 99%, 1.0, 99%, 96% and 97% respectively. The relative expression of gene transcripts were calculated by ΔCt and $2^{(-\Delta Ct)}$ formulas. Finally, the ratios of target to reference gene were determined with the Pfaffl method.[18]

Statistical Analysis

The expression levels of FOXP3, IL-10, TGF-β, EBI3 and P35 transcripts in peripheral blood were compared with the corresponding values of control samples with nonparametric Mann-Whitney test using SPSS software v. 11.5. Relative expression was also calculated and analyzed by Prism 6 software (Graphpad software Inc; 2236 Avenida de la Playa La Jolla, CA 92037 USA). The relationships between different values were examined using Pearson's correlation coefficient and Spearman's rank correlation tests. For all the statistical analysis, p values less than 0.05 was considered as statistically significant.

Results

Foxp3 Gene Expression

The result of our study provided evidence that relative expression of Foxp3 gene transcript in different grades of breast cancer patients was not significantly different (p>0.80). However, its expression was about 12-fold higher than healthy individuals (p = 0.0013). Moreover, breast cancer patients of early-stage showed a significant increase in mean relative expression of Foxp3 (p=0.0019). There was also an increased expression of Foxp3 in non-metastatic patients (p = 0.0014) as well as the patients with a low-grade tumor burden (p = 0.0012) (Figure 1A).

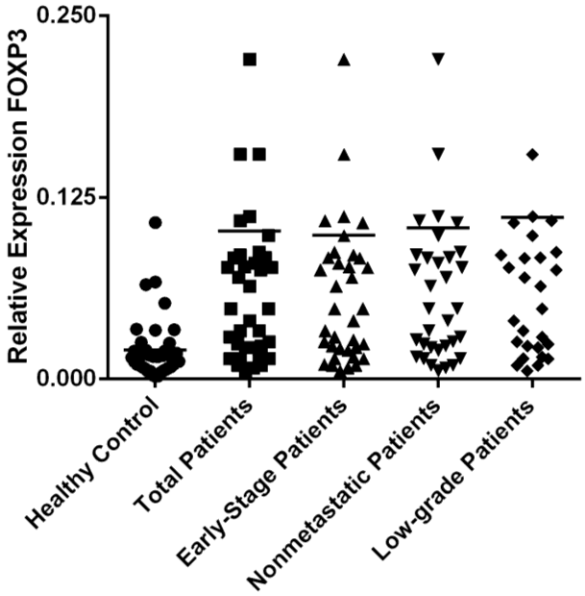

Figure 1A. Expression level of Foxp3 in peripheral blood cells of breast cancer patients and normal controls. Presented data were calculated with the $_2^{(-\Delta CT)}$ and analyzed with the nonparametric two-tailed Mann-Whitney test. Horizontal bars indicate median values.

IL-10 Gene Expression

Our findings implied that no significant differences was detected in relative expression of IL-10 among different groups of breast cancer patients (p = 0.40). Nevertheless the relative expression of IL-10 was about 20-fold higher in patients in comparison with healthy individuals (p=0.024) (Figure 1B). The expression level of IL-10 in early-stage, non-metastatic and low-grade patients were significantly increased compared to healthy individuals and p value was 0.0001, 0.0229 and 0.0271 respectively.

TGF-β Gene Expression

There was not any significant differences in relative expression of TGF-β gene transcript in different grades of breast cancer patients (p = 0.30). Yet, the relative expression of TGF-β in patients was significantly higher in relation to healthy volunteers (p = 0.0075) (Figure 1C). For patients with early stage of breast cancer, relative expression of TGF-β was about 15-fold higher when compared to control group (p = 0.0006). Similarly,

TGF-β expression in patients with non-metastatic disease and the ones with low-grade tumors increased up to 10-fold (p = 0.0081) and 12-fold (p = 0.0025) respectively when they compared to healthy control group.

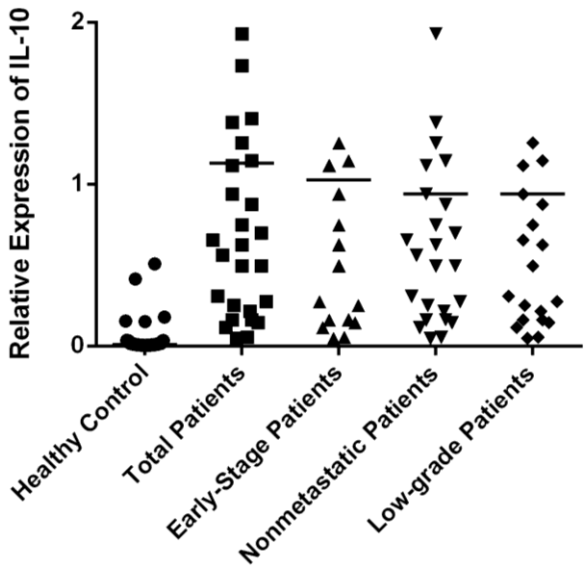

Figure 1B. Expression level of IL-10 in peripheral blood cells of breast cancer patients and normal controls. Presented data were calculated and analyzed as explained in Figure 1A.

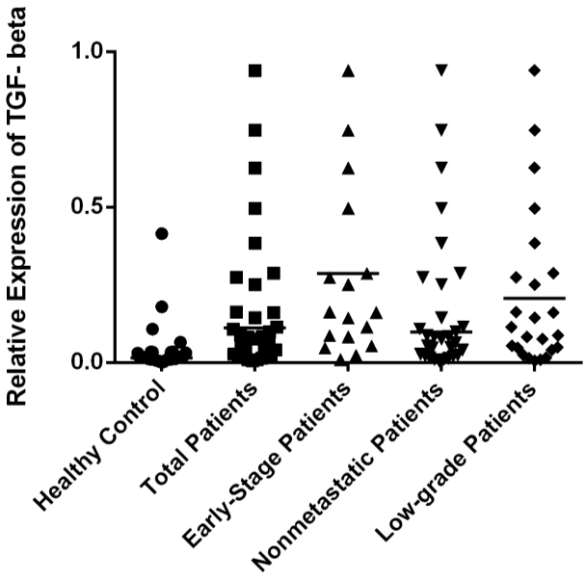

Figure 1C. Expression level of TGF-β in peripheral blood cells of breast cancer patients and normal controls. Presented data were calculated and analyzed as explained in Figure 1A.

EBI3 Gene Expression

Although the comparative statistical analysis of EBI3 gene transcript did not reveal any significant differences among different grades of breast cancer patients (p >0.50) it was significantly higher compared to normal cases (about 18-fold) (p = 0.0008). Still, Increased EBI3 expression was not found in any metastatic (p = 0.0019) or low-grade tumor burden patients (p = 0.0012) (Figure 1D). In addition, patients with the early-stage disease showed a significant increase in mean relative expression of EBI3 gene in relation to normal subjects (p = 0.0055).

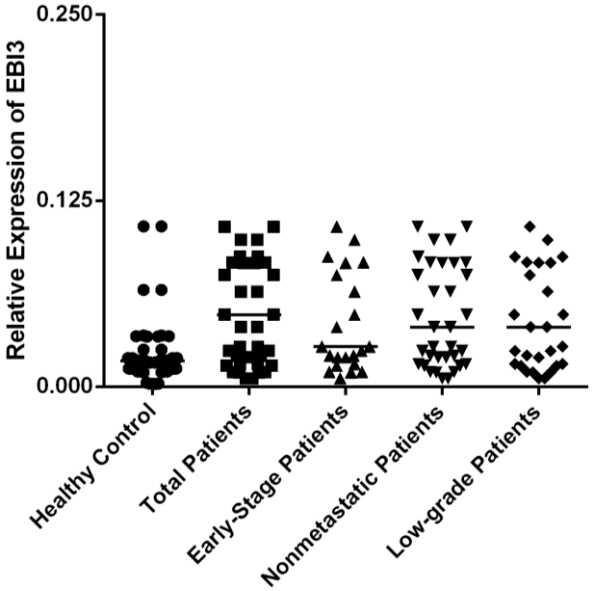

Figure 1D. Expression level of EBI3 in peripheral blood cells of breast cancer patients and normal controls. Presented data were calculated and analyzed as explained in Figure 1A.

P35 Gene Expression

We also observed no significant differences in relative expression of P35 in different grades of breast cancer patients (p >0.40) but the relative expression of P35 in all patients demonstrate 20-fold increase regarding control group (p = 0.0247). In other groups including early-stage, non- metastatic and low-grade patients high expression of P35 was noticeable as well (p = 0.0001, 0.0227, 0.0295) respectively (Figure 1E).

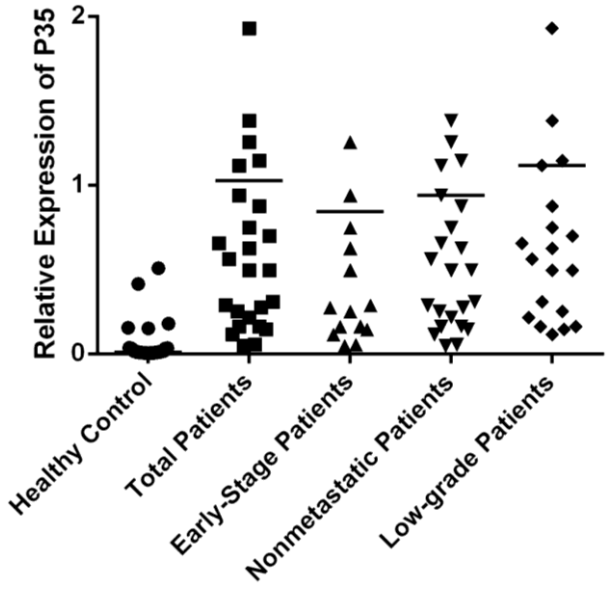

Figure 1E. Expression level of P35 in peripheral blood cells of breast cancer patients and normal controls. Presented data were calculated and analyzed as explained in Figure 1A.

Discussion

According to several researches, immune system interaction and regulatory T cells tumor immunity status, and their cytokines and stimulatory molecules are very important in peripheral blood and tumor micro-environment. One of the essential points to understand the mechanism of T_{reg} suppressive function is recognizing the T_{reg} gene expression. Likewise improving treatment effects in immune disorder also requires identifying multiple gene expression transcripts assay. Consequently in current study we investigated expression of multiple genes of T_{reg} cells in cancer patients and compared their expression with other features of clinical outcome before surgery.

Realizing the mechanisms by which T_{reg} cells exert their suppressive function is an essential step in elucidating their role in control of cancer progression and other immune system-mediated disorders. For this purpose, studying T_{reg} genes expression, by using multiple genes transcripts assay, may be a useful tool to improve the effect of different treatments for immune-mediated disorders. It has been shown that Treg cells are generated in the periphery and exert their suppressor activity mainly by producing IL-10, IL-35, and TGF-β.[8,19] The results of this study showed a significant up-regulation of IL-10, TGF-β, p-35, EBI3 and Foxp3 gene expression in peripheral blood of patients compared to normal healthy controls. These data suggest that the immune system is suppressed in breast cancer patients, which may due to an augmentation in T_{reg} cells population.

IL-10 has a complex role in tumor biology; therefore it may be an important growth factor for cancer cells.[20] IL-10 has a major role in maintenance and increase of Tregs.[21]

IL-10 induces regulatory T cells by involving several events in antigen presenting cells, such as reducing antigen presentation by trapping peptide-loaded major histocompatibility complex type II (MHCII) molecules, reducing the co-stimulatory molecules CD80/CD86 expression, and destabilizing cytokine mRNAs.[22] On the other hand, it can also strengthen the innate immune mechanisms that destroy cancer cells.[23] Wang and colleagues examined 63 lung cancer tissues in 2011. They purified tumors associated macrophages and found that there is a direct correlation between tumor progression and macrophages activity. Thus they concluded that mass production of IL10 by macrophages facilitates tumor progression and lymph node metastasis.[24] Furthermore Merendino et al, reported a correlation between IL-10 expression levels and clinical stages of breast cancer, and suggested that Neoplasia-Metastatic Disease are associated with higher IL-10 levels compared with patients with non-metastatic diseases.[25] In this study we found a significant increase in IL-10 transcripts even at early stages of breast cancer which discovered to be stage-dependent.

Tumor cells and regulatory T cells produce high levels of TGF-β in cancer patients. Besides, patients may also have high levels of plasma TGF-β after surgery. Even cancer can stimulate the production of TGF- β. It has been reported that TGF-β can facilitates the conversion of T cells into Tregs. TGF-β can turn off its expression by binding to the inhibitory Smad7 promoter region which may result in cumulative Foxp3 expression.[26]

Nam and et al, have recently revealed that TGF-β stimulates T $CD8^+$ cells to promote its penetration in the tumor environment and induce IL-17, IL17-IL17R expression which act as survival factors for tumor cells.[27] TGF-β inhibition in cancer therapy has been known so far because both radiation and chemotherapy are capable of inducing TGF-β activity. TGF-β signaling and cross-talk with other signaling networks can induce cancer cell survival and help them to become more aggressive.[28] Thus, inhibition of TGF-β signaling could be an alternative therapy for cancer patients.[29]

Many investigations have recently shown that loss of TGF-β inhibitors and increased TGF-β gene expression are associated with the tumor aggressiveness and would promotes breast, colon, endometrial, ovarian, cervical, and melanoma tumor progression. However specific mutations in components of TGF-β signal transduction pathways only occur in breast cancer.[30] In this study we found that TGF-β transcription has a significantly stage-dependent increase even during early stages of breast cancer.

IL35 as a new cytokine that is highly expressed in T_{reg} cells, can induce the differentiation and proliferation of T_{reg} cells.[31] Besides, other investigations suggested that IL-35 is essential for repressive functions of T_{reg} cells under *in vitro* and *in vivo* conditions.[8] $CD4^+$ $CD25^+$ T cells are induced by IL-35, express Foxp3 marker and produce a large amount of IL-10 which is a specific features of T_{regs}.[13] Little investigation has been done on IL-35 so far, but a recent study conducted by Collison et al. in 2010 proved that, when human and mouse T cells are in presence of IL-35, a population of T cells is induced by IL-35 which is defined as (iT_{reg} 35). These findings suggest an inhibitory function of these cells which are mediated by IL-35. Moreover they act independently from IL-10 and TGF-β, and do not express Foxp3 marker. Collison and his colleagues also reported that, IL-35 induced T_{reg} cells (iT_{reg} 35) potentially impose repressive function in IBD and EAE animal models under both *in vivo* and *in vitro* conditions. In addition human IL-35 producing $iT_{reg}35$ can secret IL-35 which displays suppressive functions.[32] Therefore it seems that IL-35; along with regulatory T cells confer suppressive function. In present study we observed significant increase of IL-35 expression in breast cancer patients.

Conclusion

Breast cancer with its heterogeneous nature and lacking well-established markers for prediction and prognosis is highly complicated disease. T_{regs} are one of the major players in this scenario. The results demonstrate that Foxp3, IL-10, TGF-β and IL-35 genes expression is positively correlated with prognosis, recurrent metastatic and effective treatment response of the disease. Further

studies are therefore required to more evidently confirm the role of Tregs markers in breast cancer.

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Ethical Issues
Not applicable.

Conflict of Interest
The authors declare no conflict of interest.

Abbreviations
EBI3 Epstein-Barr virus-induced gene 3 protein
Foxp3 Forkhead/winged helix transcription factor
IL-10 Interleukin-10
IL-35 Interleukin-35
qRT- PCR quantitative real-time PCR
TGF-β Transforming growth factor- beta

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