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Increased IL-17 and IL-6 Transcripts in Peripheral Blood Mononuclear Cells: Implication for a Robust Proinflammatory Response in Early Stages of Breast Cancer

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Abstract

Background: Several recent studies demonstrated that transforming growth factor beta (TGF-β), by stimulating T regulatory cells, and interleukins 6 and 17 (IL-6, IL-17), by inducing inflammatory reactions, may be critical factors in cancer pathogenesis.

Methods: We used quantitative real-time polymerase chain reaction assays to quantify the expression of IL-17, IL-6 and TGF-β mRNA in peripheral blood mononuclear cells and lymphocytes from draining lymph nodes of 60 women with breast cancer. The results were compared according to the patients' clinical or pathological status.

Results: Higher amounts of IL-17 and IL-6 mRNA, but not TGF-β transcripts, were found in patients compared to controls. There were no significant differences between patients with negative or positive nodes or with different histological grades or stages of disease.

Conclusion: Most women in this analysis had stage I or II disease. We thus conclude that IL-17, a prominent proinflammatory cytokine, may play an important role in recruiting and infiltrating antitumor immune responses in early stages of breast cancer.

Keywords: Breast cancer, Interleukin-6, Interleukin-17, Transforming growth factor beta

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Introduction

The classification of CD4+ T lymphocytes into either T helper 1 or T helper 2 (Th1, Th2) subsets, reported earlier by Mossman and Coffman,[1] was recently modified by the addition of Th17 as a third subset of CD4+ T cells. The main feature of this subset is its release of interleukin 17 (IL-17).[2-4] Recent reports of the

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role and function of Th17 cells indicate that this subset of CD4+ T cells plays a fundamental role in the infiltration and recruitment of inflammatory cells against intercellular parasites and fungi.[5] Recently, a role for this subset was reported in certain Th1-mediated autoimmune diseases such as rheumatoid arthritis and multiple sclerosis.[6]

Several recent studies demonstrated that transforming growth factor beta (TGF-β) and IL-6, but not IL-23, are critical factors for murine Th17 cell differentiation in vitro.[7-8] It appears that TGF-β plays an essential role in the plasticity of CD4+ T cells to T regulatory (Treg) or Th17 cells. Together, TGF-β and IL-6 have a synergistic effect on the differentiation of Th17 cells from CD4+ T cells.[7-8] In contrast, TGF-β in combination with retinoic acid may halt Th17 cell differentiation in favor of Treg cell generation.[9,10] It was recently shown that interaction between cytotoxic T-lymphocyte antigen 4 and the B7 costimulatory molecule inhibits the differentiation of Th17 from naïve CD4+ T cells.[11]

Interleukin-1β has been shown to play a critical role in murine Th17 differentiation.[12] The biological activity of Th17 cells, which show a range of activities in defense against infection, autoimmunity and more importantly in cancer, occurs through the release of IL-17, a potent multifunctional cytokine. Apart from synthesis by Th17, IL-17 may also be produced by cells such as CD8 T, natural killer T cells, epithelial cells and cells from the innate immune system (for a review see13). It seems that IL-17 acts as an interface between the inflammatory response and cell-mediated immunity in cases of cancer and infectious diseases. To promote the inflammatory reaction, IL-17 enhances the production of IL-6, IL-1β and TNF-α.[14] Inflammatory reactions in early stages of most malignancies are considered a first line of defense in the host's immune response against cancer cells.[15] Although the molecular mechanisms of this response are unknown, proinflammatory cytokines such as IL-6 and TNF-α appear to be involved. In this respect, the role of IL-17 in recruiting inflammatory cells and potentiating inflammation is indispensible. Several factors and mediators released by tumor cells or tumor stroma may be significant in triggering Th17 and consequently the synthesis and release of IL-17. Factors such as TGF-β, IL-6, progstaglandin E2, IL-21, IL-23, IL-1β and TNF-α play major roles in the induction of Th17 differentiation.[16-19]

Despite the known roles of IL-17 in enhancing inflammatory reactions, reports on the role and involvement of this cytokine in cancer cell growth and development are inconsistent.[20] Increased growth and proliferation of cervical cancer cells through IL-6,[21] increased blood vessel development in ovarian cancer,[22] and the potential to act as a prognostic biomarker for the progression of colorectal cancer have been noted.[23] Apart from its tumor-promoting activity, antitumor functions of IL-17 have also been reported. For example, Muranski et al. found that the release of IL-17 by Th17-polarized cells was more effective than by Th1 cells in the elimination of established tumors.[24] In addition, IL-17 has been shown to induce IL-6 and IL-12 from a variety of cells and to induce tumor-specific cytotoxic lymphocyte generation.[25] Other research has found that IL-17 affects the overexpression of MHC class I and II[26] and plays a role in dendritic cell maturation.[27]

Breast cancer is the most common cancer among Iranian females, with a mean age onset at least 10 years younger than in Western countries.[28] The role of IL-17 in breast cancer pathogenesis is not fully understood. In this study we report the expression of IL-17, IL-6 and TGF-β mRNA in peripheral blood mononuclear cells (PBMC) and lymphocytes from draining lymph nodes of patients with breast cancer. We compared the results according to the patients' clinical or pathological status.

Materials and Methods

Participants

The 60 women who participated in this study were diagnosed with infiltrating ductal carcinoma of the breast, confirmed by histological studies. All patients were referred to our laboratory from the

Table 1. Forward and reverse primers of β-actin, IL-6, TGF-β and IL-17 genes for real-time PCR amplification. Sequences were designed by Primer 3 software (SourceForge, Geeknet).

Primer	Sequence
β-actin forward	ACAGAGCCTCGCCTTTGCCG
β-actin reverse	CACCATCACGCCCTGGTGCC
IL-17 forward	GGACTGTGATGGTCAACCTG
IL-17 reverse	CTCCCAGATCACAGAGGGAT
IL-6 forward	CAGGTTGTTTTCTGCCAGTG
IL-6 reverse	GACCGACACTCACCTCTTCA
TGF-β forward	TGGTTGAGCCGTGGAGGGGA
TGF-β reverse	CTCGGCGGCCGGTAGTGAAG

Breast Clinic of Shiraz University of Medical Sciences (Shiraz, Iran) during a 1-year period from 2008 to 2009. All patients provided their informed consent to take part in this study.

Peripheral blood samples (2 mL), with EDTA were collected before any clinical intervention. In addition, we obtained lymph nodes from 26 patients of which 9 were positive and 17 were negative for tumor infiltration. None of the patients received chemotherapy, radiotherapy or immunotherapy before sampling. The expression of IL-6 and TGF-β genes in patients was compared to expression in 37 healthy volunteer women with no history of malignancies or autoimmune disorders. The expression of IL-17 was compared to that in 70 healthy volunteer women. Mean age was 51 years in the patients and 45 years in healthy controls.

RNA isolation and reverse transcription

Total RNA was extracted from blood and lymph nodes after lysis of the red blood cells with ammonium chloride and TRizol reagent (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. To avoid DNA contamination, RNA was treated with DNase I (Invitrogen-Gibco, Paisley, UK) before cDNA synthesis. To synthesize cDNA, we used 5 μg of total RNA and the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania).

Quantitative real-time polymerase chain reaction

The quantities and expression of IL-6, TGF-β and IL-17 gene transcripts was determined with a Bio-Rad system (Chromo4 Real-time PCR Detector, Bio-Rad, Foster City, CA, USA) for quantitative real-time PCR (qRT-PCR) and SYBER Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Expression of the β-actin housekeeping gene was used to normalize the level of target gene expression. Each PCR reaction was carried out in a final volume of 25 μL that contained 0.5 μg of the cDNA product, 2.0 pmol of each primer, and 1× reaction mixture which consisted of FastStart DNA polymerase, deoxynucleoside triphosphate, reaction buffer and SYBR Green I. Thermal cycling for all genes was done with a denaturation step at 95 °C for 10 min, followed by 40 cycles (denaturation at 95 °C for 15 s, annealing at 56 °C for 30 s and extension at 60 °C for 60 s). The qRT-PCR amplification products were examined by melting curve analysis and 1% agarose gel electrophoresis (data not shown).

Table 1 shows the forward and reverse primers for β-actin, IL-6, TGF-β and IL-17 genes. All primers were designed with Primer 3 software (SourceForge, Geeknet Inc. http://sourceforge.net/about, Mountain View, CA, USA).

Statistical analysis

The amounts of IL-6, TGF-β and IL-17 gene transcripts in peripheral blood were compared to the corresponding values from control samples with the nonparametric Mann-Whitney test in SPSS v. 11.5 software (SPSS, Chicago, IL, USA). The relative amounts of IL-6, TGF-β, and IL-17 transcripts were determined from ΔCt and 2−ΔCt formulas. Target-to-reference gene ratios were

calculated with the Pfaffl method.[29] Relative expressions were plotted and evaluated with Prism v. 5 software (GraphPad Software, San Diego, CA, USA). $P<0.05$ was regarded as significant in all statistical analyses.

Results

Clinical and pathological characteristics

Data on age, tumor histology, tumor size, clinical stage, histological grade, lymph node involvement and distant metastases were obtained from the hospital records of all 60 patients. Clinical stage was determined with the tumor-node-metastasis classification. Table 2 shows the distribution of patients according to different clinical criteria.

IL-6 gene expression

Expression of the IL-6 transcript in PBMC was significantly higher in patients than in healthy controls ($P=0.0001$, Figure 1). The level of IL-6 expression was similar in positive and negative nodes. No correlation was found between the level of IL-6 gene expression and stage, grade, estrogen receptor, progesterone receptor or human epidermal growth factor receptor expression (data not shown).

TGF-β gene expression

Expression of the TGF-β gene transcript in patients with breast cancer did not differ compared to the control group ($P=0.5$, Figure 2). Expression in positive and negative lymph nodes was similar. We noted no correlation between TGF- β gene expression and clinical or pathological characteristics in women with breast cancer (data not shown).

IL-17 gene expression

Expression of the IL-17 gene was increased 12-fold in patients compared to controls ($P<0.0001$; Figure 3). However, there were no significant differences in IL-17 gene expression between positive and negative nodes ($P>0.6$). There was no significant association between IL-17 expression and any of the clinical and pathological characteristics.

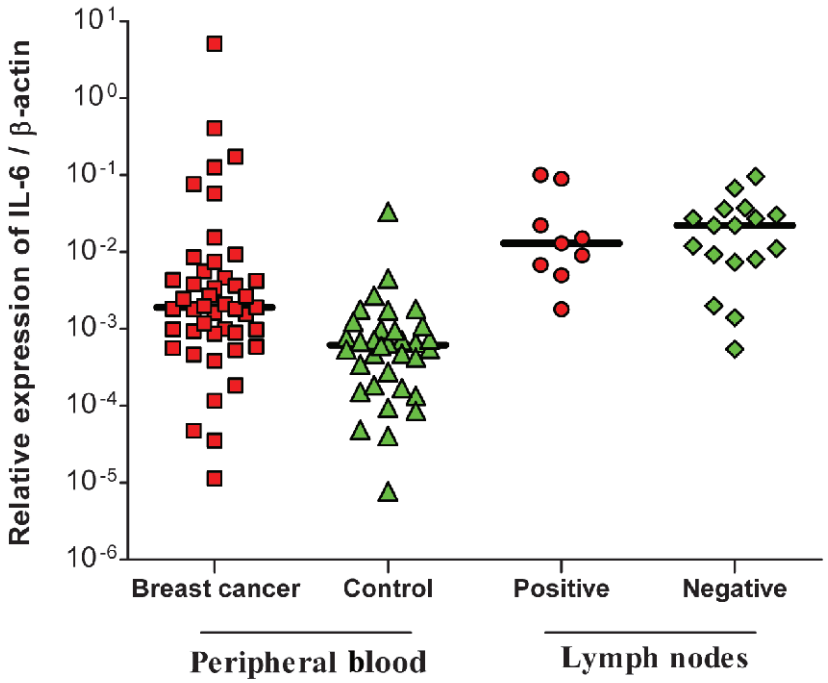

Figure 1. IL-6 gene transcripts in the peripheral blood of breast cancer patients and healthy controls, and cells from draining lymph nodes. The data were calculated with the 2−ΔCt formula and analyzed statistically with the nonparametric two-tailed Mann-Whitney test. Significant differences were found in the levels of IL-6 expression in peripheral blood mononuclear cells from patients compared to healthy controls ($P=0.0001$). However, IL-6 transcripts were similar in positive and negative lymph nodes.

Discussion

We analyzed the expression of IL-17, IL-6 and TGF-β transcripts in PBMC and lymphocytes from draining lymph nodes of patients with breast cancer. Our data indicated higher expressions of IL-17 and IL-6, but not TGF-β, in patients compared to controls. The expression of IL-17 mRNA was investigated by Zhang et al., who detected increased expression of IL-17 and IL-23 mRNA in tumor tissues from patients with gastric cancer. These authors suggested that Th17 cell differentiation may increase in gastric cancer.[30] Zhu et al. reported that IL-17 expression in breast cancer tissue is mostly restricted to macrophages, a finding which the authors interpreted as evidence of a possible role for IL-17 released by macrophages in promoting tumor progression and invasion.[31] Wang et al. reported that IL-17 might promote tumor cell growth, an effect mediated through IL-17-induced IL-6 release via activation of the signal transducer and activator of transcription 3 factor (STAT 3), both in tumor cells and nonmalignant stromal cells.[32] Kato et al. reported high expression of IL-17 mRNA in

Table 2. Patient distribution according to different clinical criteria.

Pathological characteristic		Frequency (n=60)
Grade		
	High	55
	Low	5
Stage		
	I	20
	II	27
	III	11
	IV	2
Metastases		
	Positive	2
	Negative	58
Side		
	Left	29
	Right	31
ER		
	Positive	39
	Negative	21
PR		
	Positive	41
	Negative	19
HER-2		
	Positive	35
	Negative	25

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor.

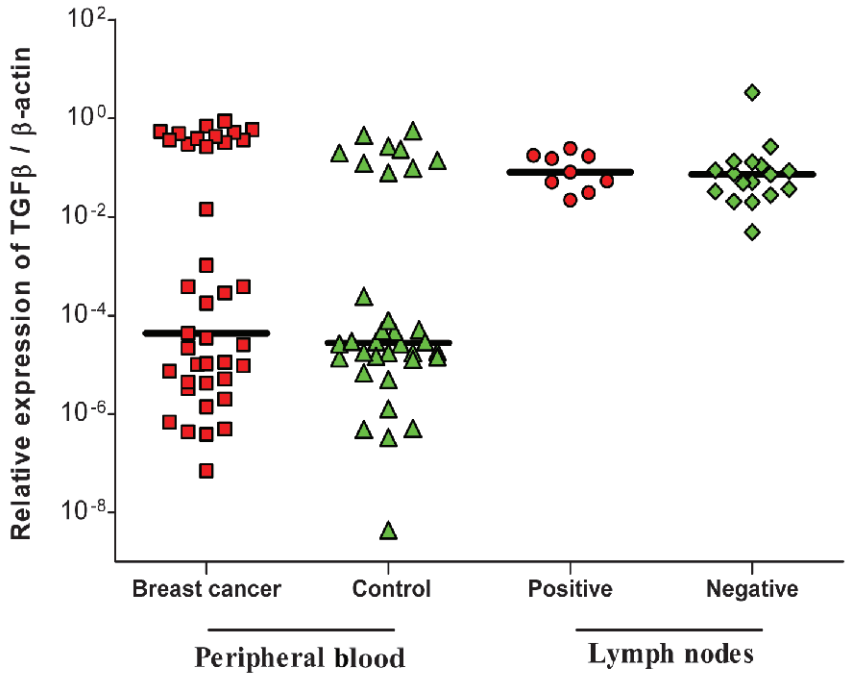

Figure 2. Expression levels of TGF-β in the peripheral blood of patients and healthy controls, and lymphocytes from draining lymph nodes. The data were calculated with the 2−ΔCt formula and analyzed statistically with the nonparametric two-tailed Mann-Whitney test. No significant differences were found in TGF-β expression between patients vs. controls or between lymphocytes from draining vs. nondraining nodes.

ovarian cancer, with a significant role in tumor growth and angiogenesis.[22] Radosavljevic et al. found no detectable IL-17 serum protein in colorectal cancer, and reported a heterogeneous pattern for IL-17 protein expression in tumor tissue.[23]

The above studies document evidence that IL-17 is a multifunctional cytokine able to promote tumor growth and expansion. The basis for this concept originated from the detection of IL-17 at the protein or transcript level. In addition, IL-17 is produced mostly by Th17 in a physiological manner in response to intercellular parasites, and consequently potentiates and enhances inflammatory cells at the site of infection or injury.

The effect of Th17 should be considered within the context of Treg function, as the two cell subsets of the immune response have evolved to fine-tune immune suppression versus immune potentiation. In this scenario, our finding of increased expressions of both IL-6 and IL-17 in PBMC from a group of patients that contained many women in the early stages of breast cancer can be interpreted as a reflection of a protective proinflammatory response. This response aims to support the systemic recruitment of the host's immune cells to the site of early initiation of a malignant transformation in breast tissue. Although the role of inflammation in the initiation of cancer is controversial, most evidence from malignancies associated with the gastrointestinal tract strongly support the importance of inflammation.[15,35]

The increased expression of both IL-6 and IL-17 in our study is unsurprising, as IL-17 is capable of inducing IL-6 from various cells to support a proinflammatory reaction. Increased amounts of IL-6 have been reported in the late stages of several malignancies, including metastatic breast cancer.[36] In this connection, the role of IL-6 in early and late stages of cancer requires further study. It remains to be determined whether IL-6 is produced by two different pathways: one during the inflammatory phase in response to early-stage tumor growth, and the other as sustained release by tumor cells during the progressive and

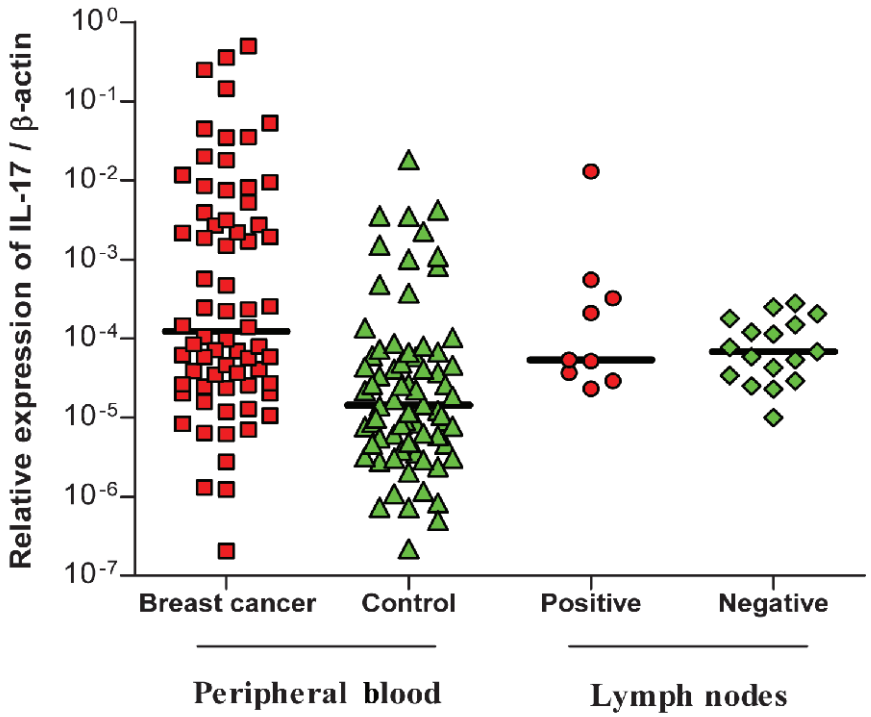

Figure 3. Expression levels of IL-17 in the peripheral blood of patients with breast cancer, controls and cells from draining lymph nodes (positive and negative). The data were calculated with the 2−ΔCt formula and analyzed statistically with the nonparametric two-tailed Mann-Whitney test. Significant differences were found in the levels of IL-17 expression in peripheral blood mononuclear cells from patients and healthy controls (P<0.0001). However, IL-17 transcripts were similar in positive and negative nodes.

metastatic phases. As cancer progresses, particularly in the late stage and possibly in metastatic phases, pressure by tumor cells, the release of self-antigens and the emergence of Treg cells occur. It may thus be inevitable for the anticancer proinflammatory reaction to be converted into an anti-inflammatory condition, resulting in disease deterioration. If this hypothesis is correct, then early immunotherapy during the early stages of breast cancer may be the most beneficial for patients, before the immune system has been placed under pressure by tumor cells or before the immunosuppression induced by Treg cells occurs.

Both tumor and Treg cells release TGF-β during the late stages of most solid cancers.[37] The absence of an increase in TGF-β expression in our study confirms that in the early stages of breast cancer, the patient's immune system is still competent and has not been influenced by the waves of Treg suppression. If reduced TGF-β expression and increased IL-6 and IL-17 expression are confirmed in the early stages of breast cancer in single- or multicenter studies with larger sample sizes, then a suitable window period could be identified on the basis of the detection of these cytokines for the immune manipulation of breast cancer.

In conclusion, the results of our study indicate that in patients with early-stage breast cancer, the expression of IL-6 and IL-17 mRNA, but not TGF-β, is significantly increased. This finding may reflect a vigorous proinflammatory reaction orchestrated by the host immune system against cancer. In early stages of the disease, the initiation of anticancer immunotherapy may delay the expected emergence of immunosuppression induced by Treg cells.

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