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ORIGINAL ARTICLE

Intratumoral FOXP3 expression in infiltrating breast carcinoma: Its association with clinicopathologic parameters and angiogenesis.

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Abstract
The activity of T regulatory cells (Tregs) is known to be closely associated with the expression of forkhead/winged helix transcription factor, FOXP3. To determine, whether accumulation and activation of intratumoral Tregs help in the progression of breast carcinoma, we have analyzed the intratumoral expression of FOXP3 in invasive breast carcinoma and compared it with its level in ductal carcinoma *in situ* (DCIS) and adjacent normal tissue with the main aim of using this factor as marker of tumor progression. Intratumoral FOXP3 levels were correlated with the levels of transforming growth factor β1 (TGF-β1), vascular endothelial growth factor (VEGF, an invasogenic and angiogenic growth factor) and intratumoral microvessel density (IMD, a prognostic marker for angiogenesis). We also analyzed whether FOXP3 gene expression correlated with other clinicopathological variables like age, tumor stage, histological grade and lymph node metastasis. Infiltrating cancers had higher FOXP3 transcription (7.43 ± 3.44) than did ductal carcinoma *in situ* (4.27 ± 1.97, $p < 0.05$) and normal tissues (3.51 ± 1.22, $p < 0.001$). Intratumoral FOXP3 expression was significantly higher in patients with stage III disease (TNM classification) compared to patients who had stage II disease ($p = 0.037$). In infiltrating carcinoma, a significant positive correlation between FOXP3 expression and TGF-β1 expression was noted ($p < 0.001$). Furthermore, a positive correlation between FOXP3 expression with VEGF expression and IMD values was also detected, however, statistically that was non-significant. A linear association of intratumoral FOXP3 expression with invasion, size and vascularity suggests a utility of FOXP3, an indicator of Treg activity as a marker of tumor progression and metastasis in breast carcinoma.

Tolerance to immune response may contribute to progression of a tumor by local invasion as well as metastatic spread. Recently, the existence of $CD4^+CD25^+CD45RA^-$ T regulatory cells (Tregs) has been described in rodents and humans which suppress the effector functions of $CD4^+CD25^-$ and $CD8^+$ lymphocytes [1,2]. In healthy humans, this population accounts for 1–2% of peripheral CD4+ T cells [3]. The studies indicate that expansion of Tregs that suppress tumor specific immune responses is associated with certain type of invasive cancers [4]. The number of Tregs has been shown to be increased in peripheral blood of breast and pancreatic cancer patients as compared to normal individuals [5]. The activity of these cells is known to be closely associated with the expression of forkhead/winged helix transcription factor, FOXP3 (foxp3 in mice). Generally, foxp3 has been shown to be expressed exclusively in Tregs and plays a very important role in the development and function of these cells [6]. When foxp3 gene is introduced via retrovirus or enforced transgene expression, naïve $CD4^+CD25^-$ T cells transform to Tregs [7]. Wolf et al. [8] demonstrated that high FOXP3 is an independent prognostic factor for overall-survival and progression-free survival in ovarian cancer. Hence, FOXP3 fulfills the criteria of a Treg-specific marker, which at least in differentiated Tregs, is not known to be substantially regulated and represents a suitable surrogate marker for the indirect quantitation of Treg induction in tissues.

Ex-vivo studies on adaptive T regulatory cells reveal a poorly proliferative cell population that secretes inhibitory cytokines such as TGF-β and IL-10 [2]. The secretion of TGF-β is further enhanced by costimulation via CTLA-4 [9]. Moreover, Tregs

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have been shown to mediate TGF-β dependent suppression of both T cell and B cell function [9]. TGF-β1 is reported to induce the expression of vascular endothelial growth factor (VEGF) in target cells [10], which is one of the most selective and potent angiogenic factors known [11].

Angiogenesis, a key phenomenon for tumor growth and metastasis is regulated by endothelial cell mitogens. Studies demonstrate that the vascularity and malignancy of tumors correlate with the expression of VEGF [12]. VEGF plays crucial role in the promotion of angiogenesis in breast cancer [13] and is directly related to intratumoral microvessel density (IMD) [14,15]. IMD in tumors, a measure of angiogenesis, has also been shown to be a prognostic indicator that correlates with an increased risk of metastasis in various epithelial cancers and with overall and relapse free survival in patients with breast cancer [13,16].

Since Tregs have been shown to help tumor progression and FOXP3 being the most specific marker of Treg activation, it is hypothesized that the level of FOXP3 expression in tumor would help to predict the progression of carcinoma. In the current piece of study, we have tried to correlate the level of FOXP3 gene expression in tumor tissues from ductal carcinoma *in situ* (DCIS) and invasive carcinoma of breast with the tumor progression factors like TGF-β1 and VEGF along with some histopathological parameters of the tumor.

Material and methods

Acquisition of samples

Fifty two patients undergoing mastectomy were included in the study after informed consent. Twenty eight of these patients were taking neoadjuvant chemotherapy. Breast carcinoma tissue samples were obtained from the surgical O.T. of the Nehru Hospital attached with Post Graduate Institute of Medical Education and Research, Chandigarh, India. The study was carried out on 52 samples of infiltrating breast carcinoma along with equal number of their adjacent normal tissue and ten samples of ductal carcinoma *in situ* (DCIS). Tissue samples from the breast biopsies were frozen in OCT compound and a diagnosis was made on a haematoxylin and eosin (H & E) stained frozen section. The diagnosis was subsequently confirmed on permanent sections. Frozen sections of tumors would contain at least 80–90% tumor. Simultaneously normal breast tissue adjacent to tumor was also obtained from each biopsy. The amount of tissue varied from 30 mg to 100 mg.

For the purpose of gene expression study, tissue specimens were identified and bisected. One portion was stored at -70°C and processed for gene expression studies by reverse transcription-polymerase chain reaction (RT-PCR) analysis and the other was processed for routine histopathological analysis. For each tissue sample studied, clinicopathological data including age, tumor stage, histological type of tumor, histological grade and axillary lymph node metastasis status was obtained (Table I). Tumor staging was done according to most widely used clinical staging system for breast carcinoma, adopted by both International Union against Cancer (UICC) and the American Joint Commission on Cancer staging. It was based on TNM system [17]. Tumor grade was assessed on routine H & E stained slides using method described by Elston and Ellis [18].

RNA isolation

Total cellular RNA was isolated from the tumor specimens and their corresponding normal tissue using GITC method [19]. Briefly, the specimens were removed from -70°C storage and weighed. Tissue was homogenized in GITC (Guanidium Isothiocyanate) solution and total RNA was isolated using phenol-chloroform extraction and isopropanol precipitation. Final RNA pallet was dissolved in 50 μl of RNase-free water. RNA quality was checked by electrophoresis and the yield was determined spectrophotometrically.

Table I. Clinicopathological characteristics of the patients

	Number of Patients
Age	
Age ≤48	28
Age >48	24
Tumor Stage (pTNM)	
Stage IIA	12
Stage IIB	28
Stage IIIA	10
Stage IIIB	2
Histological type	
Infiltrating ductal carcinoma	48
Infiltrating lobular carcinoma	2
Apocrine carcinoma	1
Papillary carcinoma	1
Tumor Grade	
*IDC 1	7
IDC 2	31
IDC 3	10
Lymph node metastasis	
Negative (N−)	25
Positive (N+)	27

*IDC- tumor grade in infiltrating ductal carcinoma

RT-PCR

FOXP3, TGF-β1 and VEGF transcripts were detected by RT-PCR method. Briefly, complementary DNA strand (cDNA) was made from 2 μg of total RNA using M-MLV reverse transcriptase enzyme (MBI fermentas, Lithuana) and random hexamers. For each 20 μl PCR reaction, 5 μl of cDNA template was mixed to a standard reaction mixture consisting of 1 X reaction buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTPs, 0.5 pmoles of each oligonucleotide primer and 1.5 units of Taq DNA polymerase (Roche, Germany). The reaction was carried out in a Thermal cycler (Eppendorf, Germany) and consisted of following steps; (a) an initial denaturation at 94°C for 3 min (b) PCR amplification by 35 sequential cycles of denaturation (94°C for 1 min), annealing (60°C, 64°C and 58°C in case of FOXP3, TGF-β1 and VEGF genes respectively for 1 min) and primer extension (72°C for 1 min), followed by a final extension at 72°C for 8 min. Amplification of β-actin transcript, a housekeeping gene was used for normalization during quantitation as well as an internal quality control. The sequence of the primers (designed as per the published gene sequences and got manufactured from Sigma Genosys, USA) used are given as:

FOXP3 sense: 5′-GAAACAGCACATTCCCAG AGTTC-3′
FOXP3 antisense: 5′-GCACTTGTGCAGACTC AGGTTG-3′
TGF-β1 sense- 5′-GCC CTG GAC ACC AAC T AT TGC T-3′
TGF-β1 antisense- 5′-AGG CTC CAA ATG TA G GGG CAG G-3′ [20]
VEGF sense: 5′-GAGGAGGGCAGAATCATC AC-3′
VEGF antisense: 5′-AGGCCACAGGGATTTT CTTGTC-3′ [21]

Semiquantitation of gene expression

Semiquantitation of FOXP3, TGF-β1 and VEGF transcripts was done by comparing the signal intensities of PCR products of these genes to those of β-actin gene from the same RNA sample using agarose gel electrophoresis. The intensities of the product bands were quantified by densitometric scanning of gels in gel documentation system (Pharmacia Biotech) using 'Total image' 1D GEL ANALYSIS software. A 100 bp DNA ladder (MBI fermentas, Lithuana) was run in every gel to confirm the size of PCR product. The quantitative expression of FOXP3, TGF-β1 and VEGF genes is given as the percentage of constitutively expressed β-actin gene for each tissue sample.

Immunohistochemistry for IMD

For microvessel density, the tissue sections were stained to highlight the blood vessels by staining endothelial cells using an anti-CD34 monoclonal antibody (Dako, Denmark). Tissue sections (5 μ thick) were taken on poly-L-lysine coated slides from formalin fixed paraffin-embedded blocks. After deparaffinization and rehydration, the sections were subjected to microwave treatment in 0.01 M citrate buffer (pH 6.0) for 15 min for antigen retrieval. The slides were then washed in phosphate-buffered saline (PBS) for 30 min at room temperature and incubated in 0.3% H_2O_2 in methanol for 10 min to block the endogenous peroxidase activity. The sections were then incubated overnight at 4°C with CD34 antibody (1:100). A secondary biotinylated anti-mouse antibody followed by streptavidin horseradish peroxidase was used sequentially for 30 min before color developement using chromogenic Diamino benzidine (DAB 0.5 mg/ml) in the presence of 0.1% H_2O_2. Sections were then counterstained with haematoxylin and mounted with DPX. Sections of primary breast carcinomas in which primary antibody were omitted served as negative controls. Individual microvessels were counted in the area of highest vascularity at 200x (20x objective and 10x ocular lens magnification and at 400x (40x objective and 10x ocular lens; 0.52 per field) magnification. Any brown stained endothelial cell or cluster that was separate from other nearby microvessels were counted [16].

Statistical analysis

For each group and subgroup of patients, FOXP3, TGF-β1 and VEGF mRNA expression as % of β-actin mRNA values (Mean and SEM) were calculated. The difference in the expression of FOXP3, TGF-β1, VEGF genes and microvessel density in normal versus tumor tissues was analyzed by two tailed t-test. Among infiltrating carcinoma, the comparison of FOXP3, TGF-β1, VEGF transcripts and microvessel density with clinicopathological variables was done with two tailed t-test. Where indicated, a one-way analysis of variance (ANOVA) was performed. Among infiltrating carcinoma, correlation analysis between FOXP3, TGF-β1, VEGF gene expression and IMD was performed with Pearson's correlation. All statistical analyses were carried out with the use of SPSS software for windows 10.0. Tests were considered as significant when their p-values were <0.05.

Results

The age of the patients ranged from 23–77 years with a mean $\pm$SD of 48.08 ± 11.87. Of the total 52

patients, twenty two (42.30%) cases were premenopausal and the rest 30 (57.69%) were postmenopausal. Twenty eight patients were given chemotherapy and only one patient had family history of breast carcinoma.

Intratumoral FOXP3 mRNA expression and clinicopathological findings

The relative expression of FOXP3 gene in infiltrating breast carcinoma (7.43 ± 3.44) was higher than in DCIS (4.27 ± 1.97) and was approximately two fold higher than in normal tissues (3.51 ± 1.22) and the differences were statistically highly significant ($p < 0.001$) (Figures 1 and 2). Thus, high levels of intratumoral FOXP3 expression appear to be associated with the invasive phenotype of human breast carcinoma as compared with the non-invasive phenotype.

Table II shows the correlation between the FOXP3 gene expression and each of the clinicopathological features analyzed in infiltrating breast carcinoma. FOXP3 transcript levels in advanced disease (stage III disease) tumors were significantly higher than in tumors with limited disease (stage II disease) ($p = 0.037$). There was no significant correlation of FOXP3 gene expression and any of the other clinicopathological features, including age, histological tumor grade and lymph node metastasis.

Correlation of FOXP3 gene expression with levels of TGF-β1, VEGF gene expression and IMD in infiltrating breast carcinoma

Table III shows the correlation matrix of Pearson's correlation coefficients depicting the association between mRNA expression of FOXP3, TGF-β1, VEGF genes and IMD scores in infiltrating breast carcinoma samples. A significant positive correlation was noted between FOXP3 and TGF-β1 expression ($p < 0.001$). Correlation analysis of intratumoral expression of various genes viz. FOXP3 vs VEGF, TGF-β1 vs VEGF, FOXP3 vs IMD scores, TGF-β1 vs IMD scores, VEGF vs IMD scores revealed a positive correlation, and among these pairs none was statistically significant.

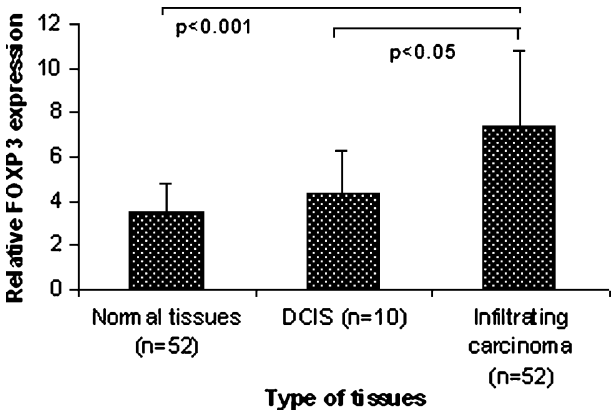

Figure 2. Distribution of relative mRNA expression of FOXP3 gene in normal tissues (n = 52), DCIS (n = 10) and infiltrating breast carcinoma (n = 52), normalized to the content of β-actin in each sample (e.g. expressed as the % of β-actin gene).

Discussion

The presence and activation of $CD4^+CD25^+$ Tregs, known to suppress the activity of effector cells, is shown to help invasion in some malignant neoplasms. Their number was found to be significantly raised in cancer patients, when compared to normal donors [5]. Forkhead/winged helix transcription factor, FOXP3, a single most important factor associated with activation and differentiation of Treg cells, is therefore, particularly an interesting molecule to study functional status of these cells in any carcinoma.

In the present study, we have analyzed the intratumoral expression of FOXP3 in invasive breast carcinoma and compared it with its level in DCIS and normal tissue with the main aim of using this factor as marker of tumor progression. We found a significantly higher intratumoral FOXP3 gene expression as checked by their mRNA levels in tumor bed than normal breast tissue. These findings suggest that high intratumoral expression of FOXP3 gene is correlated with the invasive phenotype of breast carcinoma. Our findings further confirm that malignant epithelial tumors are enriched with FOXP3-expressing Tregs and might contribute to tumor induced shutdown of an effective antitumor immune response [22]. Viguier and colleagues [23] have also reported that $FOXP3^+$ $CD4^+$ $CD25^+$ T cells are

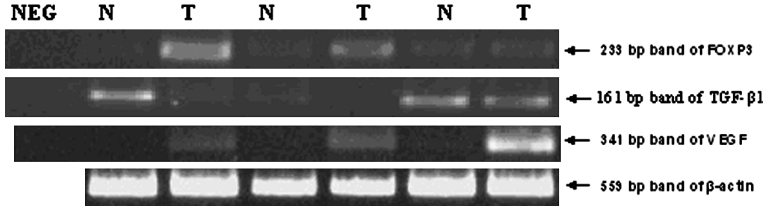

Figure 1. (A) 2.0% Agarose gel showing RT-PCR product of 233 bp of FOXP3 gene, (B) 2.5% agarose gel showing 161 bp band of TGF-β1 gene, (C) 2.0% agarose gel showing 341 bp of VEGF gene in paired normal and tumor breast tissues.

Table II. Analyses of FOXP3 expression in different tumor lesion types, their relationship with lymph node metastasis, tumor stage and histological tumor grade in infiltrating breast carcinoma

Parameter	Relative FOXP3 mRNA expression	p-value
Tumor Type		I vs II = NS, I vs III = <0.001, II vs III = 0.004
Normal tissue (I)	3.51±1.22	
DCIS (II)	4.27±1.97	
Infiltrating carcinoma (III)	7.43±3.44	
Age		NS
≤48	6.80±3.40	
>48	8.12±3.41	
Lymph node metastasis		NS
Node negative (N−)	8.22±3.82	
Node positive (N+)	6.70±2.93	
Tumor stage		0.037
IIA and IIB	7.06±3.41	
IIIA and IIIB	9.26±3.54	
Infiltrating ductal tumor grade		NS
Grade I	5.67±3.40	
Grade II	7.43±3.46	
Grade III	7.28±2.88	

Values tabulated are means±standard errors of mean. NS: non-significant.
Tumor type: I vs III, $p < 0.001$ (Two tailed t-test); II vs III, $p < 0.05$ (Mann-Whitney U test).
Tumor stage: $p < 0.05$ (Mann-Whitney U test).

overexpressed in metastatic lymph nodes with a two fold frequency compared to both tumor-free LNs and autologous PBMCs.

Stage of clinical advancement and tumor grade, are recognized prognostic factors in breast carcinoma [24,25]. Pathological studies from the National Surgical Adjuvant Breast Cancer Project indicate that tumor size is also an independent prognostic indicator for both overall survival and disease free survival in breast cancer [26]. Within the group of invasive cancers, our study revealed a significant linear association of increasing FOXP3 expression with advanced tumor stage (increasing size) of the tumor ($p < 0.05$).

The angiogenesis depends on the production of several factors such as TGF-β1 and VEGF, by tumor cells and normal cells [27]. TGF-β1, a multifunctional growth factor, is known to help the growth of malignant tumors in an autocrine as well as paracrine fashion, resulting in increased cell-matrix interaction, suppressed immune surveillance, or increased angiogenic activity [28,29]. Donovan et al., [10] reported a positive correlation between serum and tumor tissue levels of VEGF and TGF-β1 and concluded that there is a positive relationship between TGF-β1 and VEGF in breast carcinoma patients. Various studies have indicated a positive correlation of the expression of VEGF with the vascularity and malignancy of tumors. In our study, the intratumoral levels of FOXP3, TGF-β1 and VEGF were found to be positively correlated besides the IMD values. Similarly the intratumoral levels of TGF-β1 were also found to be positively related to the levels of VEGF and IMD. The results lead to the suggestion that activated Tregs release excessive levels of TGF-β1, which indirectly induces the expression of VEGF leading to increased vascularity and tumor progression. This implies that levels of FOXP3, an indicator of Treg activity, might also be an indicator of breast tumorigenesis.

Since invasion, size and vascularity are prognostic parameters in breast cancer, the finding of a positive correlation between FOXP3 expression and these parameters suggests a role of FOXP3 as a marker of progression for breast carcinoma to an aggressive tumor phenotype. However, a study based on a larger sample group and determining whether the subset of tumors with high FOXP3 overlaps with subsets of tumors with other biological indicators of adverse prognosis, such as HER/*neu* oncoprotein, p53 gene mutations and ER/PR status would further strengthen this fact.

Table III. Correlation matrix (Pearson's correlation coefficients) of the association between mRNA expression of FOXP3, TGF-β1, VEGF genes and IMD scores in 52 infiltrating breast carcinoma samples

	FOXP3	TGF-β1	VEGF
TGF-β1	0.702*		
VEGF	0.072	0.111	
IMD	0.008	0.062	0.057

*$p \leq 0.001$

Current research on immunotherapy for cancer mainly focuses on generation of T cell mediated tumor lysis by using vaccination strategies. Explanations offered for this marginal success of immunotherapy includes "tolerance" development due to lack of costimulatory molecules on tumors, down-regulation of signal transduction molecules in T cells, apoptosis of T cells upon contact with tumor, tumor-induced dysfunction of antigen presenting cells, secreted immunosuppressive proteins such as TGF-β1 by tumors, and emergence of antigen loss variants. Addition of FOXP3 to the list of important markers associated with invasive behavior of breast carcinoma would go a long way in development of new anti-cancer targets in future.

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