

The Prognostic Role of Tumor-Infiltrating Lymphocytes CD8 and Foxp3 and their Impact on Recurrence in Breast Cancer Patients

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Abstract

Background: The presence of tumor-infiltrating lymphocytes (TIL) within tumor epithelium or stroma of breast cancer is a surrogate of an immune response to cancer development, but their significance remains controversial. We conducted this study to evaluate the correlation of CD8+ (cytotoxic T) lymphocyte infiltration and Foxp3+ Tregs and tumor characteristics and their impact on recurrence in patients with invasive breast cancers.

Patients and methods: CD8+ T cells and Foxp3+ Tregs were detected by immunohistochemistry using the paraffin-embedded tumor tissues from 68 patients with stage (I to III) breast cancer. Clinicopathological data including patient's age, tumor size and grade, stage, lymph node metastasis, estrogen and progesterone receptor status, Ki-67, and human epidermal growth factor receptor-2/neu, and recurrence were reviewed.

Results: The decreased mean number of CD8+ T cells was significantly associated with tumors with lymph node metastasis (P=0.02), and immune-positivity of Ki-67 (P=0.00). The increased number of Foxp3+ Tregs was significantly correlated with tumors with lymph node metastasis (P=0.01) higher stage (stage III, P=0.03), and immune-positivity for Ki-67 (P=0.02).

Further analysis of the correlation using CD8+ T-cell/Foxp3+ Treg ratio showed significant correlation with tumors with lymph node metastasis (P=0.01), and immune-positivity of Ki-67 (P=0.00). Also, there were significant correlations between the increased Foxp3+Treg /CD4+ T-cell ratio and lymph node metastasis (P=0.00), and immune-positivity of Ki-67 (P=0.02).

Conclusions: Data showed that lymph node metastases, tumor stage, immunopositivity of Ki67, and non-triple-negative tumors were associated with high regulatory T-cell infiltration. The prognostic role of immunologic balance as a marker for recurrence must be evaluated more clearly in a larger study.

Keywords: Early breast cancer; Foxp3+ Treg; CD8+ T cell

Introduction

The presence of tumor-infiltrating lymphocytes (TILs) either within the tumor stroma or tumor epithelium reflects the immune response of the host against the tumor, defined as cancer "immunoediting" [1]. Large tumors are in the "escape" phase during which immune cells are not able to stop tumor growth but their presence at least denotes stand-by immunocompetency [1-3] which can be reactivated by treatment.

Many TILs have been recognized. Of those, fork head box P3-positive (Foxp3+) regulatory T cells (Tregs), CD8+ T cells, and CD4+ T cells are known to be the main keys for immune surveillance and tolerance, respectively [4]. CD8+ T cells are mediators of antitumor immunity and can lyse tumor cells directly [5]. The clinical importance of CD8+ T cells has been suggested by many recent studies that reported a survival benefit in correlation with an increase in CD8+ T cells in large cohorts of various human cancer patients [6-12].

CD4+ T cells have an important role in antitumor immunity that was implicated by their helper or memory cell functions. CD4+ T cells may have several effector functions, such as priming tumor-specific cytotoxic T cells or macrophages that are involved in clearance of tumor cells [5]. In contrast, Tregs are known to have a very important role in escape of antitumor T-cell response in cancer cells [13], due to their ability to potently suppress immune reaction against tumors *in vivo* [14,15]. Tregs are a subset of T lymphocytes, constituting 5%-10% of CD4+T cells. Tregs regulate the immune response by suppressing the cytokine production and proliferation of effector T cells [16,17].

Tregs constitutively express CD25 (IL-2Ra-chain) on their surface

[18,19]. More recent studies have shown that the transcription factor Foxp3 is a key intracellular marker that has additional crucial functional and developmental importance for CD4+CD25+ Tregs [20].

Many studies have reported the presence of higher numbers of Tregs in the peripheral blood of patients with breast, gastric, esophageal, and liver cancer, compared with healthy controls [21-24]. Higher numbers of Tregs were associated with poor clinical outcome in many studies [21,25,26]. An increased Tregs population both in tumor tissues and peripheral blood was also reported in breast cancer, and a recent study demonstrated a significant infiltration of Foxp3+ Tregs intratumoral in high-risk breast cancer patients [21,27].

Different techniques are used for assessment of TILs. These techniques include simple counting of mononuclear cell infiltrate on routine hematoxylin and eosin (H&E) stained slides [28-30]; typing of an immune cell with immunohistochemistry [30-32]; immune-related gene expression [33-35] and weighing of the digital immune cell [36].

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Recent recommendations for the morphological assessment of TILs on H&E sections have been published to bring TILs closer to clinical application [30]. The aim of this study is to evaluate the correlation of CD8+ (cytotoxic T) lymphocyte infiltration and Foxp3+ Tregs and tumor characteristics and their impact on recurrence in patients with invasive breast cancers.

Patients and Methods

Patient selection

The present study included 68 patients who had been diagnosed with invasive ductal carcinoma, and underwent surgical excision followed by appropriate adjuvant treatment as indicated at Tanta University Hospital at the period from January 2010 to December 2011.

All patients provided an informed consent before surgery or core biopsy. Patients who received neoadjuvant chemotherapy or hormonal therapy were excluded.

All H&E stained slides for each patient were retrospectively reviewed, and we selected the most representative tumor block. All specimens were fixed in 10% formalin solution and embedded in paraffin for routine histopathologic analysis. The following Clinicopathological parameters were assessed for each patient, age, lymph node metastasis, tumor size, histologic grade, {was assessed using the Nottingham grading system [37]}, nuclear grade, hormonal receptor status including estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) status, Ki-67, tumor recurrence, distant metastasis, and survival. All tumors stages were classified according to the American Joint Committee on Cancer (AJCC)-TNM (tumor, node, metastases) classification (seventh edition) [38].

The median follow-up period after surgery was 24 months. This protocol was approved by our ethical committee at Tanta University Hospital.

Immunohistochemistry

For immunohistochemical analysis, breast carcinomas sections with a serial 4-mm were treated on electrostatic slides. Xylene first used for deparaffinization at formalin-fixed and paraffin-embedded tissues then treated by ethanol for rehydration. After quenching of endogenous peroxidase activity with 3% hydrogen peroxide for 30 min, the slides were subjected to EDTA antigenic retrieval buffer (pH 8.0) then used a microwave oven for adequate heating.

Bovine serum albumin/1x tris-buffered saline (TBS) was used at a concentration of 0.3% for incubation of the given slides for 20 minutes to minimize nonspecific and unwanted background staining. At normal room temperature, a primary antibody was added for only 30 minutes. After a series of TBS rinse, the bound antibody was detected by using a polymer secondary antibody from the DAKO EnVision+system (DAKO, Carpinteria, CA).

The slides were rinsed with TBS series and visualized with a 10 minute incubation of liquid D, 3,3'-diaminobenzidine in buffered substrate (DAKO) for 10 minutes followed by hematoxyline used for counterstaining.

Staining of lymphocytes was considered as internal positive controls, with given sections. Tonsils can be used as external controls. Negative controls were done by the omission of the antibodies.

Assessment of immunohistochemical staining

Immunohistochemical staining for CD4/CD8 was considered positive when lymphocytes showed evident membranous immunoreactions. Immunohistochemical staining for Foxp3 was considered positive when lymphocytes showed evident nuclear immunostaining.

The average scoring of the number of Foxp3+lymphocytes, CD8+ and CD4+ T positive cells present within the malignant epithelium and just adjacent stroma (within the same high-powered field; HPF) was counted manually in 3 HPFs (x400) then the average of each marker-positive cells was calculated.

Expression patterns of Ki-67 were determined in a semi quantitative manner by light microscopy. Cases with >14% of ki-67-positive cancer cells were considered as those with higher proliferative activity (categorized as ki-67-positive cancers).

Statistical analysis

The correlations between subsets of TILs (CD8+ T cells, Foxp3+ Tregs, CD8+ T-cell/Foxp3+ Treg ratio, and CD4+ T-cell/Foxp3+ Treg ratio) and clinicopathological characteristics listed before were analyzed by Spearman test. The statistical significance of the difference between the 2 groups was determined using the Mann-Whitney test. Simple logistic regression analysis was used for comparisons of independent variables with the dependent variable. Disease-free survival was measured from the date of diagnosis to the time of recurrence.

The Kaplan-Meier method was used for calculation of Univariate analysis. A P value of less than 0.05 was considered significant. The log-rank test was used for confirmation of significance.

Cox proportional hazards regression model was used for calculation of multivariate analysis.

Results

Study population

The clinicopathological characteristics of our patients are demonstrated in Table 1. The median follow-up duration of the study population was 24 months (range; 6-42). The median age of the patients was 54 years (range, 28-70), and 41.7% of the patients were postmenopausal. All of the patients had operable early stage breast cancer; 53 patients (78%) were clinical stage I or II and 15 patients (22%) were stage III. Forty-five patients (66.1%) had estrogen receptor-positive cancers, 15 patients (22%) had HER-2 amplified, and 24 patients (35.2%) had triple-negative cancers. All of the patients are still alive, and 7 patients (10.3%) had recurrent disease.

Correlation of TIL subtypes with clinicopathological features

In this study, we observed that TILs were frequently found in both the tumor epithelium and stroma in the form of lymphoid aggregates. The representative pictures of lymphoid cells are shown in Figure 1. The correlations among the subtypes (CD4+, CD8+ T cell, and Foxp3+ Tregs) of tumor infiltrating lymphocytes were analyzed. There was a significant correlation between the expressions of each pair subtype by linear correlation analysis (Figure 2). The relationships between the mean numbers of each subtype of TILs (CD8+ T cells, Foxp3+ Tregs) per HPF with clinicopathological features of our study are shown in Table 1. Further analysis of ratio of the subtypes of tumor infiltrating lymphocytes (CD8+ T-cell/Foxp3+ Treg ratio and CD4+ T-cell/Foxp3+ Treg ratio) were also performed (Table 2).

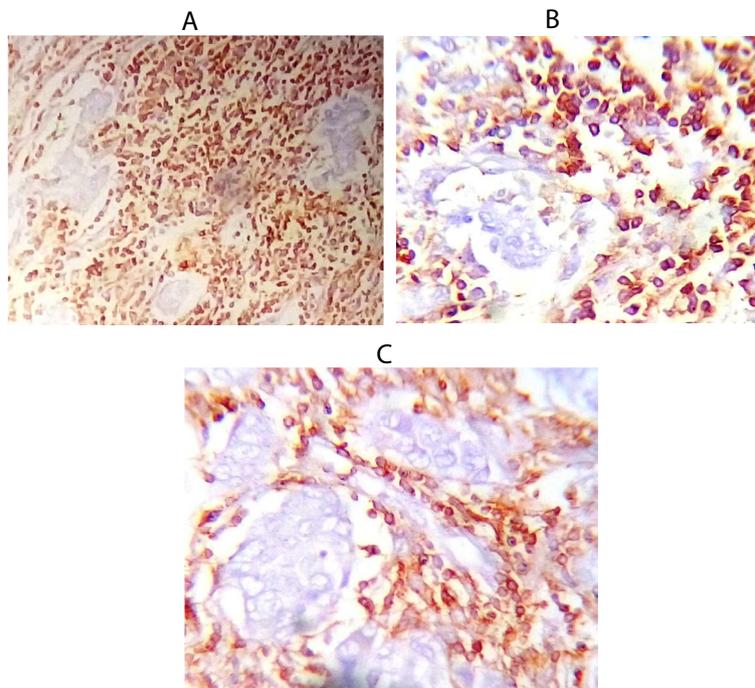


Figure 1: (1A) Fox: Strong nuclear immunostaining of Fox with negative immunoreaction of malignant ductal cells inbetween (x200). (1B) CD8: Strong positive membranous immunoreaction for CD8, with negative immunohistochemistry staining of malignant ductal cells in between (x400). (1C) Strong positive membranous immunoreaction for CD4, with negative immunohistochemistry staining of malignant ductal cells in between (x400).

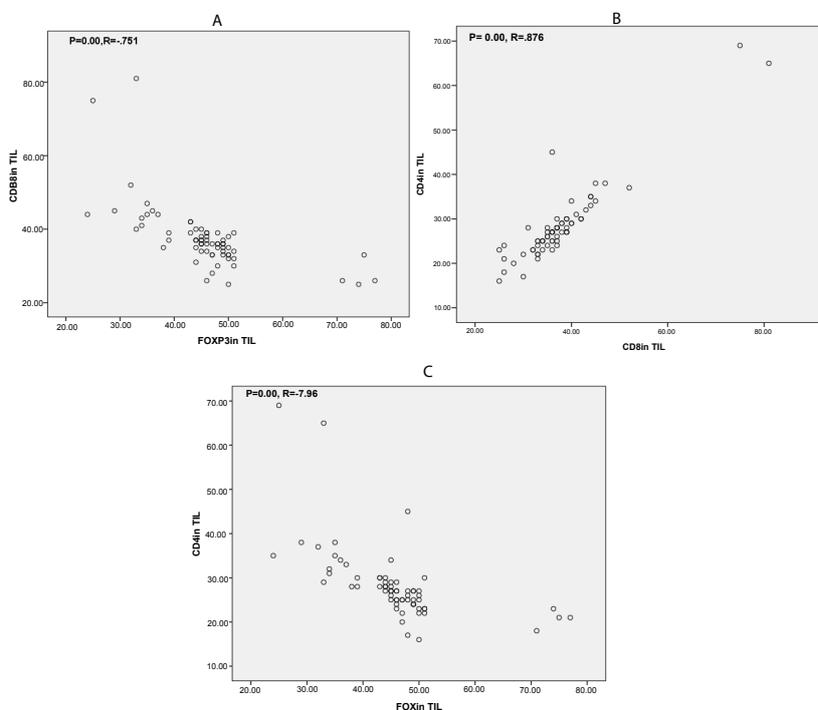


Figure 2: (A,B,C) Correlation between CD 4+, CD8+ T lymphocytes and FOXP3+ Tregs.

The decreased mean number of CD8+ T cells was significantly associated with tumors with lymph node metastasis ($P=0.02$), and immune-positivity of Ki-67 ($P=0.00$). The increased number of Foxp3+ Tregs was significantly correlated with tumors with lymph

node metastasis ($P=0.01$) higher stage (stage III, $P=0.03$), and immune-positivity for Ki-67 ($P=0.02$).

Further analysis of the correlation using CD8+ T-cell/Foxp3+ Treg ratio showed significant correlation with tumors with lymph node

metastasis(P=0.01), and Ki-67 (P=0.00). Also, there were a significant correlations between the increased Foxp3+Treg /CD4+ T-cell ratio and lymph node metastasis (P=0.00), and Ki-67 (P=0.02).

Prognostic significance of TIL subtypes in breast cancer

To investigate the prognostic impact of tumor infiltrating lymphocytes on disease-free survival (DFS), 68 patients who were diagnosed with early breast cancer were analyzed (Figure 3).

Although CD8+T cells, the number of Foxp3+ Tregs/HPF, CD8+ T-cell/Foxp3+ Treg ratio, and CD4+ T-cell/Foxp3+ Treg ratio were applied to various dichotomous cut-off points, none of them showed a significant correlation with overall DFS.

Patients with higher CD8+T-cell/Foxp3+ Treg ratio showed better disease free survival with borderline significance (P=0.06).

We also investigated the prognostic value of TIL subtypes. Univariate analysis indicated that DFS was significantly different according to age (P=0.024), LN (P=0.012), and KI 67 (P=0.012) (Table 2). CD8+ T cells, Foxp3+ Tregs, CD8+T-cell/Foxp3+ Treg ratio, and CD4+ T-cell/Foxp3+ Treg ratio had no significant impact on DFS. However, age, LN, and Ki-67 were no longer significant in multivariate analyses.

Discussion

The immune system use complicated balance between different positive and negative signals to protect the body from foreign agents

Characteristics	No.cases	CD8+	Foxp3+	CD8+/Foxp3+	Foxp3+/CD4+
Age					
≤50	23	39.34	46.43	0.95	1.82
>50	45	36.62	45.17	0.84	1.69
Tumor size					
≤5	39	38.69	44.33	0.95	1.69
>5	29	36	47.31	0.78	1.79
Grade					
1-2	55	38.18	45.01	0.9	1.66
3	13	34.84	48.07	0.8	2.02
LN					
0-1	51	39.33	43.41	0.96	1.57
2-3	17	32.17	52.17	0.63	2.22
Stage					
I/II	53	37.28	45.92	0.86	1.76
III	15	38.46	44.46	0.95	1.63
ER					
Neg	23	36.92	45.04	0.86	1.74
pos	45	37.91	45.88	0.89	1.73
PR					
Neg	35	38.62	45.57	0.93	1.73
pos	33	36.39	46.63	0.82	1.73
Her2					
Neg	53	35.88	45.84	0.8	1.75
pos	15	43.4	44.73	1.16	1.65
Ki					
Neg	60	40.3	43.51	1.01	1.57
pos	8	33.82	48.41	0.7	1.95
TN					
Yes	21	41	41.57	1.07	1.5
no	47	36	47.4	0.8	1.84

Table 1: Correlation between clinicopathological characteristics and subtype of TILs.

Characteristics	Univariate Analysis		Multivariate Analysis	
	P	HR (95% CI)	P	
Age				
≤50	0.024	2.46	0.311	
>50		0.431-14.098		
Tumor size				
≤5	0.489			
>5				
Grade				
1-2	0.077	3.457	0.161	
3		0.611-19.556		
LN				
0-1	0.012	2.105	0.353	
2-3		0.438-10.128		
Stage				
I/II	0.083	0.000	0.974	
III		0.000-5.691		
ER				
Neg	0.21			
pos				
PR				
Neg	0.304			
pos				
Her2				
Neg	0.245			
pos				
Ki				
Ki	0.012	2.18	0.502	
Neg		(0.224-21.252)		
TN				
Yes	0.172			
no				
CD8+				
≤37	0.157			
>37				
Foxp3+				
≤45	0.129			
>45				
CD8+/Foxp3+				
CD8+/Foxp3+	0.065	0.000	0.976	
≤0.88		0		
Foxp3+/CD4+				
≤1.7	0.577			
>1.7				

Table 2: Prognostic factors for disease-free survival in univariate and multivariate analysis.

[40]. Tumor growth and spread depends mainly on the interaction between the host immune system and malignant tumors.

The immune response of the host against tumor has been shown to change the response to chemotherapy. Denkert et al. reported that tumor infiltration by a high number of lymphocytes at diagnosis is associated with a higher likelihood of achievement of complete pathological response after neoadjuvant chemotherapy.

Besides clinical and treatment parameters, the host immune response affects the prognosis of cancer patients after standard treatment. TILs with their various types represent the reaction of the host to tumor antigens. Among subtypes of TILs, CD8+ T cells and Foxp3+ Tregs are the main keys for immune tolerance and surveillance, respectively [39]. Numerous recent studies have shown the importance of the balance between immune surveillance and immune evasion in tumor microenvironment [40-42].

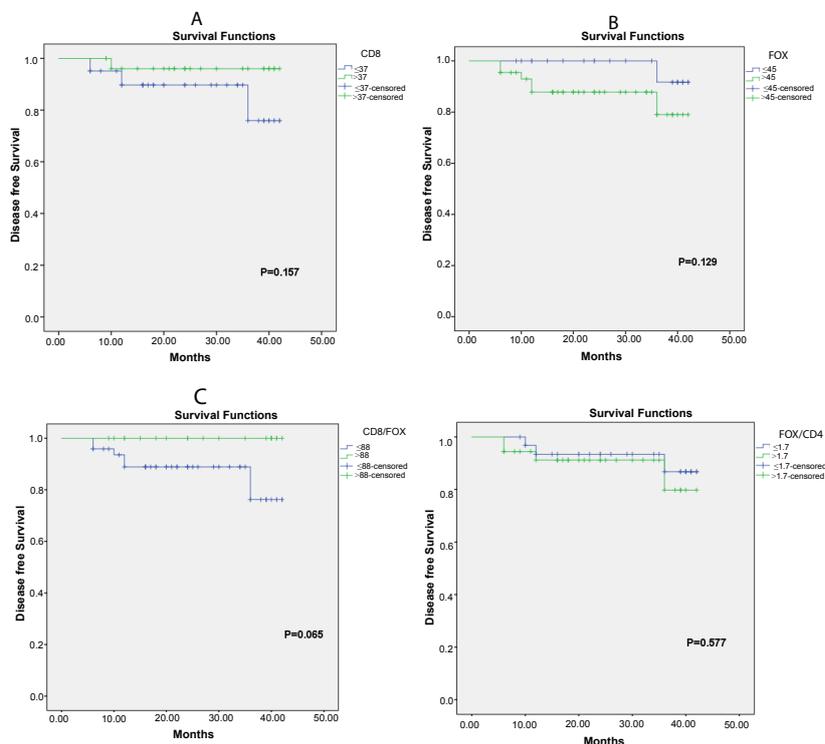


Figure 3: A Kaplan-Meier curve for disease-free survival of all patients stratified by low or high numbers of CD8+ T lymphocytes (A) Foxp3+ Tregs (B) CD8+ T lymphocytes/FOXP3+ Tregs ratio (C) and FOXP3+ Tregs /CD4+ T lymphocytes ratio (D) P values <0.05 were considered significant.

Many randomized adjuvant clinical trials had evaluated the prognostic role of tumor-infiltrating lymphocytes (TIL) in breast cancer. In summary, TIL at baseline are associated with high-grade, high-proliferative, ER-negative tumors and represent a strong prognostic factor for some molecular subtypes, mainly for triple-negative breast cancer (TNBC) [43]. In all these trials, however, patients were treated with adjuvant chemotherapy; so, the prognostic role of TIL in chemotherapy-naïve patients is unknown. This information is important before defining the role of TIL in clinical practice. Moreover, few studies have studied the role of tumor-infiltrating lymphocytes in predicting sensitivity to specific treatments.

In our study, we investigated the infiltration of Foxp3 Treg, CD4, and CD8 T cells separately in the tumor epithelium and stroma, and assessed its association with clinical and pathological parameters, biologic subtypes, and patients' outcome. Immunohistochemical analyses were performed using full tissue sections instead of tissue microarrays to reduce selection bias. Each type of lymphocyte was counted manually on the immunostained slides. In our study, the decreased number of CD8+ T cells was significantly associated with poor prognostic factors including lymph node metastasis ($P=0.02$), and immunopositivity of Ki-67 ($P=0.00$). The increased number of Foxp3+ Tregs was significantly correlated with aggressive clinical characteristics as lymph node metastasis ($P=0.01$), higher stage (stage III, $P=0.03$), and Ki-67 ($P=0.02$).

In the further analysis, there were significant correlations between the increased Foxp3+ Treg/CD4+ T-cell ratio and lymph node metastasis ($P=0.01$), and immunopositivity of Ki-67 ($P=0.02$). The relation between tumor-infiltrating lymphocytes and aggressive biology of tumors shown here is in good correlation with the results from other studies, although there are still some contradictory data [44,45].

Breast cancer is a very heterogeneous disease, even after the recent molecular classification into 4 main subtypes according to the hormone receptor status, HER-2 expression, and Ki-67 [46-49]. Due to strong heterogeneity within the subgroups, this new classification, although it is based on biological and clinical behavior, is still unsatisfactory. TNBC has been known to be more heterogeneous than other subclasses and has poor prognosis compared with any other molecular subtype of tumors [50,51].

Although most patients with TNBC show aggressive clinical behavior and shorter survival, some TNBC patients have better clinical outcome. Recently, an immune response gene expression cluster (IR+) was used to identify a subgroup of ER-negative disease with a good prognosis [52]. Teschendorff et al. reported that the differences in clinical outcome of patients with ER-negative tumors are mainly related to differentially expressed genes in the complement and immune response pathways and that its association with prognosis may be independent of lymphocytic infiltration and lymph node status. Thus, the quality (IR+) of immune response might be more important than the quantity (lymphocytic infiltration) in the prognosis of ER-negative patients [52].

The results of our study showed that TNBCs may have favorable immune cell profiles than non-TNBCs: lower Foxp3+ Treg ($P=0.02$) in TNBCs. However, our results are inconsistent with data from the previous studies in western countries, which reported an association of a significant number of Foxp3+ Tregs infiltration with ER-negative tumors [21,53]. These apparent discrepancies may be due to the different technical methods used for assessment of TIL; Bates et al used tissue microarray for TILs examination that can limit systemic examination of TILs while Bohling counted Foxp3+ cells manually instead of the digital image analyzer. Another factor should be

considered that is ethnic differences between our study and others. Thus, our observations should be validated in a large cohort of Egyptian patients and in different ethnic group.

Although the prognostic significance of immune cell infiltrations including Foxp3+ Tregs were demonstrated in previous studies, [21] no immune cell subtypes or their ratio has been identified as an independent prognostic factor in our study. It might be partially explained by relatively short median follow-up time (24 months); thus, the number of events is still small to give a significant difference according to potential prognostic factors.

Conclusion

In conclusion, we demonstrated that higher number of CD8+ T cells and lower number of Foxp3+ Tregs and Foxp3+Treg /CD4+ T-cell ratio were associated with favorable clinic-pathologic features, whereas higher numbers of Foxp3+ Tregs were associated with aggressive tumor behavior. In this study, patients with TNBCs had a lower number of Foxp3+ Treg. Although prognostic significance according to the subtypes and balance of tumor infiltrating lymphocytes remains unclear, immunologic microenvironment according to biological subtypes of breast cancer should be more clearly characterized in future studies.

References

- Schreiber RD, Old LJ, Smyth MJ (2011) Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* 331: 1565-1570.
- Andre F, Dieci MV, Dubsky P, Sotiriou C, Curigliano G, et al. (2013) Molecular pathways: involvement of immune pathways in the therapeutic response and outcome in breast cancer. *Clin Cancer Res* 19: 28-33.
- Bianchini G, Gianni L (2014) The immune system and response to HER2-targeted treatment in breast cancer. *Lancet Oncol* 15: e58-68.
- Yu P, Fu YX (2006) Tumor-infiltrating T lymphocytes: friends or foes? *Lab Invest* 86: 231-245.
- Toes RE, Ossendorp F, Offringa R, Melief CJ (1999) CD4 T cells and their role in antitumor immune responses. *J Exp Med* 189: 753-756.
- Nakano O, Sato M, Naito Y, Orikasa S, Aizawa M, et al. (2001) Proliferative activity of intratumoral CD8(+) T-lymphocytes as a prognostic factor in human renal cell carcinoma: clinicopathologic demonstration of antitumor immunity. *Cancer Res* 61: 5132-5136.
- Naito Y, Saito K, Shiiba K, Ohuchi A, Saigenji K, et al. (1998) CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. *Cancer Res* 58: 3491-3494.
- Zhang L, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, et al. (2003) Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med* 348: 203-213.
- Chiba T, Ohtani H, Mizoi T, Y Naito, E Sato, et al. (2004) Intraepithelial CD8+ T-cellcount becomes a prognostic factor after a longer follow-up period in human colorectal carcinoma: possible association with suppression of micrometastasis. *Br J Cancer* 91: 1711-1717.
- Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, et al. (2006) Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 313: 1960-1964.
- Sharma P, Shen Y, Wen S, Yamada S, Jungbluth AA, et al. (2007) CD8 tumor-infiltrating lymphocytes are predictive of survival in muscle-invasive urothelial carcinoma. *Proc Natl Acad Sci USA* 104: 3967-3972.
- Piersma SJ, Jordanova ES, van Poelgeest MI, Kwappenberg KM, van der Hulst JM, et al. (2007) High number of intraepithelial CD8+ tumor-infiltrating lymphocytes is associated with the absence of lymph node metastases in patients with large early-stage cervical cancer. *Cancer Res* 67: 354-361.
- Drake CG, Jaffee E, Pardoll DM (2006) Mechanisms of immune evasion by tumors. *Adv Immunol* 90: 51-81.
- Baecher-Allan C, Anderson DE (2006) Immune regulation in tumor-bearing hosts. *Curr Opin Immunol* 18: 214-219.
- Zou W (2006) Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol* 6: 295-307.
- Thornton AM, Shevach EM (1998) CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* 88: 287-296.
- Ng WF, Duggan PJ, Ponchel F, Matarese G, Lombardi G, et al. (2001) Human CD4(+)/CD25(+) cells: a naturally occurring population of regulatory T cells. *Blood* 98: 2736-2744.
- Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M (1995) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155: 1151-1164.
- O'Garra A, Vieira P (2004) Regulatory T cells and mechanisms of immune system control. *Nat Med* 10: 801-805.
- Hori S, Nomura T, Sakaguchi S (2003) Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299: 1057-1061.
- Bates GJ, Fox SB, Han C, Leek RD, Garcia JF, et al. (2006) Banham. Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *J Clin Oncol* 24: 5373-5380.
- Liyanage UK, Moore TT, Joo HG, Tanaka Y, Herrmann V, et al. (2002) Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *J Immunol* 169: 2756-2761.
- Ormandy LA, Hillebrand T, Wedemeyer H, Manns MP, Gretchen TF, et al. (2005) Increased populations of regulatory T cells in peripheral blood of patients with hepatocellular carcinoma. *Cancer Res* 65: 2457-2464.
- Ichihara F, Kono K, Takahashi A, Kawaida H, Sugai H, et al. (2003) Increased populations of regulatory T cells in peripheral blood and tumor-infiltrating lymphocytes in patients with gastric and esophageal cancers. *Clin Cancer Res* 9: 4404-4408.
- Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, et al. (2004) Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 10: 942-949.
- Wolf D, Wolf AM, Rumpold H, Fiegl H, Zeimet AG, et al. (2005) The expression of the regulatory T cell-specific forkhead box transcription factor FoxP3 is associated with poor prognosis in ovarian cancer. *Clin Cancer Res* 11: 8326-8331.
- Ghebeh H, Barhoush E, Tulbah A, Elkum N, Al-Tweigeri T, et al. (2008) FOXP3+ Tregs and B7-H1+/PD-1+ T lymphocytes co-infiltrate the tumor tissues of high-risk breast cancer patients: implication for immunotherapy. *BMC Cancer* 8: 57-68.
- Loi S, Michiels S, Salgado R, Sirtaine N, Jose V, et al. (2014) Tumor infiltrating lymphocytes are prognostic in triple negative breast cancer and predictive for trastuzumab benefit in early breast cancer: results from the FinHER trial. *Ann Oncol* 25: 1544-1550.
- Loi S, Sirtaine N, Piette F, Salgado R, Viale G, et al. (2013) Prognostic and predictive value of tumor-infiltrating lymphocytes in a phase III randomized adjuvant breast cancer trial in node-positive breast cancer comparing the addition of docetaxel to doxorubicin with doxorubicin based chemotherapy: BIG 02-98. *J Clin Oncol* 31: 860-867.
- Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, et al. (2015) Harmonization of the evaluation of tumor infiltrating lymphocytes (TILs) in breast cancer: recommendations by an international TILs working group 2014. *Ann Oncol* 26: 259-271.
- West NR, Milne K, Truong PT, Macpherson N, Nelson BH, et al. (2011) Tumor-infiltrating lymphocytes predict response to anthracycline-based chemotherapy in estrogen receptor-negative breast cancer. *Breast Cancer Res* 13: R126.
- Rathore AS, Kumar S, Konwar R, Makker A, Negi MP, et al. (2014) CD3+, CD4+ & CD8+ tumour infiltrating lymphocytes (TILs) are predictors of favourable survival outcome in infiltrating ductal carcinoma of breast. *Indian J Med Res* 140: 361-369.
- Denkert C, Loibl S, Noske A, Roller M, Muller BM, et al. (2010) Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. *J Clin Oncol* 28: 105-113.

34. Mao Y, Qu Q, Zhang Y, Liu J, Chen X, et al. (2014) The value of tumor infiltrating lymphocytes (TILs) for predicting response to neoadjuvant chemotherapy in breast cancer: a systematic review and meta-analysis. *PLoS One* 9: e115103.
35. Perez EA, Thompson EA, Ballman KV, Anderson SK, Asmann YW, et al. (2015) Genomic analysis reveals that immune function genes are strongly linked to clinical outcome in the north central cancer treatment group n9831 adjuvant trastuzumab trial. *J Clin Oncol* 33: 701-708.
36. Nawaz S, Heindl A, Koelble K, Yuan Y (2015) Beyond immune density: critical role of spatial heterogeneity in estrogen receptor-negative breast cancer. *Mod Pathol* 28: 766-77.
37. Elston CW, Ellis IO (1991) Pathological prognostic factors in breast cancer: The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology* 19: 403-10.
38. Edge S, Byrd DR, Compton CC, Fritz AG, Greene FL, et al. (2010) *AJCC Cancer Staging Manual*. Springer, New York.
39. Armstrong A, Dermime S (2001) Developing effective cancer vaccines: design and monitoring are critical. *Br J Cancer* 84: 1433-1436.
40. Fu J, Xu D, Liu Z, Shi M, Zhao P, et al. (2007) Increased regulatory T cells correlate with CD8 T-cell impairment and poor survival in hepatocellular carcinoma patients. *Gastroenterology* 132: 2328-2339.
41. Gao Q, Qiu SJ, Fan J, Zhou J, Wang XY, et al. (2007) Intratumoral balance of regulatory and cytotoxic T cells is associated with prognosis of hepatocellular carcinoma after resection. *J Clin Oncol* 25: 2586-2593.
42. Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, et al. (2005) Intraepithelial CD8+ tumor infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc Natl Acad Sci USA* 102: 18538-18543.
43. Adams S, Gray RJ, Demaria S, Goldstein L, Perez EA, et al. (2014) Prognostic value of tumor-infiltrating lymphocytes in triple-negative breast cancers from two phase III randomized adjuvant breast cancer trials: ECOG 2197 and ECOG 1199. *J Clin Oncol* 32: 2959-2966.
44. Lee WS, Park S, Lee WY, Yun SH, Chun HK (2010) Clinical impact of tumor-infiltrating lymphocytes for survival in stage II colon cancer. *Cancer* 116: 5188-5199.
45. Chang WC, Li CH, Huang SC, Chou LY, Sheu BC (2010) Clinical significance of regulatory T cells and CD8+ effector populations in patients with human endometrial carcinoma. *Cancer* 116: 5777-5788.
46. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, et al. (2000) Molecular portraits of human breast tumours. *Nature* 406: 747-752.
47. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, et al. (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A* 98: 10869-10874.
48. Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, et al. (2004) Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. *Clin Cancer Res* 10: 5367-5374.
49. Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, et al. (2003) Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci U S A* 100: 8418-8423.
50. Weigelt B, Baehner FL, Reis-Filho JS (2010) The contribution of gene expression profiling to breast cancer classification, prognostication and prediction: a retrospective of the last decade. *J Pathol* 220: 263-280.
51. Foulkes WD, Smith IE, Reis-Filho JS (2010) Triple-negative breast cancer. *N Engl J Med* 363: 1938-1948.
52. Teschendorff AE, Miremadi A, Pinder SE, Ellis IO, Caldas C (2007) An immune response gene expression module identifies a good prognosis subtype in estrogen receptor negative breast cancer. *Genome Biol* 8: R157.
53. Bohling SD, Allison KH (2008) Immunosuppressive regulatory T cells are associated with aggressive breast cancer phenotypes: a potential therapeutic target. *Mod Pathol* 21: 1527-1532.