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Accumulation of regulatory T cells in sentinel lymph nodes is a prognostic predictor in patients with node-negative breast cancer

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ABSTRACT

It has been revealed that sentinel lymph nodes (SLNs) from patients with node-negative breast cancer involve RT-PCR detected micrometastases and isolated tumour cells. However, the prognostic significance of the pathologically undetectable micrometastases is still controversial. In this study, we evaluated Foxp3 positive regulatory T cells (Treg) in SLNs as host-side immune marker that has the potential to detect these micrometastases. In the analyses of training set ($n = 30$), elevated Treg was strongly associated with the pathologically undetectable micrometastases. In the analyses of validation set ($n = 129$) in patients with node-negative, relapse-free survival in patients with elevated Treg was significantly shorter than those with lower Treg ($p = 0.005$). Furthermore, in multivariate analyses, elevated Treg was correlated with relapse-free survival ($p = 0.012$). Our data indicate that Treg may increase in the microenvironment of SLNs along with pathologically undetectable micrometastases and is a prognostic predictor in patients with node-negative breast cancer.

1. Introduction

Invasion into axillary lymph nodes remains the most important prognostic factor for breast cancer.[1–3] For patients with node-negative breast cancer, however, appropriate systemic treatment must be determined by comprehensive assessment of other prognostic factors.[4,5] Although the evaluation of malignant potency of breast cancer has improved with the advent of DNA microarray cluster analyses, treatment decisions for patients with node-negative breast cancer remain difficult. The emergence of sentinel lymph node biopsy (SLNB) as a sensitive screening technique has revealed that the sentinel lymph nodes (SLNs) can harbour a number of micrometastases currently only detectable by reverse transcription polymerase chain reaction (RT-PCR).[6–9] Although SLN analyses using multiple new RT-PCR markers are being investigated, the correlation between metastases detected by RT-PCR and prognosis in patients with node-negative

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breast cancer is still controversial.[10,11] Because of the clinical significance of these micrometastases, a reliable method for detecting or predicting them is needed.

SLNs are the nodes nearest to a primary tumour on the direct lymphatic drainage pathway of the breast and are the typical site of earliest metastasis.[6,7] In a recent immunological study of SLNs and downstream lymph nodes (called non-SLNs) from breast cancer patients, SLN was identified as an important site for anti-tumour immunity.[12–16] Furthermore, in an immune profile of SLN and non-SLN based on T cells and dendritic cells, immunological changes were observed in these lymph nodes prior to pathological invasion.[12] Although the immune profile of SLNs can be used to detect pathologically undetectable micrometastases, the relationship between the immune profile of SLNs using T cells and dendritic cells and the prognosis for a breast cancer patient has not yet been reported. Therefore, a new immune profile marker that can reliably detect pathologically undetectable micrometastases is needed.

Regulatory T cells (Treg) have important roles in maintaining immunological self-tolerance through their ability to suppress wide-ranging immunological responses, including tumour immunity.[17] A previous study reported that CD4(+) CD25(+)Treg accumulate in the main tumours of various cancers.[18–21] Since the identification of the forkhead box p3 (Foxp3) gene as the master regulator of Treg,[22] the relationship between Foxp3(+)Treg and tumour progression has been clarified.[23–26] In breast cancer, the number of Foxp3(+)Treg in a main tumour has been correlated with bad prognosis[27]; thus, Foxp3(+)Treg are under investigation as a new therapeutic target.

We hypothesise that the immune profile of SLNs using Foxp3(+)Treg, due to its specificity for tumours, may be useful for detecting the pathologically undetectable micrometastases of node-negative breast cancer and could become an important prognostic factor for node-negative breast cancer patients. The aims of this study were to evaluate the relationship between the immune profile of SLNs based on Foxp3(+)-Treg and pathologically undetectable micrometastases, and to assess this new SLNs profile as a prognostic predictor in patients with node-negative breast cancer.

2. Patients and methods

2.1. Patients and tissue samples

It the early part of this study, we examined 30 SLNs samples from patients with various clinical stages. We evaluated these samples for analyses of real time RT-PCR, immunohistochemical estimation of SLNs and obtaining the cut off of immunohistochemistry. We called these samples 'training set' in this study. Thirty patients in the training set were all cases who underwent initial surgical resection and sentinel node biopsy at the Department of General Surgery, Chiba University Graduate School of Medicine (Chiba, Japan) between October 2005 and March 2006, and from whom fresh SLNs samples were obtained for RT-PCR. Of the 30 patients in the training set, six patients had ductal carcinoma *in situ* (DCIS) and 24 patients had invasive ductal carcinoma (IDC) of the breast. The DCIS patients in the training set were diagnosed accurately by a pathologist using immunohistochemistry as described below. In this study, SLNs from these DCIS patients were considered negative controls since no metastasis was involved. Clinical and pathological data for patients in both groups were complete, and the patients' cancers were staged according to the International Union Against Cancer (UICC) tumour/node/metastasis (TNM) classification.

It the late part of this study, we examined the clinical validation based on the results of training set in another set; 129 samples from patients with node-negative breast cancer. We called these samples 'validation set'. One hundred and twenty nine patients in validation sets were all cases who underwent initial surgical resection and sentinel node biopsy between January 2000 and November 2002, and were diagnosed pathologically node-negative and have had sufficient clinical follow-up data for clinical validation. In validation set, the latest survival data were collected on 1st April, 2008, and the mean follow-up time was 70 months (range, 43–103 months) for IDC patients. The duration of relapse-free survival (RFS) was the time between initial diagnosis and first recurrence. For all oestrogen receptor-positive patients, tamoxifen (Tamoxifen Citrate) or aromatase inhibitor was prescribed as an adjuvant treatment regardless of age or any other prognostic factors. In patients younger than 70 years, adjuvant cyclophosphamide and anthracyclin were administered if tumours were high histological grade, oestrogen receptor-negative and/or 2 cm in diameter. The study protcol was approved by the Ethics Committee of our institute and written informed was obtained from all patients.

2.2. SLNB and pathology procedure

Detection of SLNs was performed during surgery using two independent methods: the radio-guided method and the blue dye-guided method as described previously.[28] All blue nodes and all nodes with 10% w/v or more of the ex-vivo count of the most radioactive LN were identified as SLNs. When multiple SLNs were obtained by SLNB from patients with node-negative breast cancer, only the most radioactive of the nodes was examined in this study.

Surgically excised SLNs were labelled and cut into multiple serial sections of approximately 2-mm thickness. Some sections were stained with haematoxylin and eosin (H–E) and evaluated by pathologists intraoperatively. When the SLN was found to contain malignant cells, axillary lymph node dissection was performed during the same surgery. Patients determined to be tumour-free in this way underwent no further axillary surgery.

For permanent sectioning, the remaining frozen SLNs were thawed, fixed in 10% formalin w/v, paraffin-embedded as tissue blocks and stained by H–E. When SLNs were found to be node-negative by H–E staining, sections were examined for cytokeratin (AE1/AE3: Dako Cytomation Co., Kyoto, Japan) by immunohistochemistry. The breast specimens from DCIS patients were cut into multiple serial sections of approximately 5-mm thickness and confirmed by the absence of microinvasion of the basal membrane as determined by immunohistochemistry using epithelial membrane antigen (Dako Cytomation Co.).

2.3. Real time RT-PCR

From patients in training set, one terminal section of the SLNs that had not been used for pathological diagnosis was frozen immediately. Total RNA was extracted from these SLN tissues using an RNeasy Mini Kit (QIAGEN, Hilden, Germany). The prepared total RNA served as the template in the first-strand complementary DNA (cDNA) synthesis using Ready-To-Go™ You-Prime First-Strand Beads (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The cDNA product was analysed at a final Mg concentration of 3 mM in a LightCycler with software (version 3.5; Roche Molecular, Mannheim, Germany) using a FastStart DNA Master SYBR Green I Kit (Roche Diagnostics). PCR was performed with the following primer sets: CD4, forward 5′-CTG TGA AGT GGA GGA CCA G-3′, reverse 5′-GGA CTC CTA CAT TGC ACT GA-3′, Foxp3, forward 5′-GAG AAG CTG AGT GCC ATG CA-3′, reverse 5′-GGA GCC CTT GTC GGA TGA T-3′; cytokeratin-19 (CK-19), forward 5′-CCG CGA CTA CAG CCA CTA CTA CAC-3′, reverse 5′-GAG CCT GTT CCG TCT CAA A-3′ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5′-GTC CTT GGC GGG TAA TTC TA-3′, reverse 5′-GCT GAA GTC TGG CTT CTT GG-3′. PCR conditions for CD4, Foxp3, CK-19 and GAPDH were as follows: 95 °C for 10 min, 40 cycles at 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. The expression of CD4, Foxp3 and CK-19 was evaluated as relative ratios against that of GAPDH.

2.4. Immunohistochemistry

From tissues of patients in both groups, 4-μm-thick serial sections were prepared from each paraffin block. For Foxp3 staining, primary antibody (236A/E7, Abcam Ltd., Newcastle, UK) was used at a dilution of 1:200. For visualisation, EnVision Kits (K4007, DAKO, Glostrup, Denmark) were used according to the manufacturer's instructions. For antigen retrieval, the SLN sections were autoclaved at 121 °C for 15 min in citrate buffer (Target Retrieval Solution, pH 9.0, S2368, DAKO). Sections were counterstained with haematoxylin. No significant staining was observed in the negative controls, which were prepared under the same conditions except without incubation with the first antibody. Quantitative evaluation of lymphocytes was done by analysing at least 10 different high-power fields at 400× magnification.

A double immunohistochemical staining was applied on 4-μm paraffin-embedded tissue sections using the following monoclonal antibodies: (a) anti-Foxp3 (236A/E7) at a dilution of 1:200, (b) anti-CD4 (1F6, Nichirei, Tokyo, Japan) at a dilution of 1:1 and (c) anti-CD8 (1A5, Invitrogen Corporation, Carlsbad, CA) at a dilution of 1:1. Antigen retrieval was achieved by treating the slides in autoclave at 121 °C for 15 min in citrate buffer (Target Retrieval Solution, pH 9.0). Foxp3 was applied for 3 h at room temperature and immunodetected, using the 2-step immunohistochemical technique of streptavidinbiotin-alkaline phosphatase (LSAB+ Kits, K0678, DAKO). Fast blue was used 5-Bromo-4-Chloro-3-Indoxyl Phosphate/Nitro Blue Tetrazolium Chloride (BCIP/NBT, DAKO). Subsequently, CD4 and CD8 were added for half an hour at room temperature and immunodetected with a polymer-linked peroxidase molecule, conjugated to secondary antibody (EnVision Kits). Diaminobenzidine/nickel was used as a chromogen. Finally, sections were counterstained with haematoxylin and mounted.

2.5. Statistical analysis

Following real time RT-PCR and immunohistochemistry analyses, we evaluated the significance of the expression of CD4, CK-19 and Foxp3 and the number of Foxp3(+)Treg among patients with DCIS, pN0-IDC and pN1-IDC using Student's *t*-test. For correlation between the number of Foxp3(+)Treg and clinico-pathological variables, chi-square analyses were used. RFS and over all survival time were calculated by the Kaplan-Meier method and compared by the log-rank test based on the number of Foxp3(+)Treg in SLNs. Univariate analyses were performed for the correlation between RFS time and various clinical characteristics included age, tumour size, nodal status, histological grade, lymphatic invasion, oestrogen receptor status, progesterone receptor status, human epidermal growth factor receptor-2 (Her2) status and the numbers of Treg in SLNs. Cox's proportional hazards regression model was used for univariate and multivariate analyses. *P* values of less than 0.05 were considered statistically significant.

Table 1 – Patient and tumour characteristics.

	Training set (n = 30)	Validation set (n = 129)
Age		
Median (range)	54 (33–71)	56 (31–74)
Nodal status		
N0	22	123
N0itc	0	6
N1	8	0
Tumour size in mm		
Median (range)	17 (0–40)	16 (1–40)
Pathological tumour status		
pT0 (DCIS)	6	0
pT1	17	105
pT2	7	24
Histological grade		
1	11	57
2	13	49
3	6	23
Oestrogen receptor status		
Negative	9	42
Positive	21	87
Progesterone receptor status		
Negative	10	49
Positive	20	80
HER2 status		
0.1	23	99
2.3	7	30

Abbreviations: N0itc, node-negative with isolated tumour cells; Her2, human epidermal receptor 2.

3. Results

3.1. Patient characteristics and sentinel node biopsy

The characteristics of patients in the training set and the validation set are shown in Table 1. SLNs could be detected in all patients in the training set and the validation set who were node-negative according to the radio-guided method. In the training set patients, the average number of resected SLNs was 1.9; all patients were stained by blue-dye. In the validation set patients, the average number of resected SLNs was 2.1, and 126 of 129 (97.7%) patients were stained by blue-dye. In the validation set patients, mastectomies were performed in 38 patients, and the other 91 patients received breast-conserving surgeries followed by whole breast radiation therapy (50 Gy ± 10 Gy boost). Among the 24 IDC patients in the training set, intraoperative examination of the SLNs indicated metastases in eight patients. Among the 129 IDC patients in the validation set who were SLN-negative intraoperatively, isolated tumour cells were found postoperatively in tissues from eight of these patients using immunohistochemistry for cytokeratin. No micrometastases or isolated tumour cells were found in any of the 24 IDC patients in the training set. No lymph metastases or isolated tumour cells were found in the six DCIS patients in the training set. In total, the training set included six patients with DCIS, 16 patients with node-negative IDC (pN0-IDC) and eight patients with node-positive IDC (pN1-IDC) for a total of 30 patients. The 129 patients in the validation set with pN0-IDC were diagnosed by UICC classification and included eight patients with isolated tumour cells (pN0itc). Of these 129 pN0-IDC patients, there have been five deaths and eight relapses. Of these eight, four relapsed with lung metastases, two relapsed with bone metastases and two relapsed with metastases in both lung and bone. Among the eight pN0itc patients there was only one death.

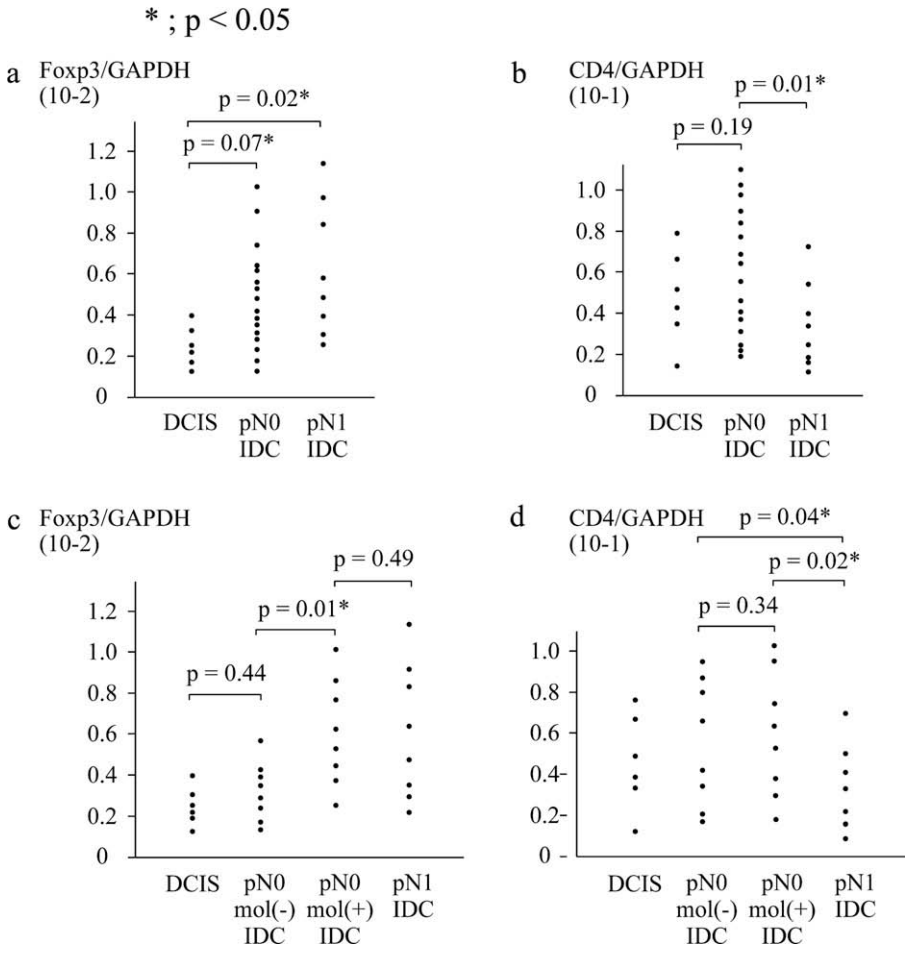

Fig. 1 – Expression of Foxp3 and CD4 detected by real time PCR in SLNs samples from breast cancer patients. (a) Foxp3 expression in SLNs from node-positive IDC patients (pN1-IDC) was significantly higher than in DCIS patients. There was no significant difference between DCIS and pN0-IDC patients. (b) CD4 expression in DCIS and pN0-IDC patients was approximately equal, and CD4 expression in pN1-IDC patients was significantly lower than in pN0-IDC patients. (c) Foxp3 expression was significantly higher in SLNs of patients with molecular metastases (pN0mol(+)-IDC) than in patients with no molecular metastases (pN0mol(–)-IDC). Foxp3 expression in DCIS and pN0mol(–)-IDC patients was approximately equal, as it was in pN0mol(+)-IDC and pN1-IDC patients. (d) CD4 expression of pN0mol(–)-IDC and pN0mol(+)-IDC groups was significantly different.

3.2. *Foxp3(+) expression in SLNs as determined by RT-PCR correlates with molecular metastases in the training set*

Using real time RT-PCR, CD4 and Foxp3 expression in SLNs were analysed in Group 1 patients with DCIS, pN0-IDC and pN1-IDC. Although Foxp3 expression in pN1-IDC patients was significantly higher than in DCIS patients, there was no significant difference between DCIS and pN0-IDC patients, and between pN0-IDC and pN1-IDC patients (Fig. 1a). CD4 expression in DCIS and pN0-IDC patients was nearly equal, and CD4 expression in pN1-IDC patients was significantly lower than in pN0-IDC patients (Fig. 1b). CK-19 expression was evaluated in the SLNs of pN0-IDC patients in order to identify pathologically undetectable micrometastases. CK-19 was considered one of the most specific marker for lymph nodes metastases of breast cancer.[29,30] In this study, we evaluated only CK-19 as micrometastatic tumour marker. Of 16 patients with pN0-IDC, eight showed high expression of CK-19 (low CK-19/GAPDH expression ratio: $3.3 \pm 1.7 \times 10^{-1}$ versus high CK-19/GAPDH expression: $35.5 \pm 21.7 \times 10^{-1}$). Next, we divided the 16 pN0-IDC patients into two groups: a molecular metastasis-positive group (pN0mol(+)-IDC) and a molecular metastasis-negative group (pN0mol(–)-IDC). Although CD4 expression in the two groups was nearly equal, the expression of Foxp3 in patients with pN0mol(+)-IDC was significantly higher than that in pN0 mol(–)-IDC patients (Fig. 1c and d). Elevation of Foxp3(+)Tregs in SLNs as determined by immunohistochemistry correlated with the presence of pathologically undetectable micrometastases. These data suggested that the accumulation of Foxp3(+)Tregs in SLNs is strongly associated with the presence of pathologically undetectable micrometastases in patients with node-negative breast cancer.

3.3. *High accumulation of Foxp3(+) in SLNs as determined by immunohistochemistry correlates with molecular metastases in the training set*

Foxp3 immunohistochemistry was performed on tissues from 30 patients in the training set in order to identify any correlation between the presence of pathologically undetectable micrometastases and the accumulation of Tregs in SLNs as determined by immunohistochemistry. Lymph node consists of cortex and medulla, and cortex consists of paracortex (T cell zone) and germinal centre (B cell zone). As shown in figure (Fig. 2a), Foxp3(+)Treg were distributed diffusely in T cell zone and few in germinal centres and medulla. Thus, in this study, we evaluated Foxp3(+)Treg in T cell zone. Although Foxp3(+)Treg approximately belong to CD4 T cells, the presence of CD8(+)Foxp3(+)Treg in lymph nodes have been reported. Thus, we performed two-colour staining of Foxp3 and CD4/8 in SLNs from breast cancer patients. Most of the cells expressing Foxp3(+) were coexpressed CD4 (Fig. 2b). Furthermore, in Foxp3 and CD8 double-staining of lymph nodes, there were remarkably few cells that coexpress Foxp3 and CD8 (Fig. 2c). Thus, we considered Foxp3(+)Treg as Foxp3(+)CD4(+)Treg in this study. In patients with DCIS, a small number of Foxp3(+)Treg were diffusely scattered in the T cell zone of SLNs (Fig. 3a). In the majority of pN1-IDC patients, a large number of Foxp3(+)Treg accumulated diffusely in the T cell zone of the SLNs (Fig. 3b). In the majority of patients with pN0mol(–)-IDC, a small number of Foxp3(+)Treg were diffusely scattered in the T cell zone of the SLNs (Fig. 3c). In contrast, in the majority of pN0mol(+)-IDC patients, a large number of Foxp3(+)Treg accumulated diffusely in the T cell zone of SLNs (Fig. 3d). Foxp3 immunohistochemistry showed that the presence of Foxp3(+)Treg in SLNs is strongly associated with the presence of pathologically undetectable micrometastases as determined by real time RT-PCR analysis. The two pN0-IDC groups, those with molecular metastases and those without, could be separated significantly ($p = 0.02$ using the χ^2 test) at the threshold of 60/hpf, which is the average of the number of Foxp3(+)Treg in the SLNs of the 16 pN0-IDC patients. The number of Foxp3(+)Treg was not above this threshold in any of the six DCIS patients (Fig. 3e).

3.4. *Presence of Foxp3(+)Treg in SLNs is a prognostic predictor in patients with node-negative breast cancer*

Based on the results from the training set, we assessed whether the presence of Foxp3(+)Treg cells in SLNs could serve as a prognostic predictor in patients with node-negative breast cancer. Foxp3 immunohistochemistry was performed in biopsied SLNs from 129 pN0 patients in the validation set. Tissues from 46 of 129 patients showed high amounts of Foxp3 (>60/hpf), while tissues from 83 patients showed lower

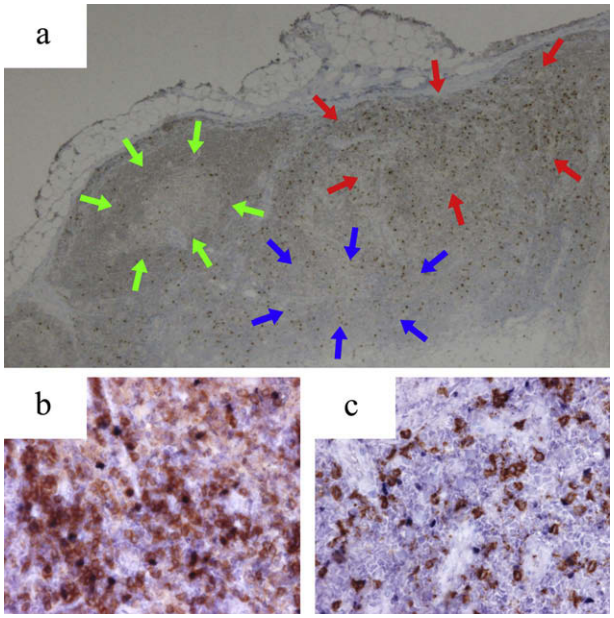

Fig. 2 – Immunohistochemistry for Foxp3 in SLNs from breast cancer patients. (a) Foxp3(+)Treg were distributed diffusely in T cell zone (framed by red arrows) and few in germinal centres (framed by green arrows) and medulla (framed by blue arrows) (×40). (b) In Foxp3 and CD4 double-staining of SLNs, most of the cells expressing Foxp3(+) were coexpressed CD4 (×400). (c) In Foxp3 and CD8 double-staining of SLNs, there were remarkably few cells which coexpress Foxp3 (blue) and CD8 (brown) (×400).

amounts (<60/hpf). Five of the eight pN0itc patients were in the high Foxp3 group (Fig. 4a); their Foxp3(+)Treg cells were distributed homogeneously rather than concentrating around the isolated tumour cells in the T cell zone of the SLNs (Fig. 4b–e). The tumour characteristics of the validation set patients are shown in Table 2. When comparing the high and low Foxp3 groups, there was no difference noted with regard to age, nodal status (pN0 versus pN0itc), histological grade (Grades 1 and 2 versus Grade 3), lymph invasion, oestrogen receptor status, progesterone receptor status and Her2 status. However, within the SLNs from the pN0-IDC patients there was a significant correlation between the number of Foxp3(+)Tregs and pathological tumour size (T1 versus T2) ($p = 0.002$). RFS in patients with elevated Foxp3(+)Tregs in SLNs was significantly shorter than those with lower Foxp3(+)Tregs (Fig. 4f). In the eight patients who had had a relapse, seven were in the high Foxp3 group. Therefore, of the eight pN0itc patients, the only patient who had a relapse also

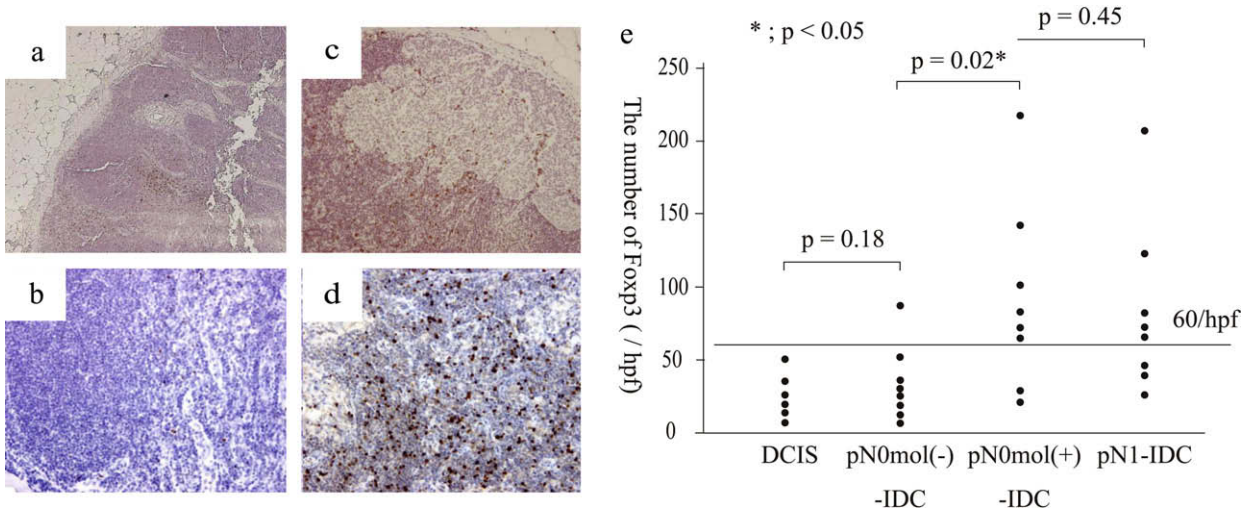

Fig. 3 – Relation between lymph node invasion to SLNs and the number of Foxp3(+)Treg detected by immunohistochemistry. (a) A small number of Foxp3(+)Tregs were present diffusely in the T cell zone of the whole lymph node in patients with DCIS. (b) In node-positive IDC patients (N1-IDC), Foxp3(+)Tregs were not localised in the vicinity of metastatic tumour cells and accumulated diffusely in the T cell zone. (c) In the majority of pN0mol(–)-IDC patients, a small number of Foxp3(+)Tregs were present diffusely in the T cell zone of SLNs. (d) In the majority of pN0mol(+)-IDC patients, a large number of Foxp3(+)Tregs accumulated diffusely in the T cell zone of SLNs. The number of Foxp3(+)Tregs in SLNs from patients with pN0mol(+)-IDC was significantly higher than in pN0mol(–)-IDC patients. (e) The number of Foxp3(+)Treg cells in DCIS and pN0mol(–)-IDC patients was not significantly different, and was approximately equal for pN0mol(+)-IDC and pN1-IDC patients. Threshold of 60/hpf, which was the average number of Foxp3(+)Tregs in SLNs from pN0-IDC patients, separated pN0mol(–)-IDC group from pN0mol(+)-IDC group significantly ($p = 0.01$).

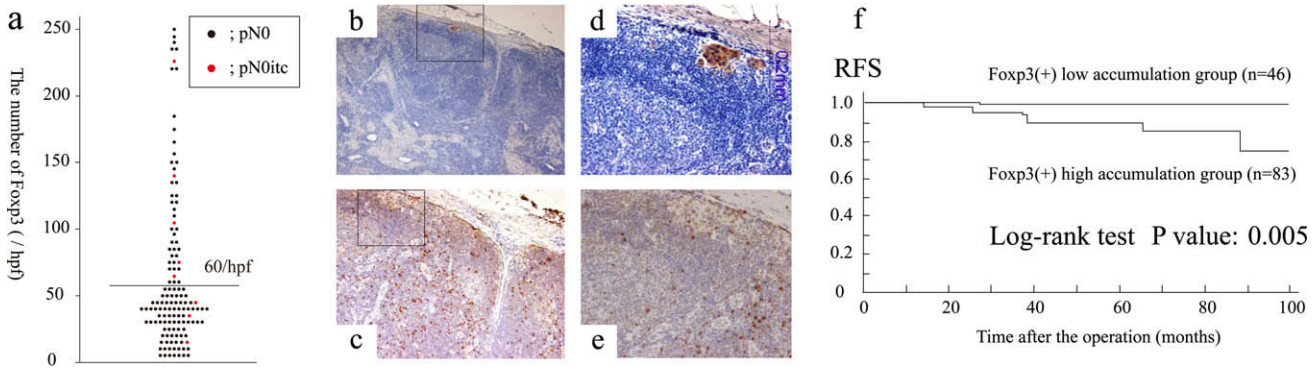

Fig. 4 – Relation between the number of Foxp3(+)Treg in SLNs and RFS. (a) Using the 60/hpf threshold of the number of Foxp3(+)Tregs in SLNs, the patients in the validation set were divided into two groups; high Foxp3(+)Treg group (>60/hpf; $n = 46$) and low Foxp3(+)Treg group (< 60/hpf; $n = 83$). Five of eight patients with pN0itc (red circle) were in the high Foxp3(+)Treg group. (b–d) Isolated tumour cells in SLNs were detected by cytokeratin immunohistochemistry (b: ×40, d: ×400). Foxp3(+)Tregs were present diffusely in the T cell zone in patients with pN0itc (c: ×40, e: ×400). (f) RFS in patients with high accumulation of Foxp3(+)Tregs in SLNs was significantly shorter than that with low accumulation ($p = 0.005$, log-rank test).

exhibited high Foxp3(+)Treg accumulation. However, there was no significant difference in overall survival for these patients (data not shown). We next analysed whether elevated Foxp3(+)Treg in SLNs was an independent prognostic predictor in patients with node-negative breast cancer (Table 3). On univariate analyses, nodal status (pN0itc), histological grade 3 and elevated Foxp3(+)Treg in SLNs correlated significantly with RFS time of the patients. Furthermore, on multivariate analyses among these three factors, histological grade 3 (Hazard ratio: 7.355, $p = 0.007$) and elevated Foxp3(+)-Treg (Hazard ratio: 7.615, $p = 0.012$) were independent prognostic predictors in patients with node-negative breast cancer. It was very interesting that elevated Foxp3(+)Treg in SLNs was an independent prognostic predictor, pN0itc was not in multivariate analyses. These data indicated that the high accumulation of Foxp3(+)Treg in SLNs can be considered a new independent prognostic predictor in patients with node-negative breast cancer.

Table 2 – Correlation analyses between low and high numbers of Foxp3(+)Treg and clinicopathologic data for 129 invasive breast cancer patients with negative sentinel lymph nodes.

Foxp3(+)Treg in sentinel lymph modes	Low ($n = 83$)	High ($n = 46$)	p Value
Age			
<50	16	10	0.745
>50	67	36	
Pathological tumour size			
pT1	70	28	**0.002***
pT2	13	18	
Histological grade			
1	39	20	0.801
2	31	17	
3	13	9	
Lymphocyte invasion			
Negative	74	41	0.987
Positive	9	5	
Oestrogen receptor status			
Negative	23	18	0.179
Positive	60	28	
Progesterone receptor status			
Negative	30	19	0.558
Positive	53	27	
Her2			
0.1	64	34	0.683
2.3	19	12	

Abbreviations: Foxp3, forkhead box p3; Her2, human epidermal receptor 2.
* Significance value $p < 0.05$ The χ^2 test was used to test for differences between categorical variables.

Table 3 – Prognostic predictors of 129 breast cancer patients with negative sentinel nodes in Cox's proportional hazard model.

Variables	Relapse-free survival					
	Univariate analysis			Multivariate analysis		
	Hazard ratio	95 % CI	p Value	Hazard ratio	95 % CI	p Value
Age (<50/⩾50)	1.087	0.225–5.242	0.917			
Tumour status (pT1/pT2)	3.479	0.932–12.99	0.064			
Nodal status (pN0/pN0itc)	6.785	1.407–32.71	**0.017***			
Histological grade (1.2/3)	5.621	1.508–20.95	**0.010***	7.355	1.659–23.20	**0.007***
Lymph invasion (–/+)	1.327	0.165–10.65	0.790			
Oestrogen receptor (+/–)	1.684	0.452–6.275	0.438			
Progesterone receptor (+/–)	1.363	0.365–5.087	0.645			
Her2 (0.1/2.3)	1.658	0.207–13.28	0.634			
Foxp3(+)Treg (low/high)	6.981	1.450–3.62	**0.015***	7.615	1.574–36.84	**0.012***

Abbreviations: CI, confidence interval; N0itc, node-negative with isolated tumour cells; Her2, human epidermal receptor 2; Foxp3, forkhead box p3.
* Significance value $p < 0.05$.

4. Discussion

To the best of our knowledge, this is the first report showing that high accumulation of Foxp3(+)Treg cells in SLNs is an independent prognostic predictor in patients with node-negative breast cancer. Although the classification of breast cancer patients has improved with the advent of DNA microarray cluster analysis, invasion into axillary lymph nodes, which is related to metastatic potential, remains the most important prognostic predictor in breast cancer.[1–3] In patients with node-negative breast cancer, the identification of pathologically undetectable micrometastases in SLNs may be of great significance. If a cellular immune reaction can be detected before pathological lymph node metastases can be detected in SLNs, a tailored treatment might be possible. Kohrt and colleagues reported a detailed immune profile based on CD4 T cells and dendritic cells in SLNs and non-SLNs. They showed that in patients with SLNs metastases a decrease in CD4 T cells and dendritic cells in non-SLNs occurs before metastasis and correlates with RFS.[12] However, an immune profile based on the same cells in SLNs showed no correlation with RFS. Matsuura and colleagues reported that the response of dendritic cells and T cells in SLNs with metastases appeared to be less active compared with that in SLNs without metastases. They showed that expression of Foxp3 was elevated in SLNs involving micrometastases detected by real time RT-PCR compared with SLNs without metastases.[16] However, they did not evaluate correlation with survival. In contrast, we evaluated CD4 T cells and Foxp3(+)Treg in four patient groups (DCIS, pN0mol(–)-IDC, pN0mol(+)-IDC and pN1-IDC) using real time RT-PCR analyses. In contrast with CD4 T cells, Foxp3(+)Treg were significantly elevated in the pN0mol(+)-IDC group compared with pN0mol(–) group. These data suggest that Foxp3(+)Treg, in contrast with CD4 T cells, were elevated in the micro environment of SLNs with pathologically undetectable micrometastases.

Immunohistochemistry was used to confirm protein levels of Foxp3(+)Treg in SLNs and to investigate possible clinical applications. Using immunohistochemistry for Foxp3, Bates and colleagues reported that elevated Treg in tumours correlates with poor prognosis in patients with breast cancer and clearly showed the relationship between expression of Foxp3(+)Treg in tumours and progression of breast cancer.[27] In this study, the number of Foxp3(+)Treg in SLNs could clearly distinguish pN0mol(+) patients from pN0mol(–) patients using as a threshold of the average number of Foxp3(+)Treg in SLNs (60/hpf). Therefore we hypothesised that in contrast to using an immune profile of SLNs based on CD4 T cells and dendritic cells, an immune profile of SLNs based on the presence of Foxp3(+)Treg may be more useful as a prognostic indicator in patients with node-negative breast cancer.

As a result of clinical validation in the validation set, elevated Foxp3(+)Treg seem to be associated with the presence of pathologically undetectable micrometastases, which significantly correlates with a poor prognosis. Furthermore, a multivariate analyses indicated that histological grade 3 and elevated Foxp3(+)Treg were independent prognostic predictors in patients with node-negative breast cancer. It was very interesting that elevated Foxp3(+)Treg in SLNs was an independent prognostic predictor, pN0itc was not in multivariate analyses.

Our data in the training set indicated that the number of Foxp3 in pN0mol(+)-IDC and pN1-IDC was nearly equal (Fig. 3e). The number of Foxp3(+)Treg was not large in SLNs with massive metastases compared to that with small metastases, because the normal T cell zone was reductive due to massive metastases. Thus we thought that the increase in Foxp3(+)Treg was a prognostic factor, the increasing amount of Foxp3(+)Treg was not.

Previously, the relationship between metastases detected by RT-PCR using cytokeratin or mammaglobin and prognosis in pN0-IDC patients was controversial. Moore and colleagues showed that this could have been due to contamination of epithelial cells into SLNs.[31] By evaluating Foxp3(+)Treg in SLNs, it was later shown that pathologically undetectable micrometastases were indirect yet a clear prognostic predictor in pN0-IDC patients. In this study, only one of the pN0itc patients had a relapse, and this patient exhibited elevated Foxp3(+)Treg in SLNs. For clinical evaluation of pathologically undetectable micrometastases in SLNs, our data suggest that assessment of the immune profile of Foxp3(+)Treg may be superior to the direct detection of epithelial cells using RT-PCR.

It will be interesting to study whether low tumour immunity is caused by elevated Foxp3(+)Treg and permits SLNs metastases, or whether metastasis to the SLNs allows Foxp3(+)Treg to accumulate there. Our data do not address this issue directly. However, in this study, the increase in Foxp3(+)Treg in the SLNs microenvironment along with pathologically undetectable micrometastases in pN0mol(+) patients but not in pN0 mol(–) patients, lends support to the latter case.

Our data indicate that Foxp3(+)Treg increase in the microenvironment of SLNs along with pathologically undetectable micrometastases and is an independent prognostic predictor in patients with node-negative breast cancer. Once the relationship between Foxp3(+)Treg and invasion into SLNs is clarified, Foxp3(+)Treg may become a new therapeutic target for regulating the metastasis of breast cancer.

Conflict of interest statement

None declared.

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