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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.).