Abstract. Background: Regulatory T-cells (Tregs) have a pivotal role not only in abrogating autoimmune disease, but also in tumor immune escape. The purpose of the present study was to evaluate the clinical significance of the relative expression of forkhead/winged helix transcription factor (FOXP3) and micrometastasis in the regional lymph nodes (RLNs) of patients with stage I non-small cell lung cancer (NSCLC). Materials and Methods: The RLNs in 131 patients who underwent complete surgical resection for stage I NSCLC were collected at the time of surgery. The relative expression levels of FOXP3 and cytokeratin 19 (CK19) in RLNs were determined by quantitative RT–PCR. Results: The pathological stage was diagnosed as stage IA in 97 patients (74.0%) and stage IB in 34 patients (26.0%). The relative expression levels of FOXP3 and CK19 in the RLNs were 0.062±0.0083% and 0.025±0.056%, respectively. The relative expression of FOXP3 tended to increase with increasing relative expression of CK19. The five-year overall survival rate of the patients with low expression of FOXP3 was better (90.3%) than that of patients with high expression (79.3%) (p=0.0419). A multivariate analysis using the significant variables (gender, age, histology and FOXP3 expression) showed that the FOXP3 expression in RLNs was a significant independent prognostic factor. Conclusion: The expression of CK19 tended to be positively correlated with the expression of FOXP3. High expression of FOXP3 in RLNs was a significant unfavorable prognostic factor in patients with stage I NSCLC.

The immune system plays a major role in the eradication of pre-cancerous cells and suppression of the development of malignancy. Traditionally, non-small cell lung cancer (NSCLC) has not been considered a good target for immunotherapy due to the presence of several tumor immune-escape mechanisms, which do not allow an adequate immune response to develop in the host. Rosenberg et al. reported that the response rate was only 2.6% in different types of clinical cancer vaccine trials (involving 440 patients) performed by the NCI Surgical Branch (1). However, recent randomized phase III trials have demonstrated that immunotherapy can prolong the survival of patients with metastatic melanoma or prostate cancer (2, 3). Furthermore, increasing evidence indicates that immunotherapy is a promising approach for treating patients with NSCLC, and this strategy might represent an alternative treatment approach for lung cancer (4, 5).

Many types of cancers suppress the immune system to enhance their survival. Malignant tumors form cellular constituents composed not only of malignant cells, but also of non-transformed cellular elements, such as stromal cells, neovasculature and various types of immune cells. Regulatory T-cells (Tregs) have a pivotal role not only in the tolerance to self-antigens and abrogation of autoimmune disease, but also in the immune-escape mechanisms employed by tumors (6, 7). An increasing number of studies have suggested that the immune cells residing within the tumor microenvironment are functionally impaired and promote tumor progression, invasion and metastasis (8). Tregs constitutively express high levels of the interleukin 2 receptor chain (CD25) and specifically express the forkhead/winged helix transcription factor (FOXP3) (9).

FOXP3 plays a pivotal role in controlling the expression of many key immunoregulatory genes (10). Our previous study using flow cytometry demonstrated that the frequency of CD4+ CD25+ FOXP3+ Tregs in the regional lymph nodes (RLNs) was a significant prognostic factor in patients who underwent surgical resection for NSCLC (11). In the present study, we evaluated the correlation of the relative expression of FOXP3 with micrometastasis in RLNs in patients with stage I
NSCLC, and also evaluated the prognostic significance of the relative expression of FOXP3+ Tregs in the RLNs.

Materials and Methods

Patients. The study was approved by the Human and Animal Ethics Review Committee of University of Occupational and Environmental Health, Japan (No.05-070), and a signed consent form was obtained from each patient before we collected the tissue samples used in this study. From 2005 to 2007, 181 patients with non-small lung cancer underwent surgery at University of Occupational and Environmental Health. Among them, those who underwent induction chemotherapy or treatment with immunosuppressive agents were excluded from the analysis. Patients who did not undergo RLN dissection were also excluded. The remaining 131 patients were evaluated in this study, and their RLNs and peripheral blood lymphocytes were collected at the time of surgery. The patients’ records, including their clinical data, preoperative examination results, details of the surgery, histopathological findings and TNM staging were also reviewed. The preoperative assessments included chest roentgenography, computed tomography of the chest and upper abdomen, magnetic resonance imaging of the brain, bronchoscopy and bone scintigraphy. All RLNs, including the dissected hilar and mediastinal lymph nodes, were examined pathologically to identify the extent of lymph node metastasis. The resected RLNs were divided into two parts for the histological diagnosis and for this study; macroscopic metastatic lymph nodes were excluded from this study. The latter part of each non-metastatic lymph node was mixed and prepared as a single cell suspension and frozen in a deep freezer at −130°C until it was used as described previously (9). The histopathological findings were classified according to the World Health Organization criteria, and the TNM staging system of international union against cancer (UICC) was employed (12, 13).

Follow-up information regarding each patient was obtained through office visits or by telephone interviews with the patient, a relative or the patient’s primary physician. The patients were evaluated every three months using chest roentgenography, and chest computed tomographic scans and bone scintigraphy were each performed using the detection of intercalated resonance imaging of the brain, bronchoscopy and bone scintigraphy. All RLNs, including the dissected hilar and mediastinal lymph nodes, were examined pathologically to identify the extent of lymph node metastasis. The resected RLNs were divided into two parts for the histological diagnosis and for this study; macroscopic metastatic lymph nodes were excluded from this study. The latter part of each non-metastatic lymph node was mixed and prepared as a single cell suspension and frozen in a deep freezer at −130°C until it was used as described previously (9). The histopathological findings were classified according to the World Health Organization criteria, and the TNM staging system of international union against cancer (UICC) was employed (12, 13).

Follow-up information regarding each patient was obtained through office visits or by telephone interviews with the patient, a relative or the patient’s primary physician. The patients were evaluated every three months using chest roentgenography, and chest computed tomographic scans and bone scintigraphy were each performed every six months for the first two years after surgery, and annually thereafter. The mean follow-up period after surgery was 47 months.

Quantitative RT–polymerase chain reaction (RT–PCR). Total RNA from the frozen tissue specimens was obtained using the RNeasy kit (QIAGEN Science, MD, USA). RNA was converted to cDNA using a First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Tokyo, Japan). These cDNAs were used as templates for PCR amplification. Quantitative RT–PCR was carried out in an ABI Prism 7000 instrument (Applied Biosystems, Foster City, CA, USA). The relative amount of FOXP3 mRNA and cytokeratin 19 (CK19) mRNA were measured by means of the detection of intercalated SYBR green. The PCR was performed using 10 μl of SYBR GREEN PCR Master Mix (Applied Biosystems), either 2 μl of cDNA or 7.4 μl of water and each primer set (described below) in a total volume of 20 μl. The PCR cycles were 95°C for 20 sec, followed by 45 cycles of 95°C for 3 seconds and 60°C for 30 sec. The sense and antisense primer sequences for FOXP3 used for quantitative RT–PCR were: 5’- CTT CAA GTT CCA CAA CAT GCG-3’ and 5’- CGT GGC GTA GGT GAA AGG G-3’, respectively. The sense and antisense primer sequences for CK19 used for the quantitative RT–PCR were: 5’- AAC GGC GAG CTA GAG GTG A-3’ and 5’- GGA TGG TCG TGT AGT AGT GCC-3’, respectively. The quantitative PCR primers used for β–actin were β–actin Control Reagents (Applied Biosystems). The threshold cycle number (CT) was defined as the fractional cycle number at which the amount of amplified target product reached a fixed threshold. The ACT was obtained by comparing the CT of FOXP3 with the CT of β–actin in same amount of templates, and 2–ACT was defined as the fold-difference in the mRNA expression of the target gene compared to the β–actin expression in the same sample. The relative expression was calculated using the following formula:

Relative expression=2–(ΔCTsample –ΔCTcontrol)

We performed a receiver operating characteristic (ROC) curve analysis of Tregs and CK19 to evaluate cut-off values for prognosis. These curves showed that the most sensitive and specific levels of 2–ACT for FOXP3 and CK19 were 0.06 and 0.01, respectively. Therefore, patients were considered to be part of the high FOXP3 expression group when their relative expression of FOXP3 exceeded 0.06. Patients were defined as being part of the high CK19 expression group when the relative expression of CK19 exceeded 0.01.

Table I. The relative expression of FOXP3 and CK19 in RLNs.

<table>
<thead>
<tr>
<th>n</th>
<th>FOXP3</th>
<th>CK19</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>Means±SD</td>
<td>Means±SD</td>
</tr>
<tr>
<td>All patients</td>
<td>131</td>
<td>0.062±0.083</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>75</td>
<td>0.066±0.104</td>
</tr>
<tr>
<td>Female</td>
<td>56</td>
<td>0.056±0.042</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>99</td>
<td>0.066±0.093</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>21</td>
<td>0.050±0.050</td>
</tr>
<tr>
<td>Other types of carcinoma</td>
<td>11</td>
<td>0.051±0.022</td>
</tr>
<tr>
<td>T Status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>97</td>
<td>0.062±0.088</td>
</tr>
<tr>
<td>T2</td>
<td>34</td>
<td>0.061±0.071</td>
</tr>
</tbody>
</table>

CK19; Cytokeratin 19; FOXP3; forkhead/winged helix transcription factor.

Statistical analysis. The Mann-Whitney U-test was used to determine the differences in the continuous variables between the two groups. The survival curve was calculated using the Kaplan–Meier method, and the data were compared using the log-rank test for a univariate analysis. The prognostic factors were analyzed using a multivariate analysis with Cox’s proportional hazard model to adjust for any potentially confounding factors. The categorical variables were compared using the chi-square test or Fisher’s exact test. Differences in the findings were considered to be significant for values of p<0.05. The Statview V software package (Abacus Concept, Berkeley, CA, USA) was used for all of the statistical analyses.

Results

Each of the 131 patients had undergone a complete resection for NSCLC. The patients included 75 males and 56 females. The mean age of patients was 70.9 years (range=28-85
years). The histological types of cancer seen in these patients included 99 adenocarcinomas (75.5%), 21 squamous cell carcinomas (16.0%) and 11 other types of carcinomas. The pathological stage was diagnosed as stage IA in 97 patients (74.0%) and stage IB in 34 patients (26.0%). The average expression levels of FOXP3 and CK19 relative to that of β-actin in the RLNs were 0.062±0.0083% and 0.025±0.056%, respectively. The relative expression levels of FOXP3 and CK19 according to the clinicopathological factors, such as gender, histology and the pathological stage are shown in Table I. A significant correlation was not observed with these clinical factors. The correlation between the relative expression levels of FOXP3 and CK19 is indicated in Figure 1. The relative FOXP3 expression tended to increase with increasing relative expression of CK19 (R=0.352, p=0.001).

The five-year overall survival rates of the patients according to the relative expression of FOXP3 were 90.3% and 79.3% in the low- and high-expression groups, respectively (Figure 2). A significant difference was observed in the survival rate between high and low FOXP3 expression groups (p=0.0419). Regarding the relative expression of CK19, the five-year overall survival rate was 88.9% in the low expression group, and 83.6% in the high-expression group (Figure 3). There was no significant survival difference between the low- and high-CK19 expression groups.

A univariate analysis for overall survival showed that gender (female vs. male, p=0.0353), age (<75 vs. ≥75, p=0.0416), histology (adenocarcinoma vs. other, p=0.0050) and the relative expression of FOXP3 (low vs. high, p=0.0492) were significant prognostic factors (Table II). A multivariate analysis using these significant variables (gender, age, histology and FOXP3 expression) showed that the hazard ratio for low relative expression of FOXP3 was 0.237 (95% confidence interval=0.180-0.902, p=0.0454). This result indicates that FOXP3 expression is a significant independent prognostic factor for patients with stage I NSCLC who undergo complete surgical resection (Table III).
On the other hand, inducible Tregs can be induced from the periphery to perform their key role in immune homeostasis. That are derived from the thymus and emigrate into the T-lymphocytes (16). The depletion of Tregs facilitated the induction of cytotoxic T-lymphocytes against autologous tumor cells, and RLNs of patients with NSCLC suppressed the induction of immunotherapies. In our previous studies, the Tregs in the major obstacle to the development of effective mechanisms of tumor-driven immune evasion, which is essential for maintaining peripheral tolerance, preventing autoimmune diseases and limiting chronic inflammatory diseases (14). Severe autoimmune diseases, such as asthma and inflammatory bowel disease, are induced by genetic or physical ablation of the Treg population (15). However, Tregs also block bowel disease, are induced by genetic or physical ablation of inflammatory diseases, such as type 1 diabetes, and chronic inflammatory diseases (14). Severe autoimmune tolerance, preventing autoimmune diseases and limiting homeostasis, which is essential for maintaining peripheral Tregs are critical for the maintenance of immune cell beneficial responses against certain pathogens and abrogate antitumor immunity. Therefore, Tregs have a crucial role in the mechanisms of tumor-driven immune evasion, which is a major obstacle to the development of effective immunotherapies. In our previous studies, the Tregs in the RLNs of patients with NSCLC suppressed the induction of cytotoxic T-lymphocytes against autologous tumor cells, and the depletion of Tregs facilitated the induction of cytotoxic T-lymphocytes (16).

There are two categories of Tregs. One is natural Tregs that are derived from the thymus and emigrate into the periphery to perform their key role in immune homeostasis. On the other hand, inducible Tregs can be induced from CD4+CD25− precursors in the periphery in the presence of IL2 and TGFβ (17). One of the immunosuppressive mechanisms is cell–cell contact; Tregs may suppress target cells via the direct interaction of receptor–ligand pairs on Tregs and target cells. Another mechanism is the local secretion of inhibitory cytokines (18). This division of labor between natural Tregs and induced Tregs is not absolute, and both subsets have similar phenotypic characteristics. Inducible Tregs regulate their function through the secretion of immunosuppressive soluble factors, such as IL9, IL10 and TGFβ (16). Lung cancer also produces immunosuppressive cytokines, including TGFβ, that may either enhance tumor growth or modify the antitumor immune responses (19). TGFβ is crucial for the maintenance of FOXP3 gene expression and the function of Tregs.

The transcription factor FOXP3 is a master gene involved in the differentiation and function of Tregs (20). FOXP3 has been used as a marker for CD4+CD25+ T-cells because it exclusively expressed in Tregs. In a previous study, the frequency of Tregs was evaluated in the RLNs and peripheral blood lymphocytes of patients with NSCLC using CD4+, CD25+ and FOXP3+ as biomarkers for Tregs by flow cytometry (11). The study showed that Tregs were present at a higher frequency in the RLNs compared to the peripheral blood lymphocytes in patients with lung cancer, and that the high frequency of Tregs in RLNs was a significant unfavorable prognostic factor.

There have been very few investigations regarding the prognostic significance of Tregs in RNLs of patients with NSCLC (11). The FOXP3+ cell density of metastatic sentinel lymph nodes in patients with malignant melanoma was associated with an unfavorable clinical outcome, but no significant correlation was found in the case of sentinel-negative patients (21). FOXP3+ cells in the metastatic sentinel lymph nodes of patients with breast cancer were significantly more frequent than in tumor-free sentinel lymph nodes (22). Our present study demonstrated that higher expression of FOXP3 in the pathologically non-metastatic lymph nodes of patients with NSCLC was a significant unfavorable prognostic factor. The discriminative power of a recurrence risk assessment by the nodal status could be improved by adding information about Tregs in RNLs.

Micrometastasis in RNLs was common in cases of resected NSCLC, even in patients with N0 disease according to routine pathological examination. Our previous study showed that the micrometastasis in RNLs detected by cyto keratin staining was also an unfavorable prognostic factor in patients with stage I NSCLC (23). Recently, micrometastasis in RNLs was evaluated in different kinds of cancers, including NSCLC, based on the expression of CK19 determined by PCR (24, 25). One-step nucleic acid amplification has been introduced as a novel standard method for the intraoperative evaluation of lymph node metastasis of breast cancer based.

**Table II. Results of the univariate analysis of the overall survival using a Cox proportional hazard model.**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Relative 95% confidence interval</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (female vs. male)</td>
<td>0.202 0.046-0.806</td>
<td>0.0353</td>
</tr>
<tr>
<td>Age (&lt;75 years vs. ≥75 years)</td>
<td>0.327 0.112-0.958</td>
<td>0.0416</td>
</tr>
<tr>
<td>Histology (adenocarcinoma vs. other)</td>
<td>0.234 0.085-0.645</td>
<td>0.0050</td>
</tr>
<tr>
<td>T Factor (T1 vs. T2)</td>
<td>0.502 0.179-1.412</td>
<td>0.1915</td>
</tr>
<tr>
<td>CK19 (low vs. high)</td>
<td>0.425 0.149-1.183</td>
<td>0.1007</td>
</tr>
<tr>
<td>FOXP3 (low vs. high)</td>
<td>0.354 0.126-0.997</td>
<td>0.0492</td>
</tr>
</tbody>
</table>

CK19: Cytokeratin 19; FOXP3: forkhead/winged helix transcription factor.

**Table III. Results of the multivariate analysis of the overall survival using a Cox proportional hazard model.**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Relative 95% confidence interval</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (female vs. male)</td>
<td>0.310 0.065-1.488</td>
<td>0.1453</td>
</tr>
<tr>
<td>Age (&lt;75 years vs. ≥75 years)</td>
<td>0.331 0.090-0.997</td>
<td>0.0493</td>
</tr>
<tr>
<td>Histology (adenocarcinoma vs. other)</td>
<td>0.293 0.096-0.893</td>
<td>0.0309</td>
</tr>
<tr>
<td>FOXP3 (low vs. high)</td>
<td>0.237 0.180-0.902</td>
<td>0.0454</td>
</tr>
</tbody>
</table>

FOXP3: Forkhead/winged helix transcription factor.

**Discussion**

Tregs are critical for the maintenance of immune cell homeostasis, which is essential for maintaining peripheral tolerance, preventing autoimmune diseases and limiting chronic inflammatory diseases (14). Severe autoimmune syndromes, such as type 1 diabetes, and chronic inflammatory diseases, such as asthma and inflammatory bowel disease, are induced by genetic or physical ablation of the Treg population (15). However, Tregs also block beneficial responses against certain pathogens and abrogate antitumor immunity. Therefore, Tregs have a crucial role in the mechanisms of tumor-driven immune evasion, which is a major obstacle to the development of effective immunotherapies. In our previous studies, the Tregs in the RLNs of patients with NSCLC suppressed the induction of cytotoxic T-lymphocytes against autologous tumor cells, and the depletion of Tregs facilitated the induction of cytotoxic T-lymphocytes (16).

There are two categories of Tregs. One is natural Tregs that are derived from the thymus and emigrate into the periphery to perform their key role in immune homeostasis. On the other hand, inducible Tregs can be induced from CD4+CD25− precursors in the periphery in the presence of IL2 and TGFβ (17). One of the immunosuppressive mechanisms is cell–cell contact; Tregs may suppress target cells via the direct interaction of receptor–ligand pairs on Tregs and target cells. Another mechanism is the local secretion of inhibitory cytokines (18). This division of labor between natural Tregs and induced Tregs is not absolute, and both subsets have similar phenotypic characteristics. Inducible Tregs regulate their function through the secretion of immunosuppressive soluble factors, such as IL9, IL10 and TGFβ (16). Lung cancer also produces immunosuppressive cytokines, including TGFβ, that may either enhance tumor growth or modify the antitumor immune responses (19). TGFβ is crucial for the maintenance of FOXP3 gene expression and the function of Tregs.

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on the quantification of CK19 mRNA because there was a 96% concordance rate with the detailed histopathology (26). Ge et al. reported that CK19 mRNA RT-PCR made the assessment of the metastatic status in RLNs more accurate, which can be helpful for screening the patients in whom early sub-clinical metastasis exists, and may be easier or more accurate than a histological examination (27).

There have been few studies regarding the micrometastasis in RLNs and the immunological response. Matsuura et al. demonstrated that the Treg cell response was induced at the micrometastasis level and persisted during the progression of metastasis in sentinel nodes in patients with breast cancer (28). Other investigators have reported that DC maturation was triggered and subsequently followed by the up-regulation of Th-1 responses along with the metastasis in sentinel nodes of breast cancer, while there was parallel upregulation of the Th-2 and Treg cell responses (29). In the present study, the relative expression of FOXP3 tended to increase with increasing relative expression of CK19, and a weak correlation was observed between the expression levels of FOXP3 and CK19.

In conclusion, the results of this study indicate that there is a weak correlation between the expression of FOXP3 and CK19 in the RLNs of patients with stage I NSCLC, suggesting that the suppression of antitumor immunity was induced at the micrometastasis level in RLNs. The higher expression of FOXP3 in RLNs was a significant unfavorable prognostic factor in patients with stage I NSCLC. The information on FOXP3 expression in RLNs might, therefore, be important for determining which individuals are at a higher risk of relapse after surgical resection, and who are indicated for more aggressive treatment and follow-up examinations. Further clinical studies are necessary to evaluate the efficacy of adjuvant therapy for the patients selected according to the FOXP3 expression in their RLNs.

Acknowledgements

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References


