Tumor-Infiltrating Foxp3+ Regulatory T Cells are Correlated with Cyclooxygenase-2 Expression and are Associated with Recurrence in Resected Non-small Cell Lung Cancer

Katsuhiko Shimizu, MD, PhD, Masao Nakata, MD, PhD, Yuji Hirami, MD, PhD, Takuro Yukawa, MD, Ai Maeda, MD, and Kazuo Tanemoto, MD, PhD

**Background:** Cyclooxygenase-2 (COX-2) is constitutively overexpressed in a variety of epithelial malignancies and is usually associated with a poor prognosis. COX-2-derived prostaglandin E2 transforms CD4+CD25+ T regulatory (Treg) cells (Tregs), and Tregs are thought to moderate the antitumor immune response. Herein, we investigated the prognostic value of tumor-infiltrating Treg cells and their correlation with COX-2 expression in resected non-small cell lung cancer (NSCLC).

**Material and Methods:** Intratumoral COX-2 and Treg expression were retrospectively assessed using immunohistochemistry in paraffin-embedded samples from 100 patients who had undergone complete resections for NSCLC. The expressions of COX-2 and Foxp3, which was most specific Treg cell marker, were compared with the clinicopathological variables, and the correlation between Foxp3+ Tregs and COX-2 expression was analyzed.

**Results:** The recurrence-free survival (RFS) of patients with elevated COX-2 expression was significantly worse than that of patients without COX-2 expression. Tumor-infiltrating Foxp3-positive lymphocytes were positively correlated with COX-2 expression. The median count for Foxp3-positive lymphocytes was 3 (minimum-maximum, 0–24) in 10 high-power fields. The RFS of patients with tumors containing ≥3 Foxp3-positive cells (Foxp3 expression group) was significantly worse than that of patients with tumors containing <3 Foxp3-positive cells. In a multivariate analysis, only nodal status was an independent predictor of a significantly shorter RFS. However, in node-negative NSCLC, Foxp3 expression was an independent predictor of a significantly shorter RFS.

**Conclusions:** Tumor-infiltrating Foxp3+ Tregs were positively correlated with intratumoral COX-2 expression and were associated with a worse RFS, especially among patients with node-negative NSCLC.

**Key Words:** Foxp3, Regulatory T cell, Cyclooxygenase-2, Non-small cell lung cancer.

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Lung cancer is a major cause of death in many developed countries. Surgical resection continues to play an important role in the treatment of this disease, especially during the early stages of lung cancer. Even when patients are diagnosed at an early stage, however, the relapse rate is high as 15 to 35% after surgical resection in Japan. Despite having identical radiologic and histologic features, many patients with presumed localized disease also have undetectable metastases at the time of diagnosis. Thus, the current clinical-pathologic staging system is inadequate.

Cyclooxygenase (COX) is the key enzyme required for the conversion of arachidonic acid to prostaglandins (PGs). Two COX isoforms have been identified and are referred to as constitutive COX (COX-1) and inducible COX (COX-2). COX-1 is constitutively expressed in many tissues and plays an important role in the control of homeostasis. Conversely, COX-2 is an inducible enzyme that is activated in response to extracellular stimuli such as growth factors and proinflammatory cytokines. Some investigators have demonstrated that COX-2 is constitutively overexpressed in a variety of epithelial malignancies such as lung, breast, pancreas, colon, esophagus, and head and neck cancers, and COX-2 overexpression is usually associated with a poor prognosis.

T regulatory (Treg) cells (Tregs) were initially characterized as having a CD4+CD25+ phenotype, and these cells are thought to modulate the antitumor immune response. Tregs can suppress the activity of cytotoxic T cells through direct cell-to-cell contact or through the release of cytokines. The most specific Treg cell marker identified to date is a nuclear transcription factor known as Foxp3. A high density of tumor-infiltrating Foxp3+ Tregs is reportedly associated with a higher risk of recurrence and a poor overall survival among patients with certain types of malignant neoplasms. Thus, Treg cells within the tumor microenvironment might play a significant role in the suppression of local antitumor immune responses.
A recent report has shown that tumor-derived COX-2/PGE₂ induces the expression of the Foxp3 gene and increases Treg cell activity in lung cancer. In this study, we investigated the prognostic value of intratumoral COX-2 expression and tumor-infiltrating Foxp3+ Tregs and determined whether a correlation exists between the expression of COX-2 and Foxp3+ Tregs in non-small cell lung cancer (NSCLC).

PATIENTS AND METHODS

Study Population

One hundred patients with NSCLC who underwent resection at our institution were retrospectively studied. All the patients had undergone a lobectomy and lymph node dissection and had followed up for at least 2 years. Patients who had received induction therapy or who had another malignancy were excluded. The baseline demographics, histopathologic data, RFS period, and pathologic specimens preserved in paraffin were available for all the patients. A written informed consent was obtained from each patient before surgery, and this study was approved by the institutional Ethics Committee of Kawasaki Medical School.

Immunohistochemistry

Immunohistochemical analyses were performed using resected, paraffin-embedded lung cancer tissues. After microtome sectioning (4 µm), the slides were processed for COX-2 and Foxp3 staining using an automated immunostainer (Nexes; Ventana, Tucson, AZ). The streptavidin-biotin-peroxidase detection technique using diaminobenzidine as a chromogen was applied. The primary antibodies were used according to the manufacturer’s instructions (COX-2: DakoCytomation clone CX-294, 1/50 dilution; Foxp3: Abcam, clone 22510, 1/100 dilution).

The slides were examined by an investigator who had no knowledge of the corresponding clinicopathologic data. To evaluate the COX-2 immunostaining, the reactions in smooth muscles and vascular endothelial cells, which were present in all the specimens, were used as internal built-in controls. Cases with tumor cells that exhibited a significantly more intense staining pattern than an internal control were classified as positive. To evaluate Foxp3 immunostaining, 10 high-power fields (HPFs) digital images of the tumor areas were selected, and the absolute number of Foxp3-positive lymphocytes in these 10 HPF digital images was determined. The number of immunostained Foxp3 cells was then determined by averaging the 10 HPF digital image cell counts (Figure 1B).

Statistical Analysis

Given that there are no widely accepted standard cutoff points for defining clinical outcome according to the number of Tregs in the present setting and consistent with the method used in previous studies, we selected the median intratumor Foxp3+ cell count of the entire group as the cutoff value. We then assessed the associations among COX-2 expression, Treg number, clinicopathologic features, and RFS. The following patient characteristics were investigated: age, sex, histology, tumor size, and nodal involvement of lung cancers. A statistical analysis examining significant differences among the categorized groups and possible correlations between COX-2 expression and the clinicopathologic features were performed using the Fisher’s exact test or the χ² test, as appropriate. An unpaired t test was used for the comparison of continuous data. Multivariate analyses were performed using a logistic regression analysis. To explore the association between RFS and COX-2 expression or the Foxp3+ Treg count, a Kaplan-Meier survival analysis was performed by stratifying significant predictor variables identified in the Cox proportional hazards model. All data were analyzed using the Stat View software program, version 5.0 (SAS Institute Inc., Cary, NC). All statistical tests were two-sided, and probability values <.05 were regarded as statistically significant.

RESULTS

The patient characteristics and immunohistochemical variables are shown in Table 1. The tumors were staged according to the 1997 tumor, node, metastasis staging system.

Relation between the Expression Status of COX-2 and Clinicopathologic Characteristics

Of the 100 patients, 65 exhibited markedly more intense COX-2 immunoreactivities in their tumor cells, whereas the remaining 35 cases did not show an increase in COX-2 expression. A significant association between elevated COX-2 expression and nodal involvement was observed (p = 0.033), but no significant associations with age (p = 0.212), sex (p = 0.285), histology (p = 0.129), tumor size (p = 0.739), or disease stage (p = 0.130) were noted (Table 1). The RFS of patients with elevated COX-2 expres-
tion was significantly worse than that of patients without COX-2 expression (p = 0.017 according to a log-rank test; Figure 2).

Relation between the Expression Status of COX-2 and the Foxp3-Positive Lymphocyte Count

In the COX-2 positive group, the mean Foxp3-positive lymphocyte count of 10 HPF was 6.5 ± 5.5. Conversely, in the COX-2 negative group, the mean Foxp3-positive lym

TABLE 1. The Patient Characteristics by COX-2 and Foxp3 Expression

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>COX-2 Expression</th>
<th>Foxp3-Positive Cells</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
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<tr>
<td>Patients, number</td>
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<td>35</td>
</tr>
<tr>
<td>Age (yr)</td>
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<tr>
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<td>17</td>
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</tr>
<tr>
<td>Squamous cell carcinoma</td>
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<td>7</td>
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<tr>
<td>Primary tumor (pT)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>31</td>
<td>18</td>
</tr>
<tr>
<td>T2</td>
<td>25</td>
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<td>T3–4</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Nodal involvement (pN)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>43</td>
<td>31</td>
</tr>
<tr>
<td>N1</td>
<td>9</td>
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<td>Disease stage</td>
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<td>16</td>
</tr>
<tr>
<td>IB</td>
<td>16</td>
<td>13</td>
</tr>
<tr>
<td>II(A+B)</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>III(A+B)</td>
<td>15</td>
<td>3</td>
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COX, cyclooxygenase-2.

Relation between the Expression Status of Foxp3 and Clinicopathologic Characteristics

Among the 100 cases, the median count of Foxp3-positive lymphocytes was 3 (minimum-maximum, 0–24). The number of cases with Foxp3 cells <3 and the number with ≥3 (Foxp3 expression group) were 49 and 51, respectively. No significant associations between an elevation in Foxp3 expression and age (p = 0.192), sex (p = 0.999), cyte count of 10 HPF was 1.7 ± 1.5. Tumor-infiltrating Foxp3-positive lymphocytes were positively correlated with COX-2 expression (p < 0.001) (Figure 3).
histology \((p = 0.465)\), tumor size \((p = 0.844)\), nodal involvement \((p = 0.081)\), or disease stage \((p = 0.255)\) were noted (Table 1). The RFS of patients with tumors containing \(\geq 3\) Foxp3 cells (Foxp3 expression group) was significantly worse than that of patients with tumors containing \(< 3\) Foxp3 cells \((p = 0.004\) according to a log-rank test; Figure 4).

**Prognostic Value of COX-2 and Tregs**

A univariate analysis revealed that nodal status \((p = 0.001)\), COX-2 expression \((p = 0.017)\), and Foxp3 expression \((p = 0.004)\) were independent risk factors associated with RFS. Nevertheless, a multivariate analysis revealed that only nodal status was an independent risk factor \((p = 0.004)\) and that COX-2 expression \((p = 0.320)\), and Foxp3 expression \((p = 0.107)\) were not independent risk factors (Table 2). In the node-negative group \((N = 76)\), the RFS of patients with elevated COX-2 and Foxp3 expression levels was significantly worse than that of patients without COX-2 expression \((p = 0.024)\) or without Foxp3 expression \((p < 0.001)\) (Figure 5). Conversely, in the node-positive NSCLC group, the RFS of patients elevated COX-2 and Foxp3 expression levels was not significantly worse than that of patients without COX-2 expression \((p = 0.933)\) or without Foxp3 expression \((p = 0.668)\). A multivariate analysis revealed that only Foxp3 expression was an independent predictor of RFS \((p = 0.016)\) (Table 2).

**DISCUSSION**

Previous reports have shown that the total number of tumor-infiltrating lymphocytes (TILs) is positively associated with patient prognosis in lung cancer and other cancers.\(^2\)\(^-\)\(^4\) However, whether all TILs have an antitumor effect is unclear. In 2001, Woo et al.\(^2\)\(^4\) showed that large populations of CD4+CD25+ T cells were present among the TILs of patients with NSCLC. They demonstrated that CD4+CD25+ T cells selectively inhibit the host immune response and therefore could contribute to the progression of lung cancer.\(^2\)\(^5\)

Since then, malignancies such as lung cancer have also been noted to have increased numbers of Treg cells and Treg activity levels within the peripheral blood and within TIL populations.\(^2\)\(^6\)-\(^2\)\(^9\) In addition, a high density of tumor-infiltrating Foxp3+ Tregs is reportedly associated with a higher risk of recurrence and a poor overall survival in patients with stage I NSCLC.\(^1\)\(^3\) Our studies also showed that the number of tumor-infiltrating Foxp3+ Tregs was associated with a worse RFS, especially among patients with node-negative NSCLC.

Meanwhile, a report published nearly a decade ago showed that COX-2 overexpression was associated with a poor prognosis and a short survival period in NSCLC.\(^4\) To date, several investigators have intensively studied the contribution of COX-2 to tumorigenesis. Several mechanisms are thought to mediate the tumorigenic activity of COX-2 as follows: (1) PGs directly stimulate the proliferation of cancer cells\(^3\)\(^0\)-\(^3\)\(^1\); (2) COX-2 acts as an angiogenic stimulator and has been shown to increase the production of angiogenic factors and the migration of endothelial cells\(^3\)\(^2\)-\(^3\)\(^3\); (3) COX-2-derived PGs function to prevent apoptosis induced by anticancer drugs\(^3\)\(^4\),\(^3\)\(^5\); (4) COX-2 expression might increase the invasive ability of tumor cells, promoting cancer metastasis\(^3\)\(^6\); and (5) PGs are immunoregulatory molecules that can suppress antitumor activity.\(^3\)\(^7\),\(^3\)\(^8\) Our studies also showed that COX-2 expression was associated with a worse RFS.

Of note, the number of tumor-infiltrating Foxp3+ Tregs was positively correlated with intratumoral COX-2 expression in this study. To our knowledge, only one other report has shown that the number of Tregs is positively correlated with intratumoral COX-2 expression. Li et al.\(^3\)\(^9\)

**FIGURE 4.** Kaplan-Meier recurrence-free survival curve according to Foxp3 expression, log-rank \(p = 0.004\).

<table>
<thead>
<tr>
<th>TABLE 2. Prognostic Value of Recurrence-Free Survival</th>
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<tr>
<td><strong>Variables</strong></td>
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<tr>
<td></td>
</tr>
<tr>
<td>All cases</td>
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<tr>
<td>Nodal involvement</td>
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<tr>
<td>COX-2 expression</td>
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<tr>
<td>Foxp3 expression</td>
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<tr>
<td>Node-negative cases</td>
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<tr>
<td>COX-2 expression</td>
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<tr>
<td>Foxp3 expression</td>
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HR, hazard ratio; CI, confidence interval.
demonstrated that an increase in the number of peritumoral Tregs was associated with a worse prognosis and was positively correlated with intratumoral COX-2 expression in patients with renal cell carcinoma. Our results using tissues from patients with NSCLC are similar to this previous report examining renal cell carcinoma and represent the first report of these phenomena in NSCLC.

A recent study reported that tumor-induced Treg cell activity can be downregulated by COX-2 inhibition, leading to the restoration of antitumor responses. Shama et al.19 documented a COX-2-dependent immunosuppressive network in the NSCLC microenvironment in mice, and tumor-derived COX-2/PGE2 induced the expression of the Treg cell-specific transcription factor Foxp3, resulting in an increase in Treg cell activity. In addition, they documented that COX-2/PGE2 suppressed antigen presentation, decreases maturation of dendritic cells and potently induced IL-10 transcription while reducing IL-12.40 Baratelli et al.41 reported that PGE2 is involved in the modulation of T cell function and differentiation in vitro, and an increase in T cell function and differentiation could contribute to tumor-induced immunosuppression. Mahic et al.42 also described Treg cells expressed COX-2 and Foxp3 in vitro, and Treg cells produced PGE2 and suppressed effector T cell responses in a manner that is reversed by COX-2 inhibitors and PGE2 receptor-specific antagonist. Recently, they described that Treg cells expressed cell surface markers consistent with activated phenotype and secreted high levels of TGF-ß and IL-10 excluding PGE2.43 Our present study using resected tissue supported these basic experiments, confirming a correlation between Tregs and COX-2 expression in NSCLC. Future cancer treatments targeting both the control of COX-2 and Treg might be feasible. In fact, recent clinical trial by Cancer and Leukemia Group B demonstrated that patients with increased COX-2 expression receiving COX-2 inhibitor had better survival than did COX-2-expressing patients not receiving drug.44

In summary, the present results indicate that the number of tumor-infiltrating Foxp3+ Tregs is positively correlated with intratumoral COX-2 expression. Among patients with node-negative NSCLC, in particular, Foxp3 expression (tumors containing ≥3 Foxp3 positive cells in 10 HPFs) was an independent prognostic factor in a multivariate analysis. Thus, COX-2 expression might suppress antitumor activity by tumor-infiltrating Tregs. A COX-2 inhibitor might be beneficial for the treatment of patients with COX-2 overexpression. Further studies examining other types of cancer are necessary.

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REFERENCES


