Detecting tumor-infiltrating Forkhead box P3-positive T cells in the prognosis of lung adenocarcinoma: Possible role of clustering tumor interleukin-12 subunit alpha and transforming growth factor beta 1 expression

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Abstract

Background. While regulatory T cells (Tregs) are a poor prognostic factor for lung cancer, they may be detected as Forkhead box P3+ (FOXP3+) and cluster of differentiation (–CD) 4+ T cells by classifying FOXP3+CD4+ T cells into different subpopulations of CD4 cells.

Objectives. To classify clusters of tumor-infiltrating Tregs in lung adenocarcinoma based on the mRNA expression levels of interleukin-12 subunit alpha (*IL12A*) and transforming growth factor beta 1 (*TGFB1*) in tumor specimens.

Material and methods. Seventy-nine patients with lung adenocarcinoma were evaluated in this study. Clinical data were obtained from the patients' medical records, while tumor tissue samples were preserved as formalin-fixed paraffin-embedded (FFPE) tissue specimens. Immunohistochemical staining for CD4, CD8 and FOXP3 was performed and stained cell counts were obtained under 5 high-power fields. cDNA was synthesized from total RNA extracted from FFPE tissue specimens and amplified with Taqman probes for *FOXP3*, *IL12A*, *TGFB1*, and the glyceraldehyde-3-phosphate dehydrogenase gene.

Results. Two clusters were identified: *IL12A*low*TGFB1*low (Cluster 1: n = 44) and *IL12A*high*TGFB1*high (Cluster 2: n = 39). Although no significant difference in the FOXP3+ cell/CD4+ cell ratio was observed between the 2 clusters (p = 0.921), the high FOXP3+/CD4+ cell ratio group showed a significantly poorer relapse-free survival rate than the low FOXP3+/CD4+ cell ratio group in Cluster 1 (p = 0.031).

Conclusions. Although the results revealed no direct association between Tregs and prognosis according to each subtype, these results suggest that if a lung cancer specimen contains low levels of *IL12A* and *TGFB1*, the FOXP3+/CD4+ cell ratio is useful for predicting the prognosis of lung cancer.

Key words: adenocarcinoma, prognosis, T-lymphocytes, regulatory

Introduction

Tumor-infiltrating Forkhead box P3+ (FOXP3+) and regulatory T cells (Tregs) are poor prognostic factors for many types of malignant tumors,¹ including lung cancer.^{2–8} Recently, FOXP3+ and cluster of differentiation (CD) 4+ T cells in peripheral blood lymphocytes were classified into 3 subpopulations based on the expression levels of *FOXP3* and *CD45RA*: subpopulation I, *FOXP3*^{low}*CD45RA*+; subpopulation II, *FOXP3*^{high}*CD45RA*–; and subpopulation III, *FOXP3*^{low}*CD45RA*–. Subpopulation II, known as effector Tregs, exhibits suppressive activity and increased numbers of these cells are found among tumor-infiltrating lymphocytes compared to peripheral blood lymphocytes.^{9,10} In colorectal cancer (CRC), tumor infiltration, predominantly by *FOXP3*^{high}/CD4+ Tregs, is strongly associated with the prognosis of malignant tumors.¹¹ In biomedical laboratories, flow cytometry is used to identify Tregs due to the difficulty in assessing effector Tregs using only formalin-fixed, paraffin-embedded (FFPE) tissues.

Saito et al. described a classification system based on mRNA expression of interleukin-12 subunit alpha (*IL12A*) and transforming growth factor beta 1 (*TGFBI*) in CRC specimens to distinguish subpopulations of (non-Treg) poor cases from Fr-III rich cases.¹¹ They classified CRC into 2 groups using this method and performed a survival analysis based on the *FOXP3* mRNA expression level in the same specimens. The current study used the same approach to evaluate lung adenocarcinoma (LAC) using only FFPE tissue. As tumor cells frequently express *FOXP3* in lung cancer, this method revealed *FOXP3* mRNA expression in lung cancer specimens, as well as the number of tumor-infiltrating Tregs.^{3,7,12–14} In flow cytometry, Tregs are assessed as a fraction using the ratio of each Fr Treg/CD4+ mononuclear cell count.¹¹ Thus, the FOXP3+/CD4+ ratio in FFPE tissue specimens was also assessed using immunohistochemical analysis.

Lung adenocarcinoma contains several histological subpopulations that include lepidic, acinar, papillary, solid, mucinous, and micropapillary subpopulations.¹⁵ Cell density of lepidic LAC is lower than that of other subpopulations. Consequently, the stained cell counts of lepidic subpopulations tend to be lower than that of other subpopulations. Adenocarcinomas in situ and minimally invasive adenocarcinomas have been associated with positive prognoses,¹⁶ which may be partly due to the generally lower numbers of FOXP3+ cells and tumor cells in these subpopulations, compared with others. However, this feature alone does not enable adequate assessment of the relationship between cell counts and prognosis. Furthermore, *FOXP3* may appear in lung tumor cells, suggesting that the number of tumor-infiltrating Tregs is not adequately reflected by *FOXP3* levels in tumor tissue.

Against such a background, the objective of this study was to classify clusters of tumor-infiltrating Tregs

in LAC based on the mRNA expression levels of *IL12A* and *TGFBI*. This study was conducted to determine whether the FOXP3+/CD4+ cell ratio is a better estimator of the number of tumor-infiltrating Tregs in a tissue sample, and whether FOXP3+-rich regions are better for evaluating stained cell counts without being affected by the biased association with histological subpopulations.

Material and methods

Patients and samples

Seventy-nine patients with LAC who underwent pulmonary resection and regional lymph node dissection at University of Yamanashi hospital. between 2004 and 2011 were included in this study. Patients were excluded if they had other malignancies 5 years before or 2 years after LAC, complications related to autoimmune disease, had undergone treatment with non-radical excision, or had been treated using adjuvant chemotherapy. Clinical and pathological data was obtained from the patients' medical records at the hospital. Pathological data were re-evaluated according to the 8th edition of the Tumor-Node-Metastasis Classification for lung cancer.¹⁷

Ethical considerations

Tumor tissue samples were obtained during pulmonary resection and preserved as FFPE tissue specimens. All participants provided informed written consent prior to pulmonary resection. The study was approved by the Ethics Committee of University of Yamanashi. The research was conducted in accordance with the 2013 Declaration of Helsinki.

Immunohistochemical staining

The FFPE tissue blocks were sliced into 3 µm-thick tissue sections using a sliding microtome (Yamato Kohki Industrial Co., Ltd., Asaka, Japan). Following deparaffinization in xylene and dehydration using increasing concentrations of alcohol, sections were pretreated by heat-mediated antigen retrieval in a 95°C water bath with Target Retrieval Solution (DAKO, Glostrup, Denmark) at pH 6.0 for 30 min (CD4), pH 9.0 for 30 min (CD8) and pH 9.0 for 40 min (FOXP3). Prior to the application of CD8 and FOXP3 antibodies, endogenous peroxidase activity was inhibited by incubating the samples in 3.0% hydrogen peroxidase for 15 min at 37°C. For CD4, endogenous peroxidase activity was inhibited following primary antibody incubation using the same method. After protein blocking with Blocking I (Nacalai Tesque, Kyoto, Japan) for 15 min at 37°C, sections were incubated for an additional 2 h at 37°C with anti-CD4 (rabbit monoclonal, dilution 1:100; ab133616), anti-CD8 (mouse monoclonal, dilution 1:50; ab17147) and

anti-FOXP3 (mouse monoclonal, dilution 1:100; ab20034) antibodies (Abcam, Cambridge, UK). After being washed 3 times with phosphate-buffered saline (PBS), sections were incubated for 30 min at 37°C with secondary antibodies: Histofine Max-PO(M) (horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin polyclonal) and Histofine Max-PO(R) (HRP-conjugated anti-rabbit immunoglobulin polyclonal; Nichirei Biosciences, Inc., Tokyo, Japan). A 3,3'-diaminobenzidine peroxidase stain kit (Nacalai Tesque) was used as the chromogen. Sections were counterstained using hematoxylin. Positive and negative staining controls for CD4/8 and FOXP3 were prepared using lymph node and tonsil sections, respectively. Staining of the positive control was performed as described above. The negative control was stained using PBS rather than with primary antibodies.

Evaluation of immunohistochemical staining

For CD4 and FOXP3, continuous sections were stained and counted. FOXP3+-rich regions in the tumor stroma were selected for counting CD4+ and FOXP3+ cell numbers. CD8+-rich regions in the tumor stroma were selected for counting CD8+ cell numbers. Each count was based on 5 fields from high-powered digital images (×400 magnification). The average cell count was used for statistical analysis. FOXP3+ tumors were defined as >20% staining of tumor cells in a section as previously described.³

Quantitative real-time polymerase chain reaction

Total RNA was extracted from FFPE tumor specimens using an Ambion™ MagMAX™ FFPE Total Nucleic Acid Isolation Kit (Invitrogen, Carlsbad, USA). Next, cDNA was synthesized from 0.5 µg of total RNA using a SuperScript III Reverse Transcriptase Kit with random primers (Invitrogen) and amplified via 10.0 µM each of Taqman probe (*FOXP3*, *IL12A*, *TGFB1*, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*); Applied Biosystems, Foster City, USA) and 10.0 µL of Taqman Gene Expression Master Mix (Life Technologies, Carlsbad, USA) according to the manufacturer's instructions. Relative mRNA expression was evaluated after normalization to *GAPDH* expression.

Tumor cell content analysis

Tissue sections continuous to FFPE tumor specimens used to extract RNA were stained with hematoxylin and eosin (H&E) and analyzed using ImageJ software (National Institutes of Health, Bethesda, USA; <http://rsb.info.nih.gov/ij/>). Analysis was based on 5 fields of view randomly chosen within the limits of RNA extraction in high-powered digital images (×400 magnification). The margins

of the tumor cells were marked and the enclosed area was calculated as the area of the tumor cells. Total cell area was calculated by excluding any cell-free areas from the overall area of each image. The tumor content ratio was determined by the tumor cell area divided by the total cell area.

Statistical analyses

Categorical variables were compared using Fisher's exact tests and continuous variables were compared using non-parametric Mann–Whitney U tests or Kruskal–Wallis one-way analysis of variance (ANOVA) and post hoc Tukey's tests. All statistical analyses were conducted using SPSS v. 24.0 software (IBM Corp., Armonk, USA). Statistical significance was set at $p < 0.05$. The cut-off value for experimental data was defined using Youden's index (J) as follows; $J = \text{sensitivity} + \text{specificity} - 1$, for variables with $p < 0.05$ (recurrence: "yes" vs "no"). Remaining continuous variables were divided by the median value. Survival curves were estimated using the Kaplan–Meier method and compared using the log-rank test. Cox proportional hazards models were used for univariate and multivariate analyses. Relapse-free survival was used as a dependent variable. Independent variables were selected from categorical variables that were significant according to univariate analysis ($p < 0.05$).

Results

Patient characteristics

A total of 79 LAC patients were included in this study. The mean age was 67.2 years (range: 32–88 years) and 38.0% (30/79) were males. Nineteen percent (15/79) of these patients were relapsed LAC cases. The mean total tumor size was 2.48 cm (range: 0.6–8.0 cm), and 81% (64/79) of patients were at the pathological stage of 0–1. Histological subtypes of the tumors were lepidic in 30/79 cases, acinar in 17/79, papillary in 16/79, solid in 9/79, and mucinous in 7/79. Fifty-four percent (43/79) of tumor samples were positive for FOXP3 staining.

Immunohistochemical staining analysis

In the positive controls, the cytoplasm of lymphocytes in lymph node tissue specimens was stained with CD4 and CD8 antibodies, whereas nuclei were stained with FOXP3 antibodies. For positive controls, cells displaying nuclear FOXP3 staining were confirmed as lymphocytes using CD4/CD8 antibodies, while cells displaying cytoplasmic FOXP3 staining were confirmed as non-lymphocytes. In negative controls, it was confirmed that tonsil tissue specimens were not stained by secondary antibodies in the absence of primary antibodies.

Fig. 1. Forkhead box P3 (FOXP3) antibody staining of lung cancer tissues. A. Staining of lymphocyte cytoplasm for CD4. B. Staining of nuclei for FOXP3. C. Staining of cytoplasm for CD8. D. FOXP3 staining was observed in nuclei of tumor cells (red arrow) and nuclei of lymphocytes (black arrow). E. FOXP3 staining was observed in the cytoplasm of tumor cells (red arrow) and nuclei of lymphocytes (black arrow). The red arrows indicate stained cancer nuclei (D) and cytoplasm (E). The black arrows indicate stained lymphocytes. All images were acquired using tumor tissues

Forty-three LAC samples (54.4%) were positively stained with FOXP3 antibodies (Fig. 1A–E). In these samples, FOXP3 was mainly concentrated in areas rich in CD4+ and/or CD8+ cells and was localized to the nuclei of lymphocytes, as well as to either the nuclei or cytoplasm of non-lymphocytic tumor cells (Fig. 1D,E). The mean FOXP3+ cell count in tumor samples from relapsed patients ($n = 15$) was significantly higher than that in samples from non-relapsed patients ($n = 64$) at 63 cells compared to 37 cells, respectively ($p = 0.018$). There were more CD4+ cells than FOXP3+ cells in all LAC samples, and therefore FOXP3+/CD4+ cell count ratios ranged between 0.05 and 0.48. The mean FOXP3+/CD4+ cell count ratio was significantly higher in relapsed than in non-relapsed patients (0.23 vs 0.16, respectively; $p = 0.012$). FOXP3+/CD8+ cell count ratios varied between samples, within a range between 0.04 and 1.94.

mRNA expression and clustering analysis

The median relative mRNA expression levels of *FOXP3*, *IL12A* and *TGFBI* in lung tumor specimens were set to 1.0. The mRNA expression level ranges were 0.10–5.12, 0.07–5.58 and 0.14–3.36, respectively. Two clusters were identified by unsupervised hierarchical clustering analysis based on *IL12A* and *TGFBI* expression levels. A heatmap was generated, where red indicated high levels of mRNA expression and blue indicated low levels of mRNA expression for each gene of interest. Most Cluster 1 analyses exhibited low levels of *IL12A* and *TGFBI* expression, while Cluster 2 analyses exhibited high mRNA expression levels of *IL12A* and *TGFBI* (Fig. 2).

Although lung tumor *FOXP3* expression was significantly higher in Cluster 2 than in Cluster 1 ($p < 0.001$), for *FOXP3*+ cases compared with *FOXP3*– cases ($p < 0.05$)

Fig. 2. Expression levels of interleukin-12 subunit alpha (*IL12A*) and transforming growth factor beta 1 (*TGFB1*) in Cluster 2. Heatmap images were created using RNA expression to *GAPDH* expression ratios (mean value of 3 samples was used in the analysis). Logarithmic transformation was used to determine the normal distributions of RR values, which were standardized using Z-scores. "0" is shown in white and $\pm 2SD$ is shown in red and blue, respectively

Fig. 3. *FOXP3* expression levels in clusters, *FOXP3*+, and *FOXP3*– cases. *IL12A*^{low}*TGFB1*^{low} (Cluster 1: n = 44); *IL12A*^{high}*TGFB1*^{high} (Cluster 2: n = 39)

FOXP3 – Forkhead box P3.

Fig. 4. Correlation analysis for *FOXP3* mRNA expression. Correlation between *FOXP3* mRNA expression and *FOXP3*+ cell counts (A) or *FOXP3*+/CD4+ cell ratio (B) in lung tumor tissues was not significant

(Fig. 3), Spearman's rank-order correlation indicated that *FOXP3* mRNA expression was unrelated to *FOXP3*+ cell counts (correlation coefficients: 0.171, $p = 0.131$; Fig. 4A) or the *FOXP3*+/CD4+ cell ratio (correlation coefficients: 0.0819, $p = 0.472$; Fig. 4B). Survival curves for all groups are shown (Fig. 5). Cluster 1 showed a higher survival rate compared to Cluster 2, when clusters of all cases were compared.

Association between clinicopathological characteristics and experimental data

The *FOXP3*+ cell counts and *FOXP3*+/CD4+ cell ratios were significantly higher in lung tumor samples from relapsed cases than from non-relapsed cases ($p = 0.018$ and $p = 0.012$, respectively), and expression level of each mRNA was significantly lower in the relapsed groups than in the non-relapsed group ($p < 0.01$) (Table 1).

Clusters 1 and 2 comprised 44 and 35 patients, respectively. Cluster 1 patients showed unfavorable pathological characteristics, such as large tumor diameter, lymph node metastasis and vascular invasion, compared with

Cluster 2 patients. Although Cluster 1 patients showed a significantly higher risk of recurrence than Cluster 2 patients ($p = 0.039$), no significant differences in *FOXP3*+ cell counts or *FOXP3*+/CD4+ cell ratios were observed between the 2 clusters. Cluster 1 comprised 14 relapsed patients and 30 non-relapsed patients. *FOXP3*+ cell counts and *FOXP3*+/CD4+ and *FOXP3*+/CD8+ cell ratios were significantly higher in lung tumor samples from relapsed cases than in those from non-relapsed cases of Cluster 1. *FOXP3* mRNA expression was significantly lower in samples from relapsed cases than in non-relapsed cases (Table 2).

Association between the tumor cell content and other experimental data

The mean tumor content ratio was 0.373 (range: 0.088–79.4). When samples were divided into 3 evenly sized groups according to tumor cell content ratio, no significant differences in cell counts or mRNA expression levels were observed. However, the amount of mRNA extracted from the low tumor content ratio group was significantly less than in the high tumor content ratio group (Table 3).

Fig. 5. Survival curves for all groups. The upper left panel shows the FOXP3+/CD4+ ratio of lung tumor tissue samples for all cases. The upper right panel shows the comparison between clusters of all cases. The bottom left panel shows the FOXP3+/CD4+ ratio of Cluster 1. The bottom right panel shows the comparison of FOXP3+/CD4+ ratio of Cluster 2

FOXP3 – Forkhead box P3; CD – cluster of differentiation. Survival curves were estimated using the Kaplan–Meier method and compared using the log-rank test.

Univariate and multivariate analysis of risk factors affecting relapse-free survival rate

Univariate analysis of all cases revealed older age (>73 years), larger total tumor size (>2.9 cm), larger invasion size (>2.4 cm), advanced T/N factor, advanced pathological stage, pleural invasion, advanced tumor grade, lymphatic invasion, vessel invasion, solid adenocarcinoma, higher tumor FOXP3+/CD4+ cell ratio, FOXP3– tumor, lower tumor *FOXP3*, as well as *IL12A*, and *TGFBI* mRNA expression levels and larger tumor cell content ratio (>0.55) as poor prognostic factors for relapse-free survival. In Cluster 1 patients, univariate analysis revealed that larger total tumor size (>3.5 cm), larger invasion size (>2.4 cm), advanced N factor, advanced pathological stage, lymphatic invasion, vessel invasion, higher tumor FOXP3+/CD4 cell ratio (>0.192), higher tumor FOXP3+/CD8+ cell ratio (>0.400), as well as lower tumor *FOXP3* and *TGFBI* mRNA expression levels were poor prognostic factors affecting relapse-free survival (Table 4).

In multivariate analysis of all cases, advanced N factor, advanced pathological stage, and vessel invasion, were confirmed as independent prognostic factors for relapse-free survival. In Cluster 1 patients, multivariate analysis confirmed larger total tumor size (>3.5 cm), advanced N factor, vessel invasion, and higher tumor *FOXP3*+/CD4+ cell ratio (>0.192) as independent prognostic factors for relapse-free survival (Table 5).

Discussion

Tregs play a key immunosuppressive role in malignant tumors.^{18–20} High tumor-infiltrating Tregs and peripheral blood Treg counts are poor prognostic factors for lung cancer.^{2–8} Treg fractions have been described previously. Flow cytometry is required to distinguish between fractions. In CRC, Saito et al. reported that FOXP3+ non-Tregs in tumor samples were associated with inflammation and that their development depended on the secretion of *IL12A* and *TGFBI* from tumor cells or tumor-associated cells.¹¹

Table 1. Comparison of *FOXP3*, *IL12A* and *TGFB1* mRNA expression levels and patient characteristics

Characteristics	Mean relative mRNA ^a expression level (range)					
	<i>FOXP3</i>	p-value ^b	<i>IL12A</i>	p-value ^b	<i>TGFB1</i>	p-value ^b
Age [years] <73 (n = 29) ≥73 (n = 50)	6.76 (0.68, 29.14) 4.88 (0.70, 33.18)	0.32	0.25 (0.05, 1.15) 0.15 (0.01, 0.88)	<0.001	0.89 (0.12, 2.57) 0.53 (0.11, 2.53)	0.04
Sex male (n = 30) female (n = 49)	4.91 (0.68, 29.01) 7.45 (1.06, 33.18)	0.03	0.19 (0.02, 1.15) 0.23 (0.01, 1.01)	0.45	0.72 (0.11, 2.57) 0.88 (0.11, 2.46)	0.29
Emphysema yes (n = 13) no (n = 66)	4.59 (2.43, 18.90) 6.76 (0.68, 33.18)	0.47	0.14 (0.04, 0.42) 0.23 (0.01, 1.15)	0.03	0.53 (0.15, 1.16) 0.88 (0.11, 2.57)	0.05
Tumor factor Tis,1 (n = 57) T2–4 (n = 32)	7.12 (0.68, 33.18) 4.16 (0.70, 18.90)	0.03	0.24 (0.01, 1.15) 0.13 (0.02, 0.35)	0	0.94 (0.11, 2.57) 0.47 (0.11, 1.41)	<0.001
Nodal factor N0 (n = 70) N1,2 (n = 9)	6.97 (0.68, 33.18) 2.95 (1.82, 9.82)	0.01	0.23 (0.02, 1.15) 0.09 (0.01, 0.22)	0	0.85 (0.11, 2.57) 0.45 (0.11, 1.01)	0.01
Pathological stage stage 0, I (n = 64) stage II, III (n = 15)	6.97 (0.68, 33.18) 3.52 (0.70, 18.90)	0.03	0.24 (0.01, 1.15) 0.12 (0.02, 0.31)	0	0.92 (0.11, 2.57) 0.45 (0.11, 1.01)	0
pl factor pl0 (n = 69) pl1–3 (n = 10)	6.83 (0.68, 33.18) 3.12 (1.06, 14.52)	0.01	0.23 (0.01, 1.15) 0.15 (0.05, 0.35)	0.13	0.87 (0.11, 2.57) 0.45 (0.22, 1.05)	0.02
Lymphatic invasion negative (n = 61) positive (n = 18)	6.83 (0.68, 33.18) 5.32 (0.70, 14.52)	0.14	0.24 (0.05, 1.15) 0.13 (0.01, 0.31)	0	0.94 (0.12, 2.57) 0.37 (0.11, 1.01)	<0.001
Vessel invasion negative (n = 62) positive (n = 17)	6.76 (0.68, 33.18) 4.46 (0.70, 15.57)	0.1	0.24 (0.04, 1.15) 0.13 (0.01, 0.35)	0	0.93 (0.12, 2.57) 0.45 (0.11, 1.21)	<0.001
Grade G1 (n = 37) G2,3 (n = 42)	7.47 (0.68, 33.18) 5.81 (0.70, 19.29)	0.04	0.27 (0.07, 1.15) 0.17 (0.01, 0.88)	0	1.05 (0.29, 2.57) 0.50 (0.11, 2.53)	<0.001
Histological subtype lepidic (n = 30) mucinous (n = 7) acinar (n = 17) papillary (n = 16) solid (n = 9)	8.17 (0.68, 33.18) 5.28 (1.28, 10.55) 5.61 (1.06, 19.29) 6.54 (2.43, 15.57) 2.95 (0.70, 9.82)	0.01	0.28 (0.07, 1.15) 0.18 (0.09, 1.01) 0.20 (0.05, 0.88) 0.18 (0.05, 0.43) 0.08 (0.01, 0.49)	0.01	1.11 (0.29, 2.57) 0.76 (0.45, 1.50) 0.69 (0.22, 2.53) 0.52 (0.12, 1.21) 0.33 (0.11, 1.55)	<0.001
Tumor FOXP3 staining negative (n = 36) positive (n = 43)	4.38 (0.68, 29.01) 7.67 (1.06, 33.18)	0	0.20 (0.02, 1.15) 0.23 (0.01, 1.01)	0.57	0.73 (0.11, 1.96) 0.90 (0.11, 2.57)	0.22

FOXP3 – Forkhead box P3; *IL12A* – interleukin-12 subunit alpha; *TGFB1* – transforming growth factor beta 1; G – grade. ^amRNA level relative to *GAPDH*; ^bmean expression levels for each mRNA type was compared between patient characteristic subgroups. Categorical variables were compared using Fisher's exact tests and continuous variables were compared using nonparametric Mann–Whitney U tests or Kruskal–Wallis one-way ANOVA and post hoc Tukey tests.

Particularly, *TGFB1* influences metastasis through epithelial-to-mesenchymal transition by transactivating epidermal growth factor signaling.²¹

Cluster analysis performed as previously described¹¹ revealed 2 clusters of tumor samples. Cluster 1 was characterized by *IL12A*^{low}*TGFB1*^{low} and Cluster 2 by *IL12A*^{high}*TGFB1*^{high}.¹¹ Although no significant differences in FOXP3+ cell counts or FOXP3+/CD4+ cell ratios were observed between these 2 clusters, Cluster 1 patients showed a significantly higher risk for recurrence than Cluster 2 patients. Moreover, among Cluster 1 patients, the high FOXP3+/CD4+ cell ratio group was associated with a significantly poorer relapse-free survival rate than that of the low

FOXP3+/CD4+ cell ratio group. Although there was no direct association between Tregs and prognosis according to each subtype, clustering based on tumor *IL12A* and *TGFB1* mRNA expression may be useful to exclude cases in which Tregs are not associated with disease prognosis.

The findings indicate that this method may be useful for excluding groups without immune escape by Tregs, thereby increasing the accuracy of analysis. Secondly, using Treg evaluation as a marker for treatment adaptation and effective prediction will likely enhance case exclusion accuracy and enable administration of treatments targeting Tregs (such as CTLA-4 inhibitors) in clinical practice.

FOXP3 staining of tumor cells has been reported

Table 2. Comparison of clinical and experimental characteristics among relapsed and non-relapsed and Cluster 1 and 2 patients (n = 79) with lung adenocarcinoma

Characteristics	All patients					
	non-relapsed	relapsed	p-value	Cluster 1	Cluster 2	p-value
Number of patients	64	15		44	35	
Age [years] (range)	67 (32–78)	74 (36–88)	0.024	73 (32–88)	65 (48–83)	0.039
Gender (%)						
male	21 (32.8)	9 (60.0)	0.075	20 (45.5)	10 (28.6)	0.163
female	43 (67.2)	6 (40.0)		24 (54.5)	25 (71.4)	
Emphysema (%)						
yes	8 (12.5)	5 (33.3)	0.114	12 (27.3)	1 (2.9)	0.005
no	56 (87.5)	10 (66.7)		32 (72.7)	34 (97.1)	
total size [cm] (range)	2.0 (0.6–5.5)	3.8 (1.2–8.0)	<0.001	2.5 (1.2–8.0)	1.8 (0.6–3.8)	<0.001
invasion size [cm] (range)	1.2 (0–5.4)	3.0 (0.8–8.0)	<0.001	2.4 (0.1–8.0)	0.6 (0.0–3.8)	<0.001
Tumor factor (%)						
Tis,1	52 (81.2)	5 (33.3)	0.001	25 (56.8)	32 (91.4)	0.001
T2–4	12 (18.8)	10 (66.7)		19 (43.2)	3 (8.6)	
Nodal factor (%)						
N0	63 (98.4)	7 (46.7)	<0.001	36 (81.8)	34 (97.1)	0.039
N1,2	1 (1.6)	8 (53.3)		8 (18.2)	1 (2.9)	
Pathological stage (%)						
stage 0, I	60 (93.8)	4 (26.7)	<0.001	30 (68.2)	34 (97.1)	0.001
stage II, III	4 (6.2)	11 (73.3)		14 (31.8)	1 (2.9)	
pl factor (%)						
pl0	60 (93.8)	9 (60.0)	0.002	35 (79.5)	34 (97.1)	0.037
pl1–3	4 (6.2)	6 (40.0)		9 (20.5)	1 (2.9)	
Lymphatic invasion (%)						
negative	56 (87.5)	5 (33.3)	<0.001	27 (61.4)	34 (97.1)	<0.001
positive	8 (12.5)	10 (66.7)		17 (38.6)	1 (2.9)	
Vessel invasion (%)						
negative	56 (87.5)	6 (40.0)	<0.001	30 (68.2)	32 (91.4)	0.014
positive	8 (12.5)	9 (60.0)		14 (31.8)	3 (8.6)	
Grade (%)						
G1	36 (56.2)	1 (6.7)	<0.001	11 (25.0)	26 (74.3)	<0.001
G2,3	28 (43.8)	14 (93.3)		33 (75.0)	9 (25.7)	
Histological subtype (%)						
lepidic	29 (45.3)	1 (6.7)	0.001	8 (18.2)	22 (62.9)	<0.001
mucinous	6 (9.4)	1 (6.7)		3 (6.8)	4 (11.4)	
acinar	15 (23.4)	2 (13.3)		12 (27.3)	5 (14.3)	
papillary	10 (15.6)	6 (40.0)		14 (31.8)	2 (5.7)	
solid	4 (6.2)	5 (33.3)		7 (15.9)	2 (5.7)	
Tumor FOXP3 staining (%)						
negative	25 (39.1)	11 (73.3)	0.022	22 (50.0)	14 (40.0)	0.496
positive	39 (60.9)	4 (26.7)		22 (50.0)	21 (60.0)	
Cell count						
CD4+ (range)	236 (64–636)	256 (127–556)	0.626	246 (106–636)	236 (64–556)	0.782
CD8+ (range)	142 (66–418)	194 (67–284)	0.127	169 (67–418)	129 (66–276)	0.009
FOXP3+ (range)	37 (8–193)	63 (9–111)	0.018	44 (9–163)	40 (8–193)	0.594
Cell count ratio						
CD4+/CD8+ (range)	1.57 (0.35–7.60)	1.50 (0.57–5.35)	0.565	1.49 (0.47–7.60)	1.92 (0.35–4.18)	0.129
FOXP3+/CD4+ (range)	0.16 (0.05–0.48)	0.23 (0.07–0.44)	0.012	0.17 (0.05–0.44)	0.19 (0.05–0.48)	0.921
FOXP3+/CD8+ (range)	0.23 (0.07–1.94)	0.40 (0.04–1.57)	0.127	0.23 (0.04–1.94)	0.3 (0.08–1.20)	0.319
mRNA expression levels						
<i>IL12A</i> (range)	1.18 (0.07–5.58)	0.62 (0.09–1.39)	0.006	0.64 (0.07–1.67)	1.57 (0.86–5.58)	<0.001
<i>TGFBI</i> (range)	1.16 (0.14–3.36)	0.58 (0.14–1.37)	0.001	0.65 (0.14–1.96)	1.52 (0.92–3.36)	<0.001
<i>FOXP3</i> (range)	1.12 (0.10–5.12)	0.54 (0.11–2.24)	<0.001	0.74 (0.11–2.98)	1.29 (0.10–5.12)	<0.001
Cluster (%)						
Cluster 1	30 (46.9)	14 (93.3)	0.001			
Cluster 2	34 (53.1)	1 (6.7)				

(+) – positive; CD – cluster of differentiation; *FOXP3* – Forkhead box P3; *IL12A* – interleukin-12 subunit alpha; mRNA – messenger RNA; pl – pleural invasion; *TGFBI* – transforming growth factor beta 1; G – grade.

Table 3. Characteristics of tumor samples by tumor cell content (high >46%, intermediate 27–46%, low <27%)

Parameter	All patients			p-value
Tumor cell content	high (n = 27)	intermediate (n = 26)	low (n = 26)	
Patients per cluster (%)				
Cluster 1	16 (59.3)	16 (16.5)	12 (46.2)	0.482
Cluster 2	11 (40.7)	10 (38.5)	14 (53.8)	
Cell count				
CD4+ (range)	237 (91–481)	247 (64–597)	242 (106–636)	0.942
CD8+ (range)	156 (67–418)	152 (66–331)	136 (83–336)	0.566
FOXP3+ (range)	40 (8–193)	43 (9–104)	42 (14–163)	0.79
mRNA expression levels				
FOXP3 (range)	1.00 (0.11–5.12)	0.88 (0.10–4.48)	1.22 (0.45–4.50)	0.183
IL12A (range)	0.92 (0.07–4.91)	0.97 (0.18–5.58)	1.19 (0.25–4.74)	0.272
TGFB1 (range)	0.96 (0.14–2.36)	1.00 (0.16–2.32)	1.25 (0.31–3.36)	0.438
Extracted mRNA [μl/mL]	66.9 (13.5–284)	43.4 (8.7–174.0)	36.8 (11.6–94.7)	0.006

(+) – positive; CD – cluster of differentiation; FOXP3 – Forkhead box P3; IL12A – interleukin-12 subunit alpha; TGFB1 – transforming growth factor beta 1.

Table 4. Univariate analysis of clinical and experimental characteristics influencing the relapse-free survival of all patients (n = 79) and Cluster 1 patients (n = 44) with lung adenocarcinoma

Characteristics	All patients			Cluster 1 patients		
	hazard ratio	95% CI	p-value	hazard ratio	95% CI	p-value
Age; elder	5.677	1.805–17.86	0.003	1.913	0.600–6.102	0.273
Gender; male	2.578	0.917–7.248	0.073	2.347	0.785–7.018	0.127
Emphysema; Yes	2.707	0.924–7.931	0.07	1.49	0.499–4.454	0.475
Total tumor size; large	13.49	4.559–39.95	<0.001	3.861	1.133–9.466	0.012
Invasion size; large	8.532	2.711–26.85	<0.001	5.418	1.867–15.72	0.002
Tumor factor; 2–4	6.665	2.272–19.55	<0.001	2.899	0.970–8.662	0.057
Nodal factor; 1–2	21.97	7.599–63.51	<0.001	17.89	3.447–30.26	<0.001
p-stage; II–III	20.29	6.363–64.67	<0.001	8.728	2.309–23.90	<0.001
Pleural invasion (+)	6.542	2.316–18.48	<0.001	2.716	0.908–8.123	0.074
Lymphatic invasion (+)	8.932	3.037–26.27	<0.001	4.608	1.438–14.76	0.01
Vessel invasion (+)	7.182	2.546–20.26	<0.001	3.591	1.242–10.38	0.018
G; 2–3	14.9	1.959–113.4	0.009	5.373	0.702–41.12	0.105
Subtype; solid	5.279	1.796–15.52	0.002	2.684	0.839–8.583	0.096
CD4+ cell; high	1.173	0.425–3.234	0.758	1.054	0.369–3.006	0.922
CD8+ cell; high	2.009	0.687–5.878	0.203	1.322	0.458–3.811	0.606
FOXP3+ cell; high	3.11	0.989–9.775	0.052	2.947	0.922–9.421	0.068
CD4+/CD8+; low	1.173	0.417–3.175	0.758	1.039	0.364–2.963	0.943
FOXP3+/CD4+; high	4.188	1.332–13.17	0.014	7.691	2.129–27.78	0.002
FOXP3+/CD8+; high	3.099	0.986–9.742	0.053	4.745	1.625–13.86	0.004
Tumor FOXP3; (+)	0.2593	0.083–0.815	0.021	0.315	0.099–1.007	0.051
FOXP3 expression; low	4.329	1.567–11.96	0.005	3.213	1.072–9.626	0.037
IL12A expression; low	10.81	2.438–47.96	0.002	1.411	0.489–4.067	0.524
TGFB1 expression; low	9.257	2.607–32.87	<0.001	3.276	1.025–10.47	0.045
Tumor cell content ratio; low	0.2037	0.072–0.575	0.003	0.280	0.067–1.175	0.08

(+) – positive; CD – cluster of differentiation; FOXP3 – Forkhead box P3; G – grade; IL12A – interleukin-12 subunit alpha; p – pathological; TGFB1 – transforming growth factor beta 1; 95% CI – 95% confidence interval.

Table 5. Multivariate analysis of risk factors for relapse-free survival rate among all patients (n = 79) and among Cluster 1 patients (n = 44) with lung adenocarcinoma

Characteristics	Hazard ratio	95% CI	p-value
All patients			
nodal factor; 1–2	5.219	1.576–17.29	0.007
p-stage; II–III	11.37	3.101–41.71	<0.001
vessel invasion (+)	4.817	1.603–14.48	0.005
Cluster 1 patients			
total tumor size; large	3.88	1.223–12.31	0.021
nodal factor; 1–2	7.683	2.317–22.17	<0.001
vessel invasion (+)	3.945	1.005–8.889	0.022
FOXP3+/CD4+; high	4.461	1.530–21.80	0.031

(+) – positive; CD – cluster of differentiation; FOXP3 – Forkhead box P3; p – pathological; 95% CI – 95% confidence interval.

in various types of malignancies, including breast, gastric and lung cancer.^{3,7,12–14,22,23} In gastric cancer, tumor FOXP3+ staining has been reported as a favorable prognostic factor.²⁴ However, tumor FOXP3+ expression was reported as a poor prognostic factor for oral and oropharyngeal squamous cell carcinomas.²⁵ In breast cancer, the effect of tumor FOXP3+ staining reportedly differs according to location, with nuclear expression associated with a favorable prognosis and cytoplasmic expression associated with a poor prognosis.²³ One study suggested that, in lung cancer, tumor FOXP3+ staining was not a prognostic factor,³ while another study reported that high tumor FOXP3 expression was associated with a favorable prognosis.⁷ The current study did not observe a correlation between lung tumor FOXP3+ staining and FOXP3 expression. Additionally, both FOXP3+ staining as well as high FOXP3 expression in lung tumor samples were associated with a favorable prognosis.

Highly specific information can be elicited by examining the FOXP3+/CD4+ ratios and subpopulations from *IL12A* and *TGFBI*, as well as by considering non-Tregs and excluding groups that are unrelated to prognosis.

This study had some limitations. First, FOXP3 mRNA was derived from both tumor cells and other cells in the tumor microenvironment. Multivariate analysis did not reveal a significant difference in relapse-free survival. It cannot be conclusively stated that tumor FOXP3 expression is a poor prognostic factor. However, the effect of tumor FOXP3 expression on assessing tumor-infiltrating Tregs was not negligible, as indicated by mRNA expression of FOXP3 in tumor specimens. Thus, when assessing FOXP3+ T cells the use of tumor FOXP3 mRNA expression should be avoided in tumors that frequently express FOXP3. Second, although there were no significant differences in mRNA expression of FOXP3, *IL12A* and *TGFBI* between the 3 groups when divided according to the tumor cell content, higher tumor cell content ratio was related

to recurrence in univariate analysis, and the higher tumor cell content group tended to have lower mRNA expression of *IL12A* and *TGFBI*. Therefore, tumor cell content ratio can be a confounding factor of mRNA expression analysis when sampling tumor cells and interstitial tissue together.

This is the first study to apply cluster classification of *IL12A* and *TGFBI* to lung cancer. In addition, we demonstrated the possibility of selecting non-Tregs without the need for flow cytometry analysis.

Conclusions

This study suggests that assessing tumor-infiltrating Tregs in lung cancer from FFPE tissues may be useful in evaluating FOXP3+/CD4+ cell ratios and classifying tumors according to *IL12A* and *TGFBI* mRNA expression levels. However, care should be taken, as tumor cells frequently express FOXP3. Further studies are needed to clarify this diagnostic confounder.

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