ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

High Stromal TGFBI in Lung Cancer and Intratumoral CD8-Positive T Cells were Associated with Poor Prognosis and Therapeutic Resistance to Immune Checkpoint Inhibitors

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ABSTRACT

Background. We investigated whether the expression of transforming growth factor-beta-induced protein (TGFBI) and intratumoral immune cells including CD8- and Forkhead box protein P3 (Foxp3)-positive T cells in clinical lung cancer patients could predict the therapeutic response to nivolumab.

Methods. Thirty-three patients who were treated with nivolumab were enrolled in this study. Immunohistochemical analyses of TGFBI, PD-L1, CD8, Foxp3, and vimentin expression were conducted. Serum concentrations of TGFBI and transforming growth factor-beta1 (TGF-β1) were determined by enzyme-linked immunosorbent assay (ELISA).

Results. Cancer TGFBI was not associated with prognosis and therapeutic response to nivolumab, but cancer stromal TGFBI and intratumoral CD8-positive T cells were associated with them. Therefore, we evaluated cancer stromal TGFBI and intratumoral CD8-positive T cells. The high-TGFBI-expression group had poorer clinical responses than did the low-TGFBI-expression group ($p < 0.0001$). The number of times nivolumab was administered in the high-CD8-expression group was significantly higher than that in the low-CD8-expression group ($p = 0.0046$). The high-CD8-expression group had better clinical responses than did the low-CD8-expression group ($p = 0.0013$). Interestingly, all patients in the high-TGFBI/low-CD8-expression group had progressive disease (PD). In contrast, all patients in the low-TGFBI/high-CD8-expression group had PR + SD (partial response + stable disease) by the Response Evaluation Criteria in Solid Tumors (RECIST 1.1).

Conclusions. The dual evaluation of stromal TGFBI and intratumoral CD8-positive T cells could be a useful predictive marker for nivolumab.

Lung cancer is one of the leading causes of malignancy-related death globally. Non-small cell lung cancer (NSCLC) constitutes approximately 80% of lung cancer. Almost all patients with NSCLC are diagnosed with locally advanced or metastatic disease, and patients with advanced NSCLC have very low survival expectancy.[1,2] Recently, an immune checkpoint inhibitor is promising to improve the survival of NSCLC patients. Programmed cell death-1 (PD-1) and its ligand (PD-L1) play a major role in tumor immune escape, because PD-1 or its ligand PD-L1 interaction inhibits T-lymphocyte proliferation, survival, and effector functions (cytotoxicity, cytokine release), and induces apoptosis of tumor-specific cytotoxic T lymphocytes (CTL)[3–8] It was reported that NSCLC patients treated with nivolumab had good therapeutic responses and improved survival intervals via the activation of CTL.[9,10] In the clinic, the evaluation of tumoral PD-L1 expression has been focused on as one of markers of sensitivity to anti-PD-1 antibodies.[11] Clinical trials have however shown that a significant number of PD-L1-negative patients also do respond to anti-PD-1 treatment, potentially due to high limit of detection in immunohistochemistry.[12] Additionally, it is widely acknowledged that mere detection of PD-L1 expression as a biomarker may be insufficient, because the PD-L1 expression pattern is heterogeneous within the tumor and its microenvironment.[13,14] On the other hand, tumor mutation burden also is known as a candidate biomarker for immunotherapeutic approaches in NSCLC. In patients with high tumor mutation burden, progression-free survival was improved, and the objective response rate was higher in patients treated with nivolumab than in those treated with chemotherapy.[15] However, evaluation of mutational burden is difficult in the clinics, because it requires next-generation sequencing, which cannot be easily implemented on a large scale. Thus, identifying good responders in NSCLC patients treated with nivolumab more effectively requires the development of a novel sensitive and specific biomarkers that can be evaluated cost-efficiently by routine means.

Mariathasan et al. clarified that the activation of TGF-β signaling in cancer fibroblasts was associated with resistance to anti-PD-L1 antibodies and that co-administration of TGF-β-blocking and anti-PD-L1 antibodies in a mouse model facilitated tumor regression via CTL infiltration into target tumors.[16] From these observations, it was suggested that the evaluation of TGF-β signaling might be a good candidate for a nivolumab sensitivity marker. However, a suitable method for evaluating TGF-β signaling in cancer patients treated with immune checkpoint inhibitors is lacking. Genetic variations of the TGF-β pathway genes have been reported in several cancers[17–22] TGF-β signaling pathway is highly complex, time- and context-dependent,

TABLE 1 The correlation of the clinical response and clinicopathological features in 33 lung cancer patients treated with nivolumab

Factors	RECIST		P value
	PR + SD n = 19	PD n = 14	
Age			
Years	69	66	0.37
Gender			
Male	16	11	0.68
Female	3	3	
Histology			
ADC	14	10	0.89
SQC	5	4	
Nivolumab addministration times			
Times	19.58 ± 2.23	3.79 ± 2.60	< .0001*
PD-L1			
Negative	5	6	0.35
Positive	12	7	
Cancer TGFBI			
Negative	9	9	0.52
Positive	8	5	
Stromal TGFBI			
Negative	11	0	< .0001*
Positive	8	14	
CD8			
Negative	2	9	0.0013*
Positive	16	5	
Foxp3			
Negative	7	8	0.10
Positive	12	4	
VIM			
Negative	5	2	0.35
Positive	13	12	
TGFBI (serum)			
ng/ml	549.61 ± 48.28	495.22 ± 51.86	0.45
TGF-β1 (serum)			
ng/ml	17.13 ± 1.04	16.56 ± 1.12	0.71
irAE			
Negative	14	12	0.40
Positive	5	2	
Driver mutation			
EGFR	0	3	0.053
Wild	14	7	
Unknown	5	4	
Frequency of previous regimens			
Times	2.63 ± 0.55	2.91 ± 0.72	0.76

ADC adenocarcinoma, *SQC* squamous cell carcinoma, *irAE* immune-related adverse events, *EGFR* epidermal growth factor receptor, *AMI* acute myocardial infarction

*$P < 0.05$

with distinct signaling (canonical and noncanonical) in cancer and stroma cells.[23] In the current work, we focused on the transforming growth factor-beta-induced protein (TGFBI), which was first described as a strongly induced protein in TGF-β1-treated A549 NSCLC cells and one of the representative downstream genes of TGF-β signaling.[24] We and others have reported high TGFBI levels in several types of solid cancers using proteomic and genomic approaches.[25,26] TGFBI is a secreted extracellular matrix protein consisting of 683 amino acids. This protein contains four conserved fascilin-1 (FAS1) domains and a carboxyl-terminal Arg-Gly-Asp (RGD) integrin-binding sequence.[27,28] TGFBI is a transcript highly induced during epithelial-to-mesenchymal transition (EMT) in A549 NSCLC cells.[29] EMT is associated with drug resistance in NSCLC patients.[30] TGFBI is one of the representative downstream proteins triggered by TGF-β pathway. On the other hand, it is related to EMT and therapeutic resistance. However, few studies address the relationship of TGFBI, intratumoral immune cells, and the therapeutic effects of anti-PD-1 antibody nivolumab in lung cancer.

The purpose of this study was to clarify the significance of TGFBI and immune cells in clinical lung cancer patients treated with nivolumab. Therefore, we performed an immunohistochemical analysis to evaluate the expression of TGFBI, PD-L1, CD8, vimentin (VIM) as well as Forkhead box protein P3 (Foxp3) in lung cancer tissues. CD8, VIM, and Foxp3 were used as CTL, EMT, and regulatory T cell markers respectively. Additionally, we have assessed the concentration of TGFBI and TGF-β1 in corresponding serum samples before nivolumab treatment.

MATERIALS AND METHODS

Patients

We collected prospectively 33 patients with recurrent advanced NSCLC who were treated with nivolumab at Gunma University Hospital and Hidaka Hospital from February 2016 to February 2017. The study criteria were as follows: (1) recurrent NSCLC, (2) candidates for nivolumab treatment after initial chemotherapy, (3) performance status on the Eastern Cooperative Oncology Group scale corresponding to 0–2. There were 24 postoperative recurrence patients: 13 patients after the first-line chemotherapy, and 20 patients who received nivolumab as the third or later line of the chemotherapy. This study was approved by the institutional review board of Gunma University (approval no. 1404).

Immunohistochemistry

We investigated 33 serial sections, consisting of the resected specimens ($n = 24$) and needle biopsies ($n = 9$) in terms of TGFBI. We obtained 32 serial sections for CD8 and VIM, 31 serial sections for Foxp3, and 30 serial sections for PD-L1, because the cancerous part of each serial section was depleted in the process of cutting. Each specimen was cut into sections 4-μm thick and mounted on a glass slide. All sections were deparaffinized in xylene, rehydrated, and incubated for 30 min at room temperature in 0.3% hydrogen peroxide to block endogenous peroxidase activity. After rehydration through a graded series of ethanol treatments, antigen retrieval was performed in Immunosaver (Nisshin EM, Tokyo, Japan) at 98–100 °C for 45 min, and PD-L1 was retrieved using Universal HIER antigen retrieval reagent (Abcam, ab208572) at 120 °C for 20 min in an autoclave. Nonspecific binding sites were blocked by incubation with Protein Block Serum-Free (Dako, Carpinteria, CA) for 30 min. Samples were incubated in primary antibody (diluted by Dako REAL Antibody Diluent) overnight at 4 °C. TGFBI (Proteintech Group, Anti-TGFBI/BIGH3 antibody, 1:200 dilution), PD-L1 (Cell Signaling Technology, E1L3 N Rabbit mAb, 1:200 dilution), CD8 (Abcam, Anti-CD8, 1:1000 dilution), VIM (Dako, V9, 1:100 dilution), and Foxp3 (Abcam, Anti-Foxp3, 1:200 dilution) were used. Histofine Simple Stain MAX-PO (Multi) Kit (Nichirei, Tokyo, Japan) was used as the secondary antibody. Chromogen 3,3-diaminobenzidine tetrahydrochloride was applied as a 0.02% solution in 50 mM of ammonium acetate-citrate acid buffer (pH 6.0) containing 0.005% hydrogen peroxide. The sections were lightly counterstained with hematoxylin and mounted.

The tissue sections were evaluated by two independent evaluators who were blinded to the patient data. We focused on the expression of TGFBI in both cancer cells and cancer stroma, and the intensity was scored as follows: 0, no staining; 1+, weak staining; 2+, moderate staining; 3+, strong staining. Patients were assigned to the TGFBI low expression group (0, 1+) or high expression group (2, 3+), according to their staining scores (Fig. 1). We evaluated PD-L1, CD8, VIM, and Foxp3 using the same method as used previously (Supplementary Fig. 1).[31, 32]

Enzyme-Linked Immunosorbent Assay

Serum concentrations of TGFBI and TGF-β1 were determined by enzyme-linked immunosorbent assay (ELISA). All samples were collected before nivolumab therapy. For TGFBI, we used a human TGFBI (BIGH3) ELISA Kit (Thermo Fisher Scientific, Waltham, MA). Human sera were diluted at 1:3000 with PBS for ELISA.
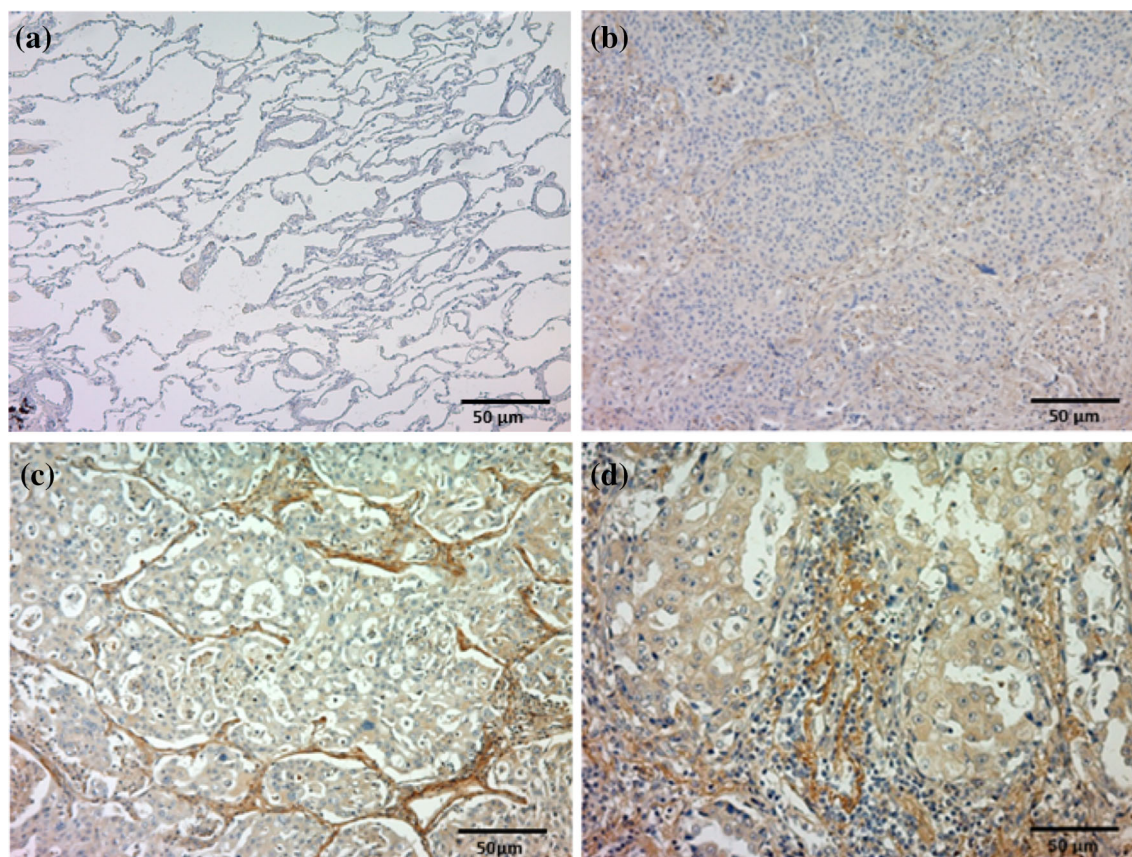
FIG. 1 Immunohistochemical staining of TGFBI of representative patients with NSCLC. **a** The expression of TGFBI in normal tissues was lower than that of lung cancer and cancer stromal tissues. **b** Both cancer cells and stromal TGFBI expressions were weak. **c** TGFBI expression in cancer cells was weak, and stromal TGFBI expression was strong. **d** Both cancer cells and stromal TGFBI expressions were strong

For TGF-β1, we used a human TGF-β1 ELISA Kit (R&D Systems, Minneapolis, MN). Human sera were diluted at 1:20 with Calibrator Diluent. All samples were activated by 1 N of HCl and 1.2 N of NaOH/0.5 M HEPES before use. Assays were evaluated as per the manufacturer's instructions, and all samples were tested in duplicate. In each ELISA kit, standard curves were constructed for each batch of ELISA using recombinant TGFBI and TGF-β1. The inter-assay coefficient of validation was less than 5%.

Statistical Analysis

Statistically significant differences were analyzed with the Mann–Whitney U test for continuous variables and the Chi squared test for categorical variables. Kaplan–Meier curves were generated for overall survival, and statistical significance was determined using the log-rank test. All differences were statistically significant at the level of $p < 0.05$, and a tendency was indicated at the level of $p < 0.1$. All statistical analyses were performed using JMP Pro 12.0 software (SAS Institute Inc., Cary, NC).

RESULTS

Immunohistochemical Staining for TGFBI in Clinical Lung Cancer Samples

A total of 33 patients were enrolled in this study. The expression of TGFBI in normal tissue was lower than that in lung cancer and cancer stromal tissue (Fig. 1a). Eleven NSCLC sections (33.3%, 11/33) were assigned to the low stromal TGFBI expression group, and 22 (66.7%, 22/33) to the high stromal TGFBI expression group (Figs. 1b, c). Thirteen (39.3%, 13/33) NSCLC sections were assigned to the high cancer TGFBI expression group (Fig. 1d) and 20 (60.7%, 20/33) to the low cancer TGFBI expression group. In our study, we could not find the correlations between the expression of TGFBI in cancer tissue and that in stromal tissue ($p = 0.62$).
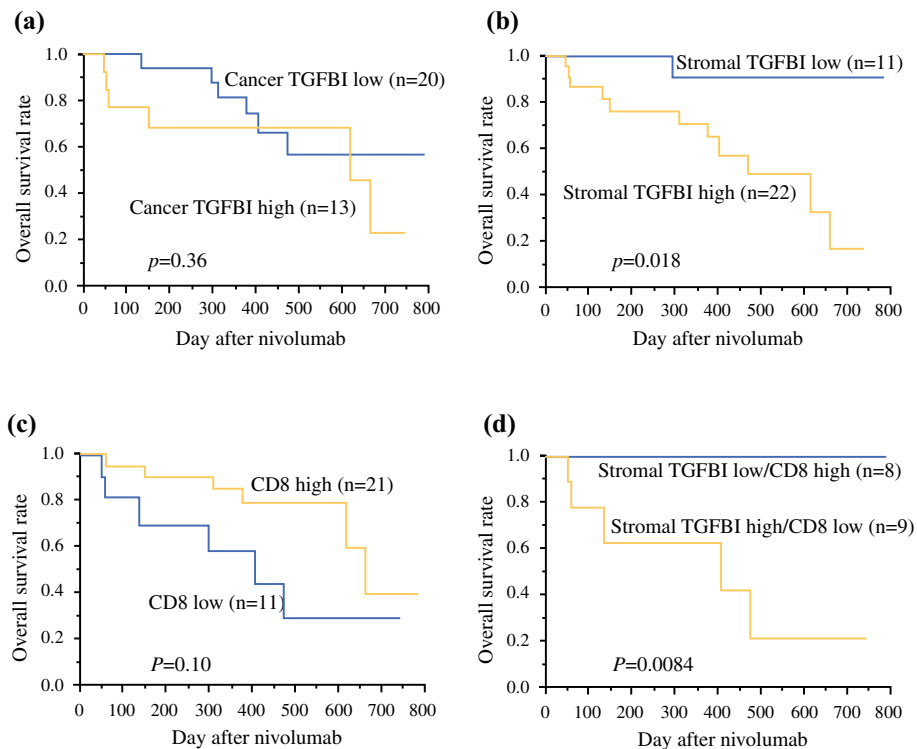
FIG. 2 Prognostic significance of the expression levels of TGFBI and CD8 in cancer and the stromal part of NSCLC samples Kaplan–Meier overall survival curves of NSCLC patients according to the level of cancer TGFBI (**a**), stromal TGFBI (**b**), intratumoral CD8 (**c**), and the combination of stromal TGFBI and CD8 (**d**)

Relationship Between Stromal TGFBI, Intratumoral Immune Cells, and Clinicopathological Features of NSCLC Patients

In Table 1, we show the correlation of clinicopathological features and clinical responses in 33 lung cancer patients as partial response (PR) plus stable disease (SD) versus progressive disease (PD) based on the Response Evaluation Criteria in Solid Tumours version 1.1 (RECIST 1.1). The definition of PD is the case that of appearance of a new lesion or diameter of target lesions increase more than 20%. Patients with PD were associated with low frequency of nivolumab administrations, high stromal TGFBI expression, and low intratumoral CD8-positive T cells in NSCLC samples (Table 1). Therefore, we focused on the relationship between nivolumab sensitivity, stromal TGFBI, and intratumoral CD8-positive T cells.

The relationship of stromal TGFBI expression and clinicopathological factors for 33 lung cancer patients is presented in Table 2. The high-TGFBI-expression group had poorer clinical responses than did the low-TGFBI-expression group ($p < 0.0001$), and the expression of stromal TGFBI was not correlated with the expression levels of CD8 ($p = 0.24$). The relationship of intratumoral CD8-positive T cells and clinicopathological factors for lung cancer patients is also presented in Table 2. The number of times nivolumab was administered in the high-CD8-expression group was significantly higher than that in the low-CD8-expression group ($p = 0.0046$). The high-CD8-expression group had better clinical responses than did the low-CD8-expression group ($p = 0.0013$). Interestingly, all patients with long-lasting responses to nivolumab were in the high-CD8-expression group ($p = 0.0043$).

Furthermore, we compared the high-TGFBI/low-CD8-expression group ($n = 9$) and the low-TGFBI/high-CD8-expression group ($n = 8$). The number of times nivolumab was administered in the high-TGFBI/low-CD8-expression group was significantly lower than that in the low-TGFBI/high-CD8-expression group ($p = 0.0032$) (Table 3). Interestingly, all patients in the high-TGFBI/low-CD8-expression group were classified as PD; in contrast, all patients in the low-TGFBI/high-CD8-expression group were classified as PR + SD by RECIST ($p < 0.0001$).

Association Between Stromal TGFBI and Serum TGFBI/TGF-β1 Concentrations

As mentioned above, it has been reported that the expression levels of TGFBI are induced by the activation of TGF-β signaling.[24] Therefore, we evaluated the

TABLE 2 The expression levels of stromal TGFBI and CD8 in tissues and clinicopathological factors

Factors	Stromal TGFBI		P value	Intratumoral CD8		P value
	Low expression n = 11	High expression n = 22		Low expression n = 11	High expression n = 21	
Age						
Years	67	69	0.65	68	68	0.98
Gender						
Male	9	18	1.00	8	18	0.38
Female	2	4		3	3	
Histology						
ADC	8	16	1.00	8	16	1.00
SQC	3	6		3	4	
Nivolumab addministration times						
	18.64 ± 3.59	10.00 ± 2.54	0.059	4.27 ± 3.35	16.90 ± 2.42	0.0046*
Response by RECIST						
PR+SD	11	8	< .0001*	2	16	0.0013*
PD	0	14		9	5	
PD-L1						
Negative	4	7	0.79	6	4	0.07
Positive	6	13		4	12	
TGFBI						
Negative	–	–	–	2	8	0.24
Positive	–	–		9	13	
CD8						
Negative	2	9	0.24	–	–	–
Positive	8	13		–	–	
Foxp3						
Negative	3	12	0.077	2	5	0.06
Positive	8	8		9	15	
VIM						
Negative	4	3	0.09	1	6	0.18
Positive	6	19		10	15	
TGFBI (serum)						
ng/ml	490.20 ± 66.38	538.02 ± 41.98	0.55	502.93 ± 57.83	535.79 ± 47.95	0.67
TGF-β1 (serum)						
ng/ml	16.47 ± 1.42	17.02 ± 0.90	0.75	17.00 ± 1.23	16.65 ± 1.02	0.83
irAE						
Negative	8	18	0.55	9	16	0.71
Positive	3	4		2	5	
Driver mutation						
EGFR	0	3	0.15	2	1	0.44
Wild	9	12		6	15	
Unknown	2	7		3	5	
Frequency of previous regimens						
Times	3.00 ± 0.72	2.58 ± 0.55	0.64	2.88 ± 0.85	2.76 ± 0.53	0.91
Reason for leading to treatment discontinuation						
Disease progression	3	14	0.11	10	7	0.0043*
irAE	2	3		1	4	
Death by AMI	0	1		0	1	

TABLE 2 continued

Factors	Stromal TGFBI		P value	Intratumoral CD8		P value
	Low expression $n = 11$	High expression $n = 22$		Low expression $n = 11$	High expression $n = 21$	
Treatment continuation	6	4		0	9	

ADC adenocarcinoma, *SQC* squamous cell carcinoma, *irAE* immune-related adverse events, *EGFR* epidermal growth factor receptor, *AMI* acute myocardial infarction

*P < 0.05

relationship of stromal TGFBI with the concentration of TGFBI and TGF-β1 in sera of corresponding 33 lung cancer patients. The mean concentration of TGFBI was 524.35 ± 185.55 ng/ml, and the mean concentration of TGF-β1 was 16.86 ± 3.96 ng/ml. Unexpectedly, the relationship of stromal TGFBI expression and serum TGFBI/TGF-β1 concentration was not detected in this cohort (Tables 2 and 3).

Kaplan–Meier Curve of Overall Survival According to the Expression of TGFBI, PD-L1, CD8, VIM, and Foxp3 in NSCLC Samples

There was no significant association between cancer TGFBI expression and the overall survival interval in the Kaplan–Meier analysis (Fig. 2a). In contrast, by focusing on the cancer stroma, the overall survival rate in the high-TGFBI-expression was significantly lower than that in the low-TGFBI-expression group ($p = 0.018$; Fig. 2b). There was no significant association between CD8 expression and the overall survival interval (Fig. 2c). The overall survival rate in the low-TGFBI/high-CD8-expression group was significantly higher than that in the high-TGFBI/low-CD8-expression group ($p = 0.0084$; Fig. 2d).

We investigated overall survival rates based on the expression of PD-L1, VIM, and Foxp3 of NSCLC tissues (Supplementary Fig. 2), but these results were not statistically significant.

DISCUSSION

In this study, we demonstrated that NSCLC patients with high stromal TGFBI and low intratumoral CD8-positive T cells were associated with poor prognosis and therapeutic resistance to nivolumab. Interestingly, all patients in the high-TGFBI/low-CD8-expression group were classified as PD, although all patients in the low-TGFBI/high-CD8-expression group were classified as PR + SD by RECIST, suggesting the possibility of TGFBI and CD8 as predictive marker candidates for the immune checkpoint inhibitor nivolumab.

We could detect TGFBI expression in not only cancer stromal tissues but also cancer cytoplasm in this study. Fong et al. reported that high TGFBI in lung cancer cells was associated with poor prognosis and progression of migration ability.[33,34] In contrast, other groups reported the cancer TGFBI function as a tumor suppressor in lung cancer.[35,36] Therefore, the significance of cancer TGFBI may be controversial. Actually, we could not detect a significant correlation of cancer TGFBI, prognosis, and nivolumab sensitivity in this study. On the other hand, TGFBI was described as a soluble TGF-β1-induced extracellular matrix-binding protein; it mediates cell adhesion to extracellular proteins, such as collagen, fibronectin, and laminins, through integrin binding. Stromal TGFBI binding to cellular integrins has been related to the activation of cell proliferation, adhesion, migration, and differentiation.[37] These observations suggested that TGFBI expression in tumor stromal tissues may be more important for the prognosis and therapeutic resistance of nivolumab than TGFBI in cancer cells.

Bhagirath et al. showed that TGFBI may have an important role in tumorigenic conditions that can be evaluated from its increased presence in the sera and urine of urothelial cancer patients.[38] TGFBI is known as a soluble endoglin, and it is induced by TGF-β signal activation. Moreover, Han et al. reported that serum TGFBI levels were significantly up-regulated in cholangiocarcinoma, hepatocellular carcinoma, and gastric carcinoma.[39] Therefore, we evaluated serum concentrations of TGFBI and TGF-β1 to clarify the significance of their expression in lung cancer with nivolumab treatment; unexpectedly, we could not find a statistically significant relationship between serum TGFBI and TGF-β1 levels. Moreover, the serum concentrations of TGFBI and TGF-β1 were not associated with prognosis, nivolumab sensitivity, or tumoral TGFBI expression. Considering these findings, it may be better to evaluate TGFBI expression in tissue specimens than body fluid specimens for predicting patient outcomes and the therapeutic efficacy of immune checkpoint inhibitors in lung cancer patients.

TABLE 3 The relationship of clinicopathological factors and tumor immune status to stromal TGFBI expression and intratumoral CD8-positive T cells

Factors	TGFBI high and CD8 low $n = 9$	TGFBI low and CD8 high $n = 8$	P value
Age			
Years	70	68	0.70
Gender			
Male	7	7	0.60
Female	2	1	
Histology			
ADC	6	5	0.86
SQC	3	3	
Nivolumab addministration times			
	3.56 ± 3.40	20.88 ± 3.60	0.0032*
Response by RECIST			
PR+SD	0	8	< .0001*
PD	9	0	
PD-L1			
Low	5	3	0.31
High	3	5	
Foxp3			
Low	5	2	0.13
High	3	6	
VIM			
Low	1	4	0.07
High	8	4	
TGFBI (serum)			
ng/ml	520.88 ± 71.20	500.06 ± 95.53	0.86
TGF-β1 (serum)			
ng/ml	16.44 ± 1.26	14.78 ± 1.69	0.45
irAE			
Negative	8	6	0.45
Positive	1	2	
Driver mutation			
EGFR	2	0	0.10
Wild	4	7	
Unknown	3	1	
Frequency of previous regimens			
Times	2.67 ± 1.20	3.13 ± 1.04	0.78

ADC adenocarcinoma, *SQC* squamous cell carcinoma, *irAE* immune-related adverse events, *EGFR* epidermal growth factor receptor

*$P < 0.05$

Our immunohistochemical analysis also showed a correlation between PD-L1 and CD8 expression in NSCLC patients, although there was no significant difference ($p = 0.07$). As in our results, several studies have shown an association between PD-L1 expression and CD8-positive T-cell density in NSCLC.[40,41] Immune checkpoint proteins, such as PD-1/PD-L1 induce immune tolerance by inhibiting T-cell proliferation and promoting apoptosis in activated T cells.[42] Although CD8-positive T-cell infiltration was appropriate for predicting the therapeutic efficacy of nivolumab, we could not find statistical significance between the intratumoral infiltration of CD8-positive T cells and stromal TGFBI expression in our experiment. However, our data showed that all patients with high stromal TGFBI/low intratumoral CD8-positive T cells could not continue nivolumab treatment due to PD and had

poorer prognoses than those with low stromal TGFBI/high intratumoral CD8-positive T cells. Good responses to immune checkpoint inhibitors are strongly correlated to the presence of intratumoral CD8-positive T cells and high PD-L1 expression in both tumor cells and stroma, and such tumors are defined as hot tumors with antitumor immunity.[43,44] In contrast, cold tumors are characterized as having low intratumoral CD8-positive T cells and PD-L1 expression in tumor tissues. EMT that causes therapeutic resistance to immune checkpoint inhibitors is accelerated in cold tumors compared with hot tumors.[45] Liu et al. demonstrated that TGFBI is highly induced during EMT in A549 NSCLC cells and acts as the EMT inducer via microRNAs regulation.[29] In this study, we could not validate the significant relationship of stromal TGFBI and mesenchymal marker VIM statistically; however, these expressions had a tendency ($p = 0.09$). Thus, it was possible to predict whether a tumor was hot or cold by investigating just two markers consisting of cancer stromal TGFBI and intratumoral CD8-positive T cells; evaluating two markers was very meaningful for predicting prognoses and the therapeutic effect of nivolumab.

There are several Food and Drug Administration (FDA) approved immune checkpoint inhibitors, such as nivolumab, atezolizumab, and ipilimumab. The EMT induction and TGF-β activation in cancer tissues was associated with therapeutic response of these immune checkpoint inhibitors[46–48] As mentioned above, TGFBI has been reported to control TGF-β-mediated EMT induction. Our data showed that high stromal TGFBI expression was significantly associated with nivolumab resistance in NSCLC patients. From these observations, TGFBI is expected to be a universal biomarker for predicting sensitivity of not only nivolumab but also other immune checkpoint inhibitors, such as atezolizumab and ipilimumab. In future, further study is needed to evaluate the potential of TGFBI as a biomarker for immune checkpoint inhibitors in patients with several cancers.

Our study has several limitations. The first limitation is that this study has a small sample size; thus, it may bias the results of our study. Further, large-scale, clinical trials will be needed to clarify the potential of stromal TGFBI/intratumoral CD8-positive T cells as a new predictive biomarker for nivolumab. Furthermore, Sato et al. suggested that PD-L1 expression in cancer cells was upregulated in response to DNA damage.[49] Chemotherapy that induces DNA damage is considered to contribute to PD-L1 expression in cancer cells. Depending on this point, we have one more limitation in our study because surgical specimens in this study were obtained before chemotherapy.

In conclusion, we clarified that the high expression of stromal TGFBI/intratumoral CD8-positive T cells in lung cancer tissue was associated with poor clinical response to nivolumab and poor prognoses. The dual evaluation of TGFBI and CD8 could be a useful predictive marker for nivolumab.

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