Original Article

Correlation of tumor microenvironment-related markers with clinical outcomes in patients with squamous cell carcinoma of the lung

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Background: Squamous cell carcinoma (SCC) is the major histological type in lung cancer (LC). The tumor microenvironment (TME) drives tumor progression and metastasis. In the TME, cancer-associated fibroblasts (CAFs) play key roles in carcinogenesis. However, the roles of CAFs in lung SCC remain unknown. In this study, we evaluated whether the CAF phenotype was determined by various CAF-related proteins and whether CAF-related protein expression contributed to clinical outcomes in patients with lung SCC.

Methods: We examined the associations of CAF- and epithelial-mesenchymal transition (EMT)-related markers expressed in CAFs, including α-smooth muscle actin (α-SMA), CD10, podoplanin, fibroblast-specific protein 1 (FSP1), platelet-derived growth factor receptor (PDGFR) α, PDGFRβ, adipocyte enhancer-binding protein 1 (AEBP1), fibroblast activation protein (FAP), tenascin-C, Zinc finger E-box binding homeobox 1 (ZEB1), and twist homolog 1 gene (TWIST1), in 108 lung SCC tissues using immunohistochemistry. In addition, cluster analysis was used to identify objective expression patterns of immunohistochemical markers. Finally, the CD3/CD8 ratio was evaluated in order to identify the associations of CAF-related proteins with the CD3/CD8 ratio using immunohistochemistry.

Results: SCC samples were classified into two subgroups (CAF-phenotype), which were significantly correlated with disease-free and overall survival using univariate and multivariate analyses. Moreover, high AEBP1 expression was identified as an independent prognostic marker in this cohort by univariate and multivariate analyses. The CD3/CD8 ratio was not correlated with the CAF-phenotype.

Conclusions: The presence of a specific subgroup defined by multiple markers could be used for prediction of prognosis in patients with lung SCC. In addition, AEBP1 overexpression played key roles in prediction of a poor prognosis in patients with lung SCC.

Keywords: Adipocyte enhancer-binding protein 1 (AEBP1); cancer-associated fibroblast (CAF); lung cancer (LC); prognosis; squamous cell carcinoma (SCC)

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Introduction

Lung cancer (LC) is a leading cause of cancer-related death worldwide (1-3). LC is histologically classified into two types: non-small cell lung cancer (NSCLC) and small cell LC (1). NSCLC is the most common histological type (1). Among LCs, small cell carcinoma is associated with poor prognosis; however, outcomes in patients with NSCLC are also poor, with a 10-year survival rate of only 5% (4,5). Moreover, NSCLC can be subdivided into squamous cell carcinoma (SCC) and adenocarcinoma (1). Lung SCC may be different from adenocarcinoma of the lung in terms of carcinogenesis (tobacco-related versus not tobacco-related) and patient survival (6-8).

The tumor microenvironment (TME) plays crucial roles in neoplastic progression and cancer metastasis (9-11). The TME is a complex region comprising cancer cells, extracellular matrix (ECM), and various types of stromal cells, including macrophages, inflammatory cells, cancer-associated fibroblasts (CAFs), and mesenchymal stem cells (6). Together, these TME-related components, particularly CAFs, contribute to tumor growth and metastasis via secretion of growth factors and cytokines (5,9). Although the contributions of CAFs to LC progression have been studied (5,9), the molecular basis of SCC (e.g., CAF-related proteins that promote tumor progression and metastasis) has not been fully elucidated.

Characterization of CAF phenotypes, including TME-related factors and their impact on outcomes, has attracted much interest (5,9,10). Many reports have also described the prognostic effects of single CAF-related markers in NSCLC (6), and various activating proteins secreted from CAFs have been shown to promote cancer invasion and metastasis (5,9). The impact of CAFs varies according to the specific marker, with contradictory results reported (5,9). This may be explained by the heterogeneity of CAFs within the same tumor, where different markers can be expressed by functionally distinct CAFs (5).

In this study, we evaluated whether the CAF phenotype could be determined based on analysis of various CAF- and epithelial-mesenchymal transition (EMT)-related markers expressed in CAFs, including α-smooth muscle actin (α-SMA), CD10, podoplanin, fibroblast-specific protein 1 (FSP1), platelet-derived growth factor receptor (PDGFR) α, PDGFRβ, adipocyte enhancer-binding protein 1 (AEBP1), fibroblast activation protein (FAP), tenascin-C, Zinc finger E-box binding homeobox 1 (ZEB1), and twist homolog 1 gene (TWIST1). We also assessed whether CAF-related protein expression contributed to clinical outcomes in patients with lung SCC. We aimed to identify heterogeneous expression of CAF-related proteins in lung SCC and evaluate whether such expression patterns affected prognosis in patients with lung SCC. These findings may contribute to evaluation of the molecular mechanisms of resistance and the associations of CAF-related marker expression patterns with prognosis. We present the following article in accordance with the REMARK reporting checklist (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-22-10/rc).

Methods

Patients

Among patients who underwent curative surgery for LC at Iwate Medical University Hospital from January 2010 to December 2016, we enrolled a consecutive series of 108 patients with lung SCC for whom paraffin-embedded tissues were relatively well preserved, medical records were complete, and patient survival had been followed-up. Tumor markers were examined. The end of the follow-up period was May 2021 (median follow-up period: 1,547 days; maximum: 4,060 days; minimum: 40 days). Histological diagnosis of SCC was performed according to the 2021 version of the World Health Organization histological classification (8,9). Patient survival was confirmed through the National Cancer Registry and by telephone interviews. The clinicopathological findings of patients with LC were further confirmed by reviewing patient medical records and pathology files. The associations of clinicopathological parameters and immunohistochemical findings with survival were investigated for all 108 patients. Clinicopathological findings of LC are summarized in *Table 1*. Only patients with pathological stages (p-stage) I–III disease were included. Patients who underwent pre-operative chemotherapy and patients with asynchronous or simultaneous LC were excluded. These findings were obtained from the medical records of patients admitted at Iwate Medical University. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the local ethics committee of Iwate Medical University (approval No. MH2021-047), and all patients provided informed consent.

Among pulmonary comorbidities, chronic obstructive pulmonary disease (COPD) was found in 43 of 108 (39.8%) cases of lung SCC, whereas interstitial lung disease (ILD),

Table 1 Clinicopathological findings (n=108)

Characteristics	Values
Age median, years [range]	72 [51–87]
Sex	
Man	100 (92.6%)
Woman	8 (7.4%)
Smoking history	
Yes	105 (97.2%)
No	3 (2.8%)
Co-morbidities	
COPD	
Yes	43 (39.8%)
No	65 (60.2%)
ILD	
Yes	24 (22.2%)
No	84 (77.8%)
Tumor marker	
CEA	
Positive	67 (62.0%)
Negative	41 (38.0%)
CYFRA	
Positive	71 (65.7%)
Negative	37 (34.3%)
p-stage	
I	55 (50.9%)
II and III	53 (49.1%)
Lymph node metastasis	
Positive	31 (28.7%)
Negative	77 (71.3%)
Vascular invasion	
Positive	32 (29.6%)
Negative	76 (70.4%)
Pleural invasion	
Positive	27 (25.0%)
Negative	81 (75.0%)
Lymphatic invasion	
Positive	14 (13.0%)
Negative	94 (87.0%)
Postoperative adjuvant therapy	
Yes	61 (56.5%)
Chemotherapy	57 (52.8%)
Chemoradiotherapy	3 (2.8%)
Radiation therapy	1 (0.9%)
No	47 (43.5%)
CD3/CD8 ratio	
High	57 (52.8%)
Low	51 (47.2%)
Disease-free survival, days, median [range]	1,281 [40–4,060]
Overall survival, days, median [range]	1,547 [40–4,060]

Data are presented as number (percentage). COPD, chronic obstructive pulmonary disease; ILD, interstitial lung disease; CEA, carcinoembryonic antigen; CYFRA, cytokeratin fragment; p-stage, pathological stage.

including interstitial pneumonia, was observed in 24 of 108 (22.2%) cases of lung SCC.

Determination of overall survival and disease-free survival

LC-specific survival, defined as cause of death from LC, was used as overall survival. In addition, recurrence-free survival, which excluded secondary cancers, was adopted to measure disease-free survival. The duration of disease-free survival was determined based on the presence/absence of metastasis, determined during the follow-up period (3–4 times/year) using computed tomography.

Sample size determination

The sample size required to identify patient outcomes was determined to be at least 100 cases using JMP Pro 16.1 software (SAS, Tokyo, Japan). The statistical power (detection power) was set to 0.8, which is commonly used in medical studies.

Chemotherapeutic treatment after surgery for lung SCC

Following surgery, tegafur uracil was administered to patients with lung SCC (29/108 cases), whereas platinum-doublet chemotherapy, including carboplatin plus gemcitabine, tegafur/gimeracil/oteracil, or paclitaxel, was

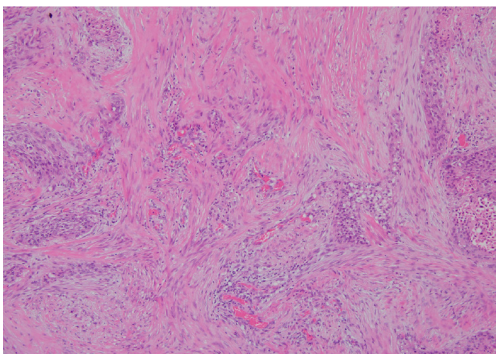

Figure 1 Representative histological figures of desmoplastic fibrosis surrounding cancer tissues (hematoxylin-eosin staining; ×200).

administered to patients with lung SCC (28/108 cases). Chemoradiotherapy was performed in three patients with lung SCC. Only one patient received radiation therapy after operation. The other 47 patients did not receive additional chemotherapy following surgery.

Construction of tissue microarrays (TMAs)

Paraffin-embedded tissues used for construction of TMAs were stored at room temperature. These materials showed sufficient quality for biological assessments, including high DNA and RNA quality. TMAs were created using a manual tissue array (Azumaya Co., Tokyo, Japan). We selected invasive areas with strong desmoplastic fibrosis for analysis by immunohistochemistry. Representative histological features of lung SCC with strong desmoplasia are shown in *Figure 1*. Five-millimeter-thick tissue cores were collected from target lesions and placed into recipient blocks containing 12 cores, including 10 cancer tissues and 2 control tissues. After construction, 3-μm-thick sections were prepared and stained with hematoxylin and eosin using the initial slides to verify the histological diagnosis. Serial sections were cut from the TMA blocks for immunohistochemical staining.

Immunohistochemistry

Three-micron-thick tissue sections were cut from paraffin-embedded blocks and placed on charged slides, which were dried and melted in an oven at 62 °C for 20 min. After deparaffinization and rehydration, the sections were heated in Envision FLEX target retrieval solution (pH 6.0 or 9.0; Dako, Glostrup, Denmark) for 20 min and washed twice for 5 min in phosphate-buffered saline (PBS). Hydrogen peroxide (3%) was used to block endogenous peroxidase activity for 5 min. Nonspecific binding was blocked with 1.5% normal serum in PBS for 35 min at room temperature. The antibodies used in this study are listed in Table S1. Immunohistochemistry was carried out using the DAKO Envision+ system (12). The specimens were heated in citrate buffer (pH 6.0) 3 times for 5 min each using a microwave (H2500; Microwave Processor; Azumaya) at 750 W before incubation with antibodies. The antigen-antibody reaction was visualized using an enhanced polymer-based detection system. Hematoxylin was used as the counterstain. Desmoplastic tissues surrounding invasive colorectal cancer (CRC) and ILD were used as a positive control. The detailed methods were described previously (12).

Assessment of immunohistochemical results

The immunopositivity of the stromal fibroblastic compartment of each tumor was examined for α-SMA, CD10, podoplanin, FSP1, PDGFRα, PDGFRβ, AEBP1, FAP, tenascin-C, ZEB1, and TWIST1. Inflammatory cells were carefully excluded from the analysis. Only nuclear positivity for ZEB1 and TWIST1 was considered significant, whereas only cytoplasmic expression of α-SMA, CD10, podoplanin, FSP1, PDGFRα, PDGFRβ, AEBP1, FAP and tenascin-C was regarded as positive. The immunostaining intensity and area were evaluated separately. The immunostaining intensity for fusiform stromal cells was classified into four categories according to staining intensity as follows: negative, weak, moderate, and strong. The immunostaining area for fusiform stromal cells was semiquantified as follows: 0%, 1–25%, 26–50%, and 51–100%. The combination of intensity and area was scored (Table S2). A score of more than 4 was considered a positive score. Determination of the score was performed by expert diagnostic pathologists (NY, MO, TS) who were blinded to the study endpoint. If results among the pathologists were discordant, a discussion meeting was held, and a consensus was reached. Finally, all markers used in the current study had been identified as CAF- and EMT-related markers in a previous study (12).

Hierarchical cluster analysis of the expression of CAF- and EMT-related markers

Hierarchical cluster analysis was performed to group the

samples according to immunohistochemical expression levels, thereby achieving maximal homogeneity for each group and the greatest difference between groups using open-access clustering software (Cluster 3.0 software; bonsai.hgc.jp/~mdehoon/software/cluster/software.htm). The clustering algorithm was set to centroid linkage clustering.

Statistical analysis

Data were analyzed using JMP Pro 16.1 software (SAS). Fisher's exact test was used to compare the immunohistochemical positivity of each marker and clinicopathological findings with each subgroup. Age distributions among subgroups were examined using Mann-Whitney U tests. Kaplan-Meier analyses were performed using log-rank tests for survival analyses. The level of significance was set at P<0.05.

Univariate and multivariate analyses were conducted with Cox proportional hazards models to identify significant differences for prediction of overall and disease-free survival. The level of significance was set at P<0.05, and the confidence interval (CI) was determined at the 95% level.

Study design

The current study was a retrospective study (Figure S1). Among the lung SCCs examined, 142 lung SCC samples were initially evaluated; 108 lung SCC samples were ultimately included after exclusion of 34 cases based on exclusion criteria (asynchronous or simultaneous cancer, 8 cases; pre-operative chemotherapy, 2 cases; prognosis unknown, 13 cases; death within 30 days, 2 cases; loss of histological specimens, 9 cases). First, 108 patients with lung SCC were used to identify the associations of 11 CAF- and EMT-related markers with patient survival using immunohistochemistry. Second, cluster analysis was performed to stratify the lung SCCs based on expression patterns to exclude arbitrary results. As a result, some subgroups were expected to be segregated. Third, we attempted to identify the examined markers to characterize the subgroups. Finally, we examined the associations of CAF- and EMT-related markers with patient outcomes, including disease-free and overall survival, using univariate and multivariate analyses. The CD3/CD8 ratio was also examined to evaluate the associations of EMT-related marker expression with immune status in the TME.

List of all candidate variables initially examined

For prediction of patient outcomes, pulmonary complications, tumor marker, p-stage, and immunohistochemical markers were examined, and subgroups were stratified using immunohistochemical expression patterns.

Assessment of the CD3/CD8 ratio is described in the Appendix 1.

Results

Basic demographic characteristics, standard prognostic variables, and tumor markers

There were no significant differences in age distribution compared with that in previous studies. The lung SCC samples examined in this study were from older patients owing to advanced smoking history in Japanese man. We compared CAF- and EMT-related markers with p-stage of LC as a standard prognostic factor. Representative immunohistochemical features are shown in *Figure 2*.

Kaplan-Meier analyses of clinicopathological findings based on disease-free and overall survival

Although the presence/absence of COPD was not correlated with disease-free and overall survival, that of ILD was closely associated with disease-free and overall survival (Figure S2A,S2B). Additionally, p-stage was also correlated with disease-free and overall survival (Figure S2C,S2D). Tumor markers, including carcinoembryonic antigen (CEA) and cytokeratin fragment (CYFRA), showed no significant association.

Hierarchical clustering based on marker scores

We performed hierarchical clustering based on marker scores to evaluate differences in the expression patterns of CAF- and EMT-related markers in patients with lung SCC. Two distinct subgroups were stratified (*Figure 3*; subgroups 1 and 2). There were significant differences in the frequencies of disease-free survival and overall survival between subgroups 1 and 2 (*Table 2*). However, no significant differences were found for other clinicopathological factors.

Kaplan-Meier analyses were performed to determine the associations of disease-free and overall survival with subgroups. Patients in subgroup 1 had poorer disease-free survival than patients in subgroup 2 (P<0.0001; Figure S2E).

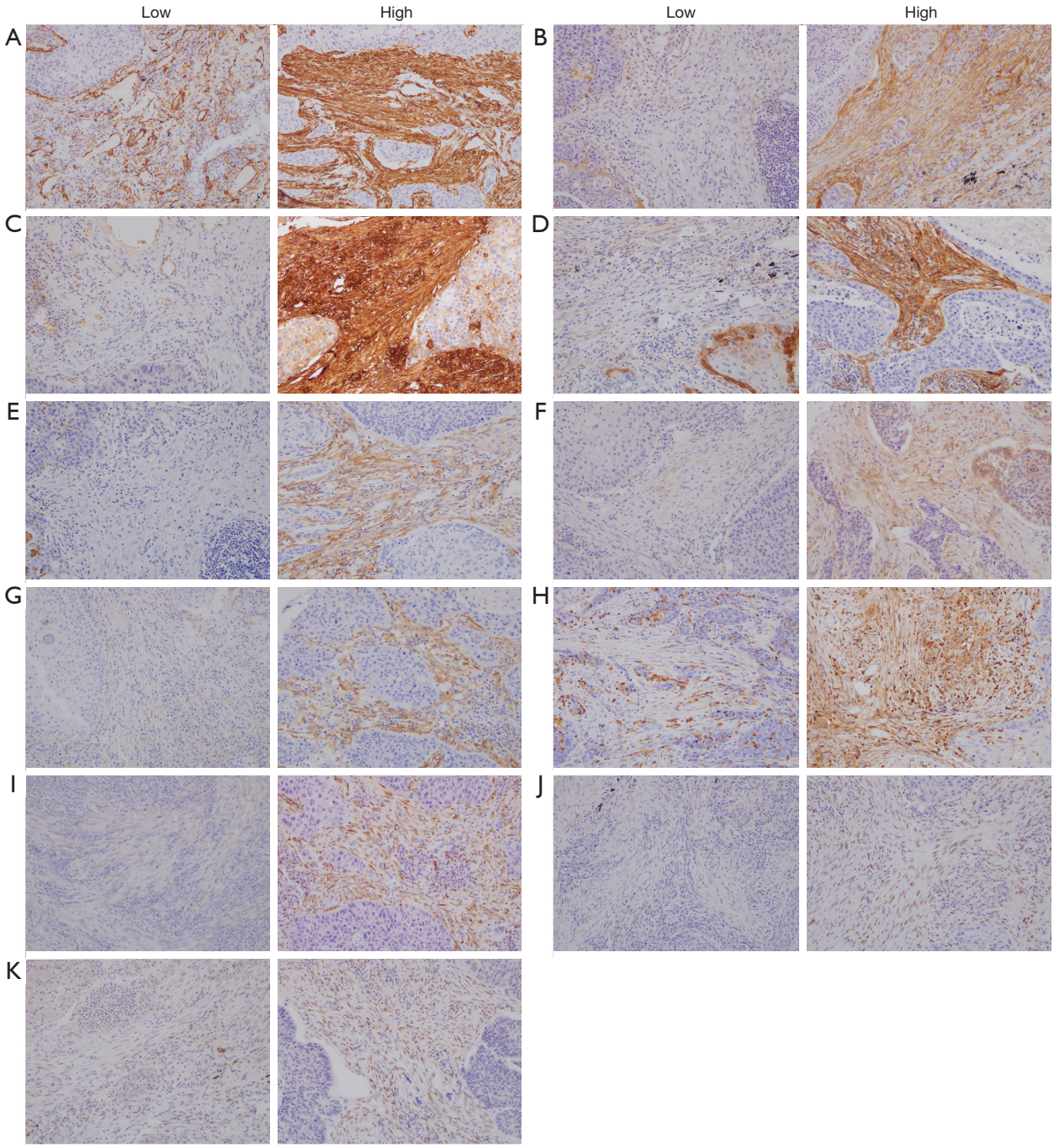

Figure 2 Representative immunohistochemical staining images of CAF-related markers in CAFs. (A) α-SMA (×200). (B) FAP (×200). (C) Tenascin-C (×200). (D) Podoplanin (×200). (E) CD10 (×200). (F) PDGFRα (×200). (G) PDGFRβ (×200). (H) FSP1 (×200). (I) AEBP1 (×200). (J) ZEB1 (×200). (K) TWIST1 (×200). CAFs, cancer-associated fibroblast; α-SMA, α-smooth muscle actin; FAP, fibroblast activation protein; PDGFR, platelet-derived growth factor receptor; FSP1, fibroblast-specific protein 1; AEBP1, adipocyte enhancer-binding protein 1; ZEB1, zinc finger E-box binding homeobox 1; TWIST1, twist homolog 1 gene.

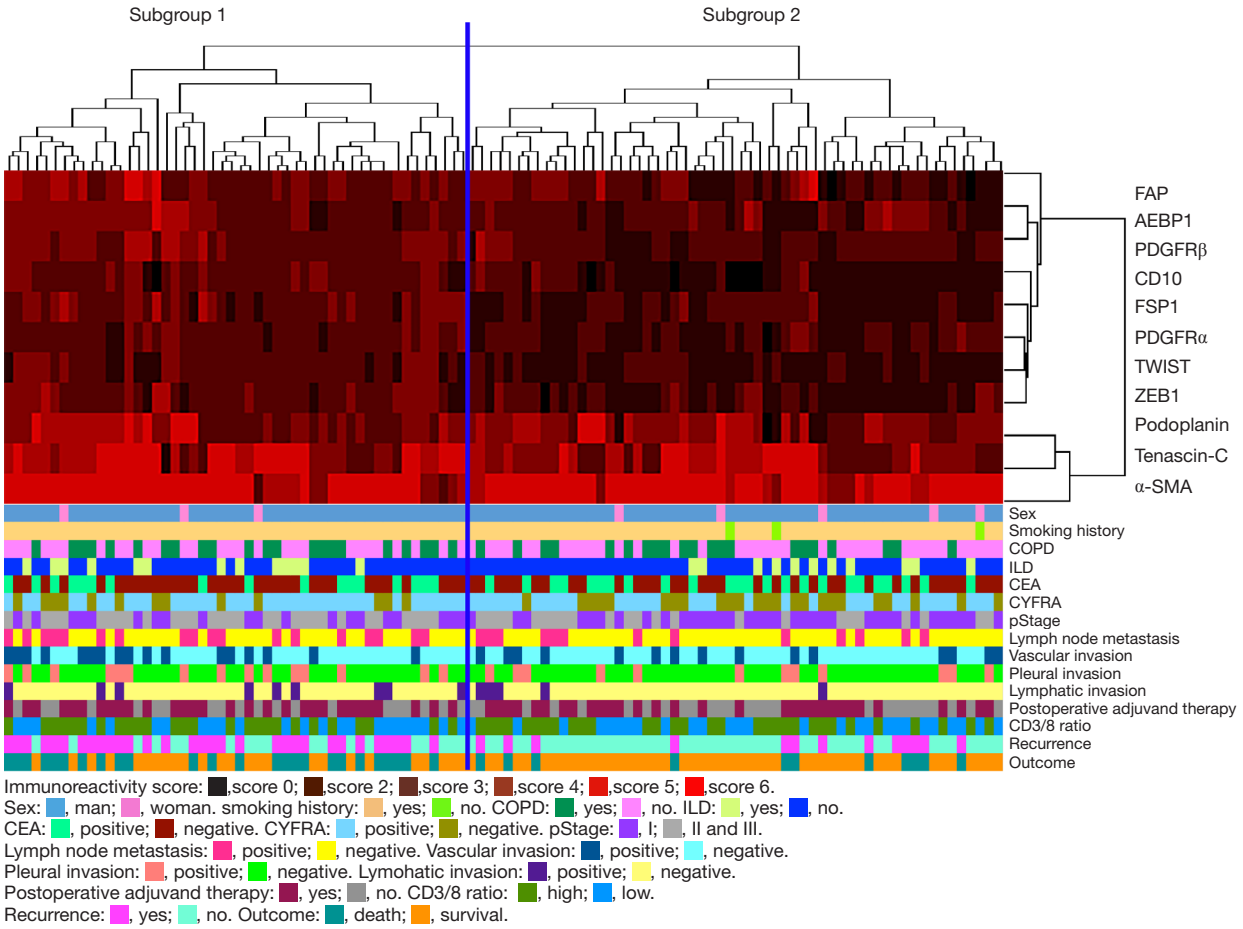

Figure 3 Hierarchical cluster analysis based on the expression patterns of CAF- and EMT-related markers. The vertical line shows the expression of each marker in fibroblasts, and the horizontal lines denote "relatedness" between samples. FAP, fibroblast activation protein; AEBP1, adipocyte enhancer-binding protein 1; PDGFR, platelet-derived growth factor receptor; FSP1, fibroblast-specific protein 1; TWIST1, twist homolog 1 gene; ZEB1, zinc finger E-box binding homeobox 1; α-SMA, α-smooth muscle actin; COPD, chronic obstructive pulmonary disease; ILD, interstitial lung disease; CEA, carcinoembryonic antigen; CYFRA, cytokeratin fragment; CAF, cancer-associated fibroblast; EMT, epithelial-mesenchymal transition.

Additionally, overall survival was correlated with subgroup 1 (P=0.0033; Figure S2F).

In our comparison of disease-free survival with clinicopathological findings, including subgroup analyses, 3 clinicopathological findings, i.e., presence of ILD, p-stage (II and III > I), and subgroup (subgroup 1 > subgroup 2), were identified in univariate analysis and retained in multivariate analysis (*Table 3*—left-sided column and second column). For overall survival, the same 3 clinicopathological findings were identified in univariate and multivariate analyses (*Table 3*—third column and right-sided column). Finally, positive values of CEA and CYFRA were not retained in univariate analysis of disease-free and overall survival.

Comparison of individual markers with each subgroup

The positive ratios of FAP (P=0.0109), CD10 (P<0,0028), PDGFRβ (P=0.0001), FSP1 (P=0.0005), AEBP1 (P=0.0005), ZEB1 (P=0.0078), and TWIST1 (P<0.0001) expression in CAFs were significantly higher in subgroup 1 than in subgroup 2 (*Figure 4*).

Association of various markers with patient survival

To determine whether the clinicopathological variables and expression patterns of the examined markers were independent predictors of clinical outcomes in patients

Table 2 Clinicopathological findings according to each subgroup

Variable	Subgroup 1	Subgroup 2	P value
Total	50	58	
Age median, years [range]	72 [51–84]	72 [54–87]	0.5958
Sex			0.7224
Man	47 (94%)	53 (91.4%)	
Woman	3 (6%)	5 (8.6%)	
Smoking history			0.2471
Yes	50 (100%)	55 (94.8%)	
No	0	3 (5.2%)	
COPD			0.4363
Yes	22 (44%)	21 (36.2%)	
No	28 (56%)	37 (63.8%)	
ILD			0.4872
Yes	13 (26%)	11 (19%)	
No	37 (74%)	47 (81%)	
CEA			0.5512
Positive	33 (66%)	34 (58.6%)	
Negative	17 (34%)	24 (41.4%)	
CYFRA			0.1072
Positive	37 (74%)	34 (58.6%)	
Negative	13 (26%)	24 (41.4%)	
p-stage			0.0531
I	20 (40%)	35 (60.3%)	
II and III	30 (60%)	23 (39.7%)	
Lymph node metastasis			0.139
Positive	18 (36%)	13 (22.4%)	
Negative	32 (64%)	45 (77.6%)	
Vascular invasion			0.402
Positive	17 (34%)	15 (25.9%)	
Negative	33 (66%)	43 (74.1%)	
Pleural invasion			0.2763
Positive	15 (30%)	12 (20.7%)	
Negative	35 (70%)	46 (79.3%)	
Lymphatic invasion			0.1641
Positive	9 (18%)	5 (8.6%)	
Negative	41 (82%)	53 (91.4%)	

Table 2 (*continued*)

Table 2 (*continued*)

Variable	Subgroup 1	Subgroup 2	P value
Postoperative adjuvant therapy			0.5613
Yes	30 (60%)	31 (53.5%)	
No	20 (40%)	27 (46.6%)	
CD3/CD8 ratio			1
High	26 (52%)	31 (53.4%)	
Low	24 (48%)	27 (46.6%)	
Recurrence			<0.0001
Yes	31 (62%)	13 (22.4%)	
No	19 (38%)	45 (77.6%)	
Outcome			0.0010
Death	25 (50%)	11 (19%)	
Survival	25 (50%)	47 (81%)	

Data are presented as number (percentage). COPD, chronic obstructive pulmonary disease; ILD, interstitial lung disease; CEA, carcinoembryonic antigen; CYFRA, cytokeratin fragment; p-stage, pathological stage.

with lung SCC, we used univariate analysis for preliminary screening of the variables, followed by a stepwise logistic regression of the risk of mortality with the significant univariate correlators (predictors). Univariate analysis (*Table 4*—left-sided column) identified 7 factors, including presence of ILD, p-stage, AEBP1, FAP, PDGFRβ, tenascin-C, and TWIST1, as being associated with disease-free survival in patients with lung SCC. Finally, we found that presence of ILD, p-stage, and AEBP1 remained significantly correlated with disease-free survival in multivariate analysis, even after controlling for the other variables (*Table 4*—second column). Next, we examined the associations of these factors with overall survival. Although presence of ILD, p-stage, FAP, tenascin-C, and TWIST1 were identified as significant factors in univariate analysis of lung SCC, only presence of ILD and p-stage were retained in multivariate analysis (*Table 4*—third column and right-sided column).

Association of subgroups with the CD3/CD8 ratio in lung SCC

The CD3/CD8 ratio was not correlated with subgroups classified based on prognosis (worse prognosis: subgroup 1 > subgroup 2; *Table 2*). Moreover, the CD3/CD8 ratio was not correlated with prognosis (not retained in univariate analysis; *Table 3*).

Discussion

The CAF phenotype, which is determined by the expression patterns of CAF- and EMT-related markers, may vary based on the type of markers selected in each specific study (5,13,14). Therefore, the CAF phenotype may depend on the type of immunohistochemical scoring system used (12). In a previous study, the dominant staining intensity was calculated in positive cells, whereas another study used an index combining both staining intensity and extent (15,16). Such differences in the scoring system may in part explain the differences observed between these two studies (15,16). Indeed, the use of different scoring criteria in biomarker research and subsequent difficulties in comparing studies are problems preventing the validation of biomarkers (5). However, in this study, the phenotype we identified could exclude the arbitrariness of classification and consequently became an objective classification of the CAF phenotype. Additionally, the current phenotype, stratified based on multiple CAF-related markers, identified the relationship between heterogeneous expression of CAF-related markers, which are affected by chemotherapy response, and patient prognosis.

The CAF phenotype is closely associated with the

Table 3 Association of clinicopathological variables and subgroups with disease-free survival and overall survival in univariate and multivariate analyses

Variable	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	HR	95% CI	P value	HR	95% CI	P value	HR	95% CI	P value	HR	95% CI	P value
Sex												
Man *vs.* woman	2.081	0.612–13.130	0.2745	–	–	–	3.268	0.661–59.691	0.1738	–	–	–
p-stage												
II and III *vs.* I	2.315	1.261–4.399	0.0066	2.243	1.199–4.197	0.0115	2.797	1.405–5.939	0.0031	2.555	1.246–5.239	0.0105
COPD												
Yes *vs.* no	0.741	0.396–1.384	0.3467	–	–	–	0.581	0.280–1.207	0.1457	–	–	–
ILD												
Yes *vs.* no	2.500	1.322–4.725	0.0048	2.539	1.338–4.818	0.0044	2.188	1.082–4.423	0.0293	2.073	1.021–4.207	0.0437
CEA												
Positive *vs.* negative	1.546	0.823–2.904	0.1754	–	–	–	1.739	0.851–3.553	0.1294	–	–	–
CYFRA												
Positive *vs.* negative	0.933	0.504–1.728	0.8262	–	–	–	1.021	0.510–2.045	0.954	–	–	–
Vascular invasion												
Positive *vs.* negative	1.798	0.935–3.361	0.0769	–	–	–	1.508	0.720–3.009	0.2661	–	–	–
Pleural invasion												
Positive *vs.* negative	1.274	0.640–2.396	0.4556	–	–	–	1.064	0.485–2.158	0.8699	–	–	–
Lymphatic invasion												
Positive *vs.* negative	1.833	0.814–3.707	0.1345	–	–	–	1.986	0.831–4.220	0.1161	–	–	–
Postoperative adjuvant therapy												
Yes *vs.* no	0.865	0.477–1.568	0.6331	–	–	–	0.893	0.462–1.727	0.7371	–	–	–
CD3/CD8 ratio												
High *vs.* low	1.186	0.650–2.164	0.5791	–	–	–	1.654	0.839–3.261	0.1464	–	–	–
Subgroup												
Subgroup 1 *vs.* 2	3.341	1.786–6.620	0.0001	3.075	1.595–5.927	0.0008	2.775	1.397–5.884	0.0032	2.523	1.264–5.371	0.0082

HR, hazard ratio; CI, confidence interval; p-stage, pathological stage; COPD, chronic obstructive pulmonary disease; ILD, interstitial lung disease; CEA, carcinoembryonic antigen; CYFRA, cytokeratin fragment.

expression patterns of the immunohistochemical markers examined in this study (12). In this study, the prognosis in patients with LC was closely associated with the immunohistochemical expression patterns of CAF- and EMT-related markers. This finding suggested that the heterogeneous expression of environmental markers

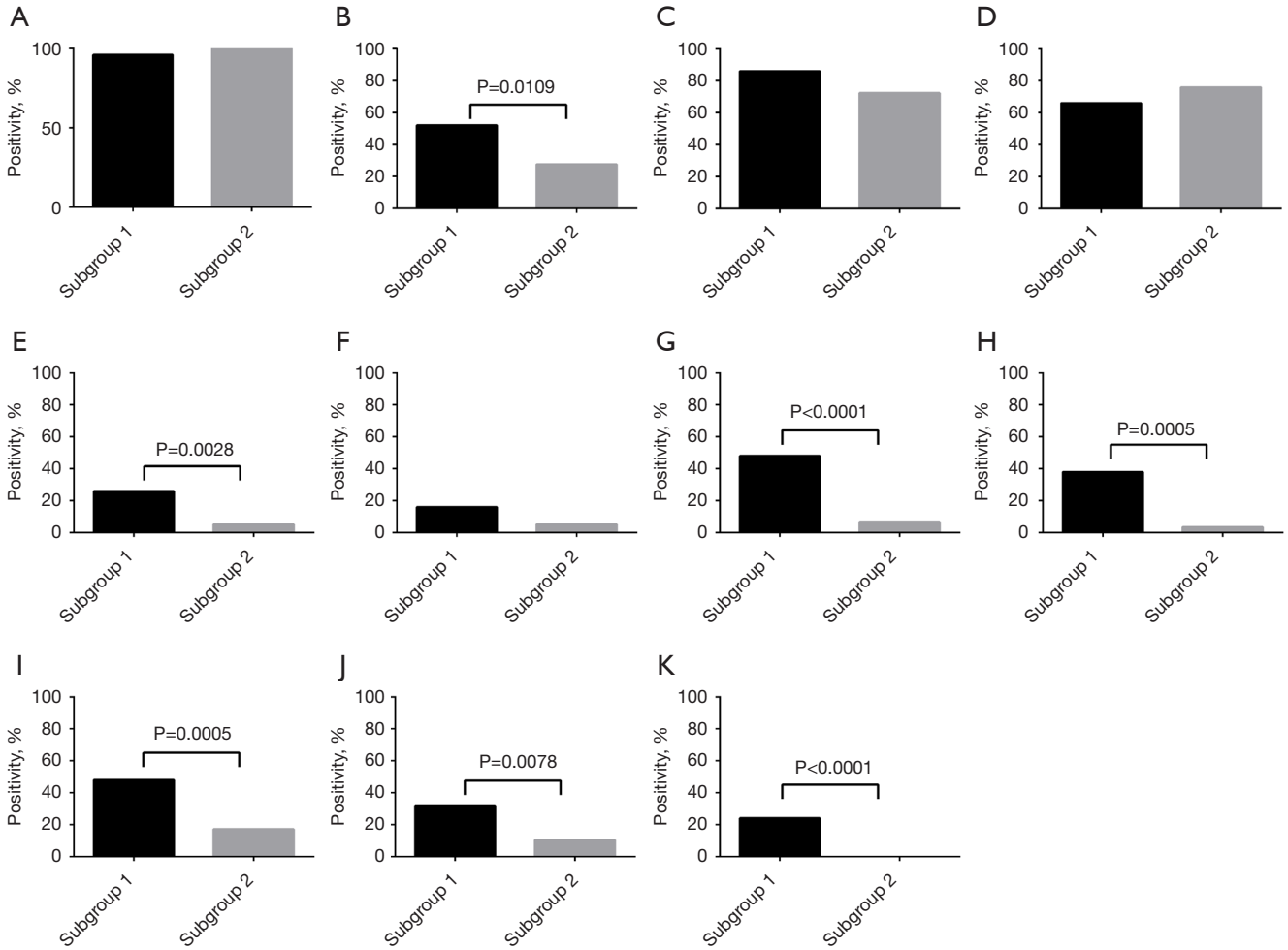

Figure 4 Expression levels of CAF- and EMT-related markers in subgroups 1 and 2. (A) α-SMA. (B) FAP. (C) Tenascin-C. (D) Podoplanin. (E) CD10. (F) PDGFRα. (G) PDGFRβ. (H) FSP1. (I) AEBP1. (J) ZEB1. (K) TWIST1. CAF, cancer-associated fibroblast; EMT, epithelial-mesenchymal transition; α-SMA, α-smooth muscle actin; FAP, fibroblast activation protein; PDGFR, platelet-derived growth factor receptor; FSP1, fibroblast-specific protein 1; AEBP1, adipocyte enhancer-binding protein 1; ZEB1, zinc finger E-box binding homeobox 1; TWIST1, twist homolog 1 gene.

may affect prediction of prognosis in patients with lung SCC. Cancer is a dynamic disease in terms of biological characteristics (17). During disease progression, cancers generally become more heterogeneous (5). As a result, the bulk tumor may contain a diverse collection of cells with distinct molecular signatures and differential levels of sensitivity to treatment, and varying patient outcomes may be observed (5). Thus, our findings suggested that clinical assessment of tumor heterogeneity may facilitate the development of more effective personalized therapies.

Histological type is correlated with prognosis in patients with lung adenocarcinoma (4,8). For example, patient outcomes were improved in papillary/acinar carcinoma with lepidic changes compared with those in solid tumors without lepidic changes (8). However, such histological markers have not yet been used in pathological diagnosis of lung SCC. In the current study, we attempted to establish histological markers predicting clinical outcomes in patients with lung SCC. Analysis of immunohistochemical expression may be a simple approach for examining biological markers using paraffin-embedded tissues, which are widely used in routine pathology diagnosis. We believe that lung SCC may be a suitable model for identifying the expression patterns of TME-related markers.

Table 4 Association of clinicopathological variables and individual marker with disease-free survival in univariate and multivariate analyses

Variable	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	HR	95% CI	P value	HR	95% CI	P value	HR	95% CI	P value	HR	95% CI	P value
p-stage												
II and III *vs.* I	2.315	1.261–4.399	0.0066	2.232	1.163–4.283	0.0158	2.797	1.405–5.939	0.0031	2.479	1.168–5.262	0.0181
ILD												
Positive *vs.* negative	2.500	1.322–4.725	0.0048	2.279	1.158–4.488	0.0171	2.188	1.082–4.423	0.0293	2.078	1.020–4.234	0.0441
AEBP1												
Positive *vs.* negative	2.971	1.617–5.525	0.0005	2.252	1.128–4.494	0.0214	1.933	0.980–3.763	0.0569	–	–	–
CD10												
Positive *vs.* negative	1.242	0.532–2.563	0.5915	–	–	–	1.074	0.402–2.415	0.8754	–	–	–
FAP												
Positive *vs.* negative	2.137	1.165–3.967	0.0144	1.258	0.631–2.510	0.5142	2.278	1.170–4.534	0.0156	1.567	0.772–3.182	0.2137
PDGFR-α												
Positive *vs.* negative	1.844	0.750–3.909	0.1684	–	–	–	1.551	0.527–3.678	0.3899	–	–	–
PDGFR-β												
Positive *vs.* negative	2.184	1.143–4.050	0.0190	1.015	0.428–2.406	0.9723	1.477	0.690–2.976	0.3024	–	–	–
Podoplanin												
Positive *vs.* negative	0.828	0.441–1.646	0.5756	–	–	–	0.937	0.464–2.047	0.8618	–	–	–
FSP1												
Positive *vs.* negative	1.118	0.502–2.244	0.7699	–	–	–	1.183	0.500–2.504	0.6822	–	–	–
α-SMA*												
Positive *vs.* negative	–	–	–	–	–	–	–	–	–	–	–	–
Tenascin-C												
Positive *vs.* negative	2.538	1.094–7.389	0.0284	1.847	0.681–5.009	0.228	3.187	1.137–13.303	0.0250	2.452	0.690–8.710	0.1655
TWIST1												
Positive *vs.* negative	2.551	1.095–5.253	0.0316	1.536	0.536–4.403	0.4245	2.649	1.059–5.784	0.0385	1.641	0.681–3.956	0.2699
ZEB1												
Positive *vs.* negative	1.151	0.535–2.261	0.7029	–	–	–	1.227	0.541–2.528	0.6050	–	–	–

*, could not analyze, why almost cases were positive expression of the marker. HR, hazard ratio; CI, confidence interval; p-stage, pathological stage; ILD, Interstitial lung disease; AEBP1, adipocyte enhancer-binding protein 1; FAP, fibroblast activation protein; PDGFR, platelet-derived growth factor receptor; FSP1, fibroblast-specific protein 1; α-SMA, α-smooth muscle actin; TWIST1, twist homolog 1 gene; ZEB1, Zinc finger E-box binding homeobox 1.

AEBP1 is a ubiquitously expressed, multifunctional protein that is expressed at particularly high levels in pre-adipocytes and macrophages (18). AEBP1 has been implicated in various human malignancies, including glioblastoma, melanoma, gastric cancer, and CRC (18). Recently, AEBP1 was found to be overexpressed in stromal cells of CRC, where it promoted proliferation, migration, invasion, and metastasis by activating nuclear factor-κB signaling (18). Furthermore, Yorozu *et al.* (18) showed that endothelial cells present in the cancer stroma are closely associated with tumor angiogenesis in CRC. In the current study, however, AEBP1 overexpression was shown to be upregulated in CAFs of patients with lung SCC. These results suggested that AEBP1 may have oncogenic effects in not only epithelial tumor cells but also stromal cells. In addition, our current findings implied that AEBP1 may be a therapeutic target in lung SCC, which was further supported by the observation that AEBP1 upregulation conferred acquired resistance to BRAF (V600E) inhibition in melanoma (19).

Few other studies have evaluated the regrading of expression patterns of CAF-related markers in CAFs. In one recent study, the expression of the extensively characterized CAF marker podoplanin in CAFs was shown to be closely associated with the immune microenvironment in early lung SCC (20). Although podoplanin is widely used as a lymphatic epithelial marker (21), podoplanin-positive fibroblasts in cancer have been shown to enhance the invasive properties of carcinoma cells and to play important roles in the remodeling of the ECM (22). In addition, podoplanin expression is significantly associated with survival, suggesting potential differences in prognostic relevance based on NSCLC histological subtype (5). Furthermore, Chen *et al.* (23) revealed that FAP-α expression is involved in microvessel and lymphatic vessel density in lung SCC. FAP is a type II integral membrane serine protease involved in ECM remodeling and tumor cell migration and has been used as a marker of activated fibroblasts in a number of studies (5,24). By contrast, one univariate study demonstrated that increased FAP expression in CAFs was associated with improved survival in NSCLC (25). However, this is the first study to identify the association of expression patterns for an extensive panel of CAF-related markers with prognosis in patients with lung SCC.

In the current study, ILD was closely associated with patient survival, including disease-free and overall survival (26). Our findings were consistent with the shared common risk factors between LC and ILD, including smoking and chemical exposure (26). According to a previous study, ILD, which is detected during surgery, is an important risk factor for lung SCC. In addition, a previous study showed that the histological distribution of ILD in LC is lower than that in adenocarcinoma, but higher than that in SCC (26). We suggest that the close association of ILD plus lung SCC with patient survival may depend on the extent of smoking. In addition, advanced fibrosis occurring in ILD may enhance the invasive ability of CAFs, as is observed in LC.

CAF-induced resistance to chemotherapy and radiotherapy in LC is closely associated with several factors, including cytokines, chemokines, growth factors, and exosomes (27,28). The molecular mechanisms mediating resistance to chemotherapy and radiotherapy have been evaluated (27-30). Cytokines and chemokines are inflammatory mediators secreted by cancer cells or CAFs in the TME and can stimulate tumor-promoting processes, including proliferation, metastasis, and progression, in an autocrine or paracrine manner (27-30). In addition, the cytokines and chemokines in the TME are strongly related to chemoresistance and poor prognosis in patients with cancer (27,28). In lung adenocarcinoma cells *in vivo* and *in vitro*, IL-11 was found to be able to protect cancer cells from cisplatin-induced apoptosis and thus promote their chemoresistance (27-29). As a result, CAFs treated with cisplatin confer chemoresistance to LC cells (27-29). Furthermore, cancer-secreted TGF-β can enhance the transition of resident fibroblasts into CAFs, and CAF-secreted TGF-β is involved in cancer therapy resistance in cancer cells (27-29). Finally, various studies have examined the roles of exosomes in cancer progression (27,30). The function of CAF-derived exosomes in cancer therapy resistance was initially investigated in CRC (27,30). Hu *et al.* (30) reported that CAF-derived exosomes promote drug resistance by mediating the activation of the Wnt signaling pathway in CSCs in CRC. Understanding the molecular mechanisms mediating chemoresistance by CAFs will become even more important in this field.

In the current study, we examined the associations of the CD3/CD8 ratio with subgroups stratified according to CAF-related proteins and with prognosis in patients with lung SCC. However, the CD3/CD8 ratio, which is closely associated with prognosis in patients with LC (31-33), was not correlated with subgroup in patients with lung SCC. This finding suggested that the expression pattern of CAF-related proteins secreted from CAFs is independent of the CD3/CD8 ratio. Furthermore, the current results implied

that the CD3/CD8 ratio may not be associated with poor patient outcomes.

There were some limitations to this study. First, heterogeneous expression of CAF- and EMT-related markers is an important issue for assessment of immunohistochemical results (34). In the current study, we evaluated immunohistochemical expression in invasive areas of tumor samples. Such invasive areas are thought to be suitable for obtaining reproducible results at the invasive area. Second, we selected markers to evaluate in the current study. Thus, subjective results may be expected. In addition, TGF-β, which plays key roles in the EMT, was not examined. However, we selected reliable and reproducible markers to identify the biological characteristics of CAFs (12). For discovery of new CAF-related markers, genome-wide analysis may contribute to comprehensive evaluation of mRNA expression occurring in the cancer stromal tissue. In our experience, isolation of the surrounding stromal cells containing CAFs may enable identification of appropriate mRNA expression that is closely associated with prognosis in patients with lung SCC. However, despite great efforts, the discovery of new CAF-related markers is actually very difficult, even when using genome-wide analysis. Further studies are needed in the near future. Third, a second cohort should be used for validation analyses, and the population size evaluated in this study was small. However, decreases in cigarette smoking in developed countries may result in fewer cases of lung SCC (8). Larger cohorts should be evaluated in the near future. Finally, in the current study, we did not perform analysis of TME-related markers of lung adenocarcinoma. In our future studies, we will plan to examine the association of immunohistochemical expression patterns with prognosis in patients with lung adenocarcinoma.

In conclusion, in this study, we examined the association of immunohistochemical expression patterns of CAF- and EMT-related markers with prognosis in patients with lung SCC. Specific subgroups stratified based on the expression patterns of the examined CAF-related markers may have applications in prediction of prognosis in patients with lung SCC following resection. More importantly, overexpression of AEBP1 was found to be an independent prognostic factor in patients with lung SCC. Overall, our findings suggested that the expression of CAF- and EMT-related markers in CAFs may be helpful for predicting patient survival in lung SCC. In addition, our findings suggested that AEBP1 signaling, particularly in the context of AEBP1 upregulation, could be a useful therapeutic target in lung SCC. Taken together, this study provides insights into the actual roles of CAF-related proteins in determination of outcomes in patients with lung SCC because of our focus on the functional aspects of CAFs present at the invasive area. Additionally, our study takes into account that the expression of specific CAF-related proteins may play important roles in cancer progression via TME formation.

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Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-22-10/rc

Data Sharing Statement: Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-22-10/dss

Peer Review File: Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-22-10/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-22-10/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the local ethics committee of Iwate Medical University (approval No. MH2021-047), and all patients provided informed consent.

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Supplementary

Table S1 Immunohistochemical markers for CAFs and EMT

Antibody	Clone	Source	Dilution	Treatment
α-SMA	1A4	Dako	Ready to use	Heat retrieval (pH 9.0)
FAP	EPR20021	Abcam	1:250	Heat retrieval (pH 9.0)
Tenascin-C	4F10TT	IBL	1:200	Heat retrieval (pH 9.0)
Podoplanin	D2-40	Dako	Ready to use	Heat retrieval (pH 9.0)
CD10	56C6	Dako	Ready to use	Heat retrieval (pH 9.0)
PDGFRα	Poly	CST	1:100	Heat retrieval (pH 9.0)
PDGFRβ	28E	CST	1:50	Heat retrieval (pH 9.0)
FSP1	Poly	Dako	1:400	Heat retrieval (pH 6.0)
AEBP1	Poly	Abcam	1:100	Heat retrieval (pH 6.0)
ZEB1	Poly	Sigma-Aldrich	1:200	Heat retrieval (pH 6.0)
TWIST1	Twist2C1a	Abcam	1:200	Heat retrieval (pH 9.0)

CAF, cancer-associated fibroblast; EMT, epithelial-mesenchymal transition; α-SMA, α-smooth muscle actin; FAP, fibroblast activation protein; PDGFR, platelet-derived growth factor receptor; FSP1, fibroblast-specific protein 1; AEBP1, adipocyte enhancer-binding protein 1; ZEB1, Zinc finger E-box binding homeobox 1; TWIST1, twist homolog 1 gene.

Table S2 Scoring method

Staining intensity	Staining area (%)			
	0 (0%)	1 (1–25%)	2 (26–50%)	3 (51–100%)
0—negative	Score 0	Score 0	Score 0	Score 0
1—weak	Score 0	Score 2	Score 3	Score 4
2—moderate	Score 0	Score 3	Score 4	Score 5
3—strong	Score 0	Score 4	Score 5	Score 6

Score 0–3 points, negative; score 4–6 points, positive.

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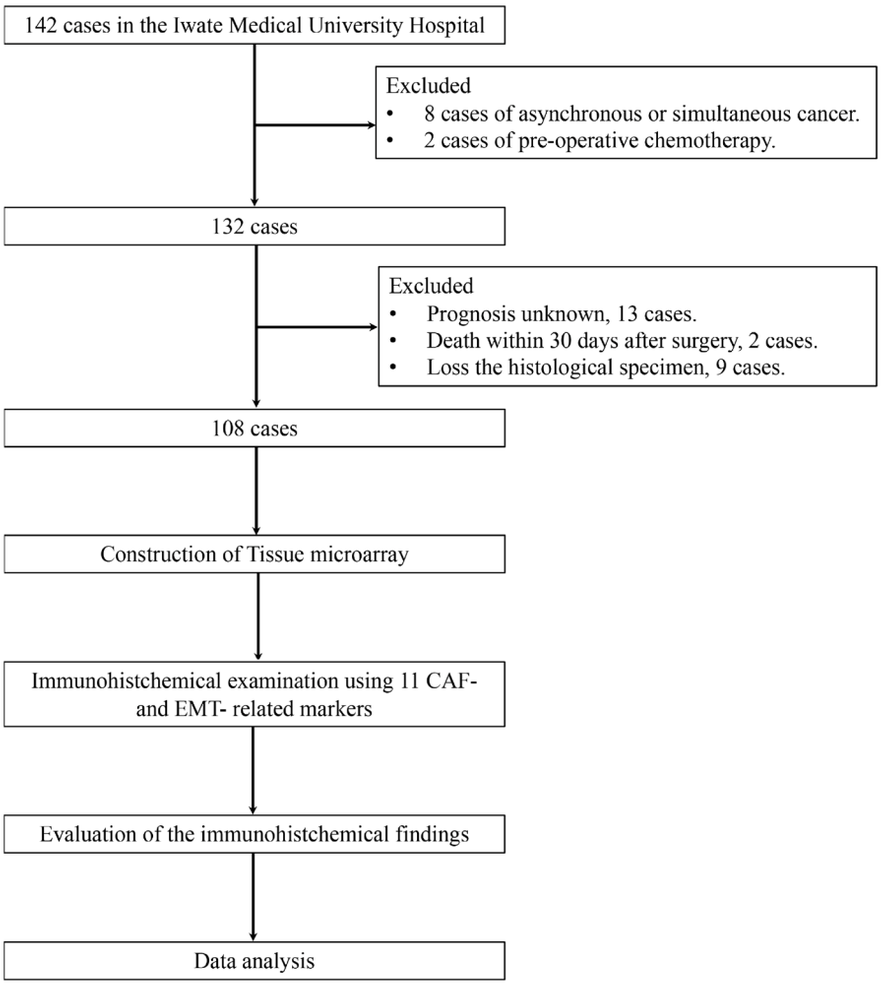

Figure S1 Study design of the present study. CAF, cancer-associated fibroblast; EMT, epithelial-mesenchymal transition.

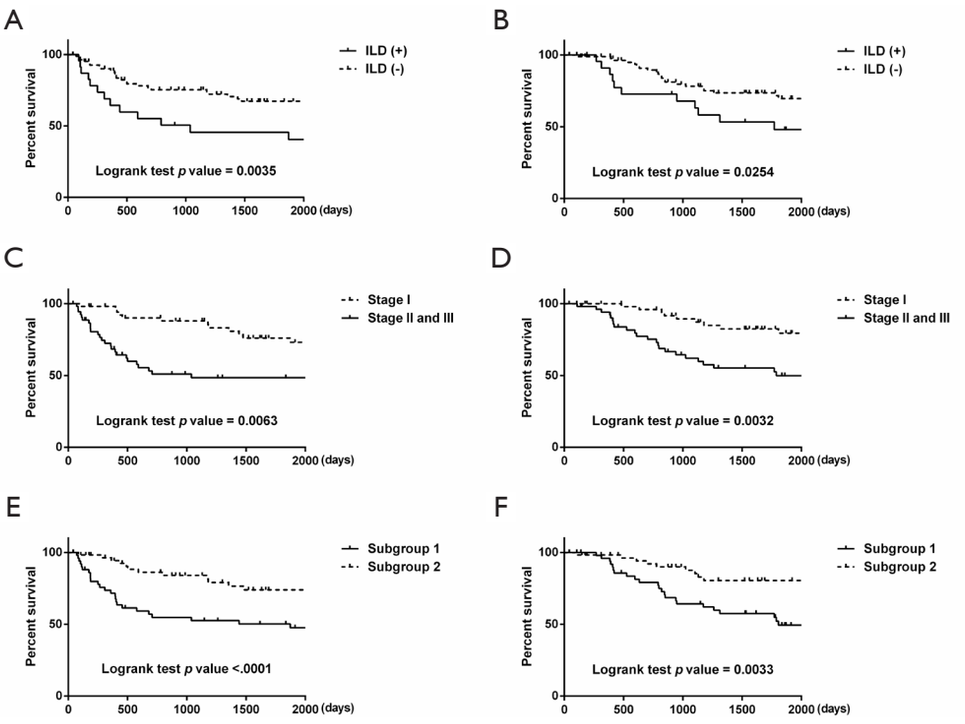

Figure S2 Kaplan-Meier analyses of patient survival. (A) Disease-free survival for ILD. (B) Overall survival for ILD. (C) Disease-free survival for p-stage. (D) Overall survival of p-stage. (E) Disease-free survival for subgroup. (F) Overall survival for subgroup. ILD, interstitial lung disease; p-stage, pathological stage.

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Appendix 1

Tian C, Lu S, Fan Q, *et al.* Prognostic significance of tumor-infiltrating $CD8^+$ or $CD3^+$ T lymphocytes and interleukin-2 expression in radically resected non-small cell lung cancer. Chin Med J (Engl) 2015;128:105-10.

Background: Altered immunoresponse is associated with tumorigenesis and cancer progression. This study assessed the levels of tumor-infiltrating $CD3^+$ or $CD8^+$T lymphocytes and interleukin-2 (IL-2) protein in radically resected non-small cell lung cancer (NSCLC) tissues to predict overall survival (OS) of the patients.

Methods: Paraffin-embedded tissue specimens from 129 NSCLC patients were retrospectively collected for immunostaining of $CD8^+$, $CD3^+$, and IL-2 expression. Clinicopathological and survival data were collected and analyzed using the Chi-squared test, Kaplan–Meier curves, and the log-rank test or the Cox regression model.

Results: The data showed a significant inverse association between $CD8^+$ T lymphocyte levels and IL-2 expression (r = –0.927; P =0.000) and between the levels of $CD8^+$ and $CD3^+$ T lymphocytes (r =–0.722; P =0.000), but a positive association between $CD3^+$ T lymphocyte levels and IL-2 expression (r =0.781; P =0.000) in NSCLC tissues. Furthermore, the levels of $CD3^+$ and $CD8^+$ T lymphocytes and IL-2 expression were associated with tumor stage (P =0.023, 0.006, and 0.031, respectively) and the level of $CD8^+$ T lymphocytes was associated with the patient gender (P =0.024). In addition, the levels of $CD8^+$ T lymphocytes were associated with an unfavorable 5-year OS, whereas patients with high levels of $CD3^+$ T lymphocytes in tumor lesions and IL-2-expressing tumors had significantly better 5-year OS rates than patients with low levels.

Conclusions: The levels of $CD8^+$ T cells in tumor lesions and IL-2 expression were both independent predictors of OS for these NSCLC patients. Thus, the detection of tumor-infiltrating $CD3^+$ or $CD8^+$ T lymphocytes and IL-2 expression could be useful to predict the prognosis of radically resected NSCLC patients.

Immunohistochemistry

Tissue sections were deparaffinized in xylene and rehydrated in a series of ethanol solutions and then subjected to antigen retrieval in a microwave using a middle-to-high power setting for 8 minutes, followed by a low-to-high temperature for 5 minutes, and cooled down to room temperature. A rabbit monoclonal anti-human CD3 or anti-human CD8 (recognizing cytotoxic T cells) antibody, a rabbit polyclonal anti-human IL-2 antibody, and a streptavidin-peroxidase-conjugated secondary antibody were obtained from Zhongshan Goldenbridge Biotechnology Co., Ltd. (Beijing, China). Immunostaining was performed according to the manufacturer's instructions. The tissue sections were then briefly counterstained with hematoxylin and mounted with a coverslip and Permount (Zhongshan Goldenbridge Biotechnology Co., Ltd.). Tissue sections with known positivity from previous experiments were used as a positive control, whereas tissue sections incubated with phosphate-buffered saline to replace the first antibody served as a negative control. The immunostained sections were then assessed by two experienced pathologists (HSM and DHC) without knowledge of patient identification.

To score the immunostaining results, we randomly selected five representative high-power microscopic fields (×400 magnification) of the tumor nest and stroma per section, counted the numbers of positively stained cells, and photographed the sections with a digital camera (Nikon Eclipse 80i; Tokyo, Japan). The mean percentages of stained cells were counted as 0 (negative), 1 (≤10%), 2 (11–50%), 3 (51–80%), and 4 (>80%). Each tissue section was scored semi-quantitatively as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong) staining intensities. Next, we multiplied them to form an immunohistochemical score (H-score) according to a previous study. [13] An H-score of 0–4 was considered as low expression, while a score of 5–12 was considered as a high expression.

Levels of $CD3^+$ and $CD8^+$ T lymphocytes and interleukin-2 expression in non-small cell lung cancer tissue specimens

Interleukin-2 protein and $CD3^+$ and $CD8^+$ T cells were present in the cancer stroma and cancer cell nests [*Figure S1*]. Specifically, 91 of these 129 tumor tissue specimens had

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$CD8^+$ T cells in the cancer nests, with a mean number of 4.65 ± 4.25 $CD8^+$ T cells; while 126 of these 129 specimens showed $CD8^+$ T cells in the cancer stromal tissues, with a mean number of 57.63 ± 23.71. Moreover, 88 of these 129 cases had $CD3^+$ T cells in the cancer nests, with a mean number of 4.95 ± 10.46 $CD3^+$ T cells; and 117 of these 129 cases also showed $CD3^+$ T cells in the cancer stromal tissues, with a mean number of 23.06 ± 21.38. In addition, IL-2 protein was detected in the cancer cells of 122 NSCLC cases and the cancer stromal cells of 79 cases, with mean numbers of 26.08 ± 21.00 and 2.00 ± 2.04, respectively.

The number of infiltrating $CD3^+$ and $CD8^+$ T cells in the cancer stroma was clearly higher than that in the cancer nests (P =0.000), whereas IL-2 protein expression was higher in the cancer nests than in the cancer stroma (P =0.000). There was a significant inverse association (r =–0.927; P =0.000) between the number of $CD8^+$ T cells and IL-2 protein expression in NSCLC tissues and between the numbers of $CD8^+$ T cells and $CD3^+$ T cells (r =–0.722; P =0.000), whereas there was a positive association between the number of $CD3^+$ T cells and IL-2 protein expression (r =0.781; P =0.000) in NSCLC tissue specimens.

Association of $CD3^+$ and $CD8^+$ T cell levels and interleukin-2 expression with clinicopathological variables from non-small cell lung cancer patients

Next, we associated these parameters with the clinicopathological data and found a significant association between the numbers of $CD3^+$ and $CD8^+$ T cells and the level of IL-2 expression with tumor stage (P =0.023, 0.006, and 0.031, respectively). There was a significant association between the number of $CD8^+$T cells and the patient gender (P =0.024). However, there was no association between the numbers of $CD3^+$ and $CD8^+$ T cells or the level of IL-2 expression and gender, age, tumor stage, lymph node or distant metastasis, tobacco smoking, or tumor histology [*Table S1*].

Association of $CD3^+$ and $CD8^+$ T cell levels and interleukin-2 expression with survival of these non-small cell lung cancer patients

The survival data from each patient were collected and stratified based on the $CD3^+$ and $CD8^+$ T cell numbers and IL-2 expression levels [*Table S2*]. We found that tumor histology was a prognostic factor for these patients (the 5-year OS rates of patients with SCC, AC, or other histological subtypes were 34.4%, 45.8%, and 20.0%, respectively; P =0.009). The same was true for the tumor pathological stage (P =0.00001). However, the patient gender, age, and tobacco smoking status as well as adjuvant chemotherapy treatment had no statistically significant impact on the OS ($P > 0.05$).

Association of $CD3^+$ and $CD8^+$ T cell levels and interleukin-2 expression with survival of these non-small cell lung cancer patients

The survival data from each patient were collected and stratified based on the $CD3^+$ and $CD8^+$ T cell numbers and IL-2 expression levels [*Table S2*]. We found that tumor histology was a prognostic factor for these patients (the 5-year OS rates of patients with SCC, AC, or other histological subtypes were 34.4%, 45.8%, and 20.0%, respectively; P =0.009). The same was true for the tumor pathological stage (P =0.00001). However, the patient gender, age, and tobacco smoking status as well as adjuvant chemotherapy treatment had no statistically significant impact on the OS ($P > 0.05$).

The multivariate analyses showed that tumor histology (P =0.000), tumor stage (P =0.000), TNM (P =0.042), number of $CD8^+$ T cells in the tumor lesions (P =0.002), and IL-2 expression levels (P =0.021) were all independent predictors of OS [*Table S2*].

Discussion

A previous study has demonstrated that TILs contain significantly higher levels of $CD8^+$ and $CD3^+$ T cells compared to those of peripheral blood. [14] In this study, we found that IL-2 protein was expressed in tumor and cancer stromal cells and that $CD3^+$ T cells and $CD8^+$ T cells were present in cancer stromal tissue and in the cancer nest. The number of $CD3^+$ and $CD8^+$ T cells and IL-2 expression were associated with the NSCLC stage, and the patients with high levels of $CD3^+$ T cells in the tumor lesion or with an IL-2-expressing tumor had a significantly better 5-year OS. In contrast, high levels of $CD8^+$ T cells were associated with an unfavorable prognosis.

$CD8^+$ T cells can recognize tumor-associated antigens as major histocompatibility complex (MHC) class I molecules on the cancer cell surface and can directly lyse cancer cells. Thus, the presence of tumor-infiltrating $CD8^+$T cells is considered as a host immunoreaction against a tumor and is associated with a better prognosis in a variety of cancers. [15] However, our current study showed that high levels of $CD8^+$ T cells in the tumor lesion were associated with a poor

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prognosis. These data contradict our current knowledge, and the reason is unclear. One possible explanation may be that some immune cells can induce immune tolerance and even promote tumor growth and metastasis, i.e. $CD8^+$ T cells *per se* might have diverse functions in a tumor microenvironment; [16] these cells could lose their antitumor activity after interacting with other B or T lymphocytes through several mechanisms, e.g. escape of immune surveillance due to secretion of immunosuppressive factors (IL-10 and transforming growth factor-β), lack of adequate T-cell costimulation, or downregulation of cell-surface MHC Class II protein expression (immunoediting). [17] A previous study has shown that nonclassical HLA-G is involved in immune escape mechanisms and could be one of the most powerful molecules for suppression of the innate and/or adaptive immune response in lung and other cancers. [18] Another possibility may be that $CD8^+$ T cells in the tumor nest are associated with survival, [19] whereas $CD8^+$ T cells in the tumor stroma are inversely associated with survival. In this study, the number of $CD8^+$ T cells was significantly higher in the stroma than inside the cancer nest. An additional possibility may be that tumor-infiltrating $CD8^+$ T cells contain high levels of T-regs in addition to cytotoxic T cells. However, further studies are needed to clarify and confirm our current data.

Furthermore, our current study demonstrated that the levels of $CD8^+$ T cells in the tumor microenvironment were associated with the pT stage, suggesting that $CD8^+$ T cells are more abundant in the tumor stroma with high cellular growth rates and malignancy potential. These $CD8^+$ T cells are anergic but cannot lyse tumor cells. Trojan *et al.* [20] found that $CD8^+$ T cells in the tumor cell nest were inadequately activated and incapable of mounting an antitumor immune effect. Tumor immunology is a very complicated field of research, and a great number of factors affect, interrupt, or interact with the favorable immune activity against tumor cells.

A high number of $CD3^+$ T cells have been associated with increased apoptosis in patients with NSCLC. [21] Al-Shibli *et al.* [22] also have reported that an increasing number of stromal and cancerous $CD3^+$ T cells were associated with a better disease-specific survival and that a high stromal density of $CD3^+$ T cells was an independent indicator for survival in patients with NSCLC. Our current univariate analysis showed a significant correlation of $CD3^+$ T cell levels with better OS of NSCLC patients, but the Cox multivariate model did not confirm the data. We speculate that mature T cells in the tumor microenvironment have an important role in tumor recurrence but might be affected by the ratio and composition of different T cell subtypes. Similar to $CD8^+$ T cells, our current data showed that the presence of $CD3^+$ T cells within the tumor microenvironment was positively associated with the pT stage; however, some $CD4^+$ $CD25^+$ Treg cells are included in these TILs, [23] which could suppress immune function. CD3 staining alone cannot identify these T-regs. This may contribute to a lack of association of $CD3^+$ T cell levels in a tumor tissue with prognosis.

In addition, IL-2 can activate T cells, NK cells, mononuclear macrophages, and marrow B cells to participate in killing tumor cells. However, activated T cells express the IL-2 receptor for IL-2 binding, leading to cell proliferation. A previous study has shown that IL-2 protein is expressed and detected in all types of lung tumor cells, [24] including atypical carcinoids, and is inversely associated with the proliferative activity of these tumor cells. An impaired immune defense or suppression of cytokine secretion capacity in cancer patients may have clinical relevance and influence patient survival. In addition, suppression of IL-2 secretion has been significantly associated with reduced survival of NSCLC. [25] The expression of different cytokines in tumor lesions may be a better predictor for prognosis and reflect antitumor immunity. Indeed, our current study showed that IL-2 was an independent prognostic parameter. An inverse correlation of $CD8^+$ T cells with IL-2 expression and $CD3^+$ T cells as well as the association of $CD3^+$ T cells and IL-2 expression showed the double-edged sword nature of immune factors as well as the complex relationship between them.

However, our current study was just a proof-of-principle study, and much more research is needed because antitumor immunology is very complex and a great number of factors and cells are involved. Future studies will precisely identify the subtypes of lymphocytes in tumor lesions and assess the expression of cytokines and chemokines in NSCLC tissues to better understand their role in NSCLC and develop some of them as biomarkers to predict the prognosis or treatment responses.

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