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.

https://dx.doi.org/10.21037/tlcr-22-10

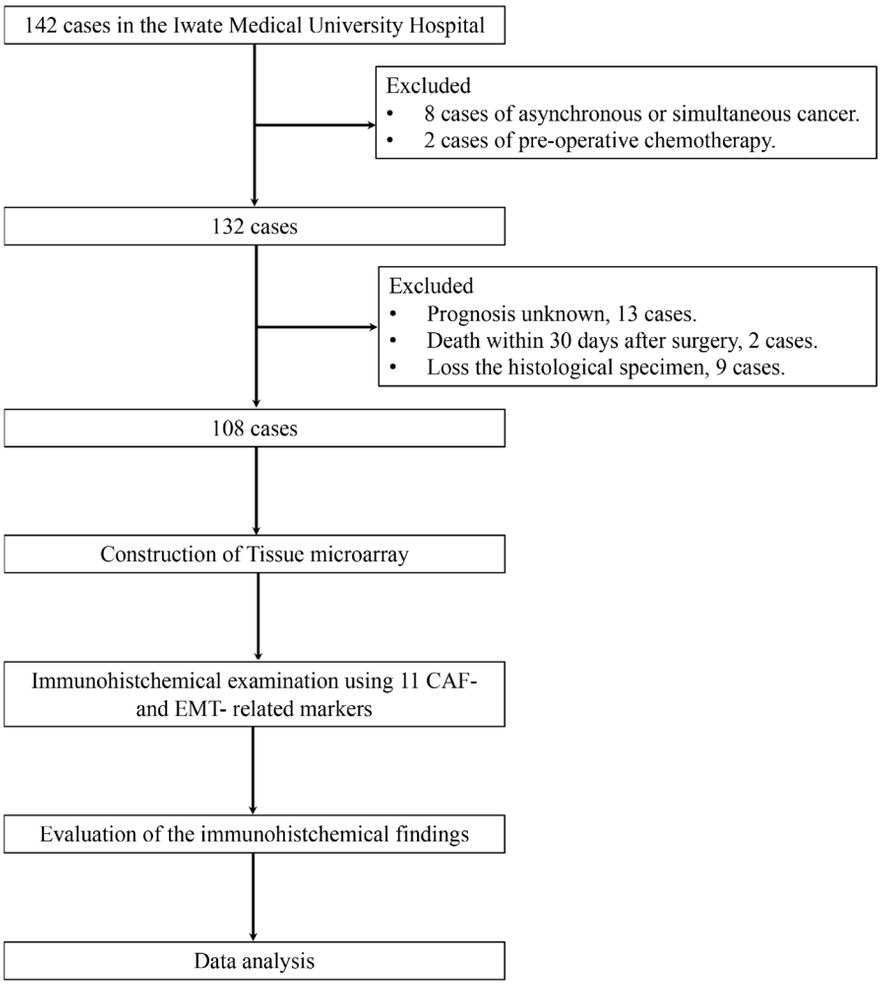

Figure S1 Study design of the present study. CAF, cancer-associated fibroblast; EMT, epithelial-mesenchymal transition.

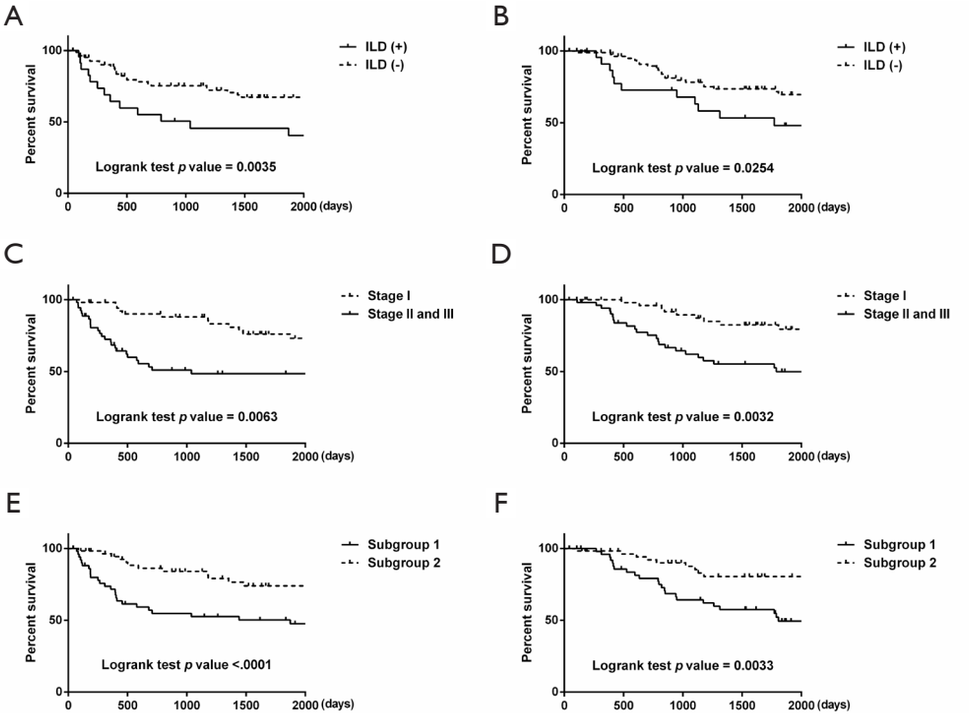

Figure S2 Kaplan-Meier analyses of patient survival. (A) Disease-free survival for ILD. (B) Overall survival for ILD. (C) Disease-free survival for p-stage. (D) Overall survival of p-stage. (E) Disease-free survival for subgroup. (F) Overall survival for subgroup. ILD, interstitial lung disease; p-stage,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

https://dx.doi.org/10.21037/tlcr-22-10

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

https://dx.doi.org/10.21037/tlcr-22-10

$CD8^+$ 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

https://dx.doi.org/10.21037/tlcr-22-10

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.

https://dx.doi.org/10.21037/tlcr-22-10