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Original Article

Infiltration of CD8+ FOXP3+ T cells, CD8+ T cells, and FOXP3+ T cells in non-small cell lung cancer microenvironment

Jianqing Hao[1], Helin Wang[1,5], Lai Song[4], Shuping Li[2], Nanying Che[3], Shucai Zhang[1], Hongtao Zhang[2], Jinghui Wang[1]

Departments of [1]Medical Oncology, [2]Central Laboratory, [3]Pathology, Beijing Chest Hospital, Capital Medical University, Beijing Tuberculosis and Thoracic Tumor Research Institute, Beijing, China; [4]Beijing DCTY Bioinformation Technology Co., Beijing, China; [5]Department of Oncology, First Affiliated Hospital of Xinxiang Medical University, Xinxiang, Henan, China

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Abstract: Background: Studies about CD8+ FOXP3+ T cells as a subtype of regulatory T cells (Treg cells) in non-small cell lung cancer (NSCLC) are few. Associations among the clinicopathologic factors of NSCLC and tumor-infiltrating lymphocytes (TILs) such as CD8+ FOXP3+ T cells, CD8+ T cells, FOXP3+ T cells and tumor PD-L1 expression are unclear. Methods: We retrospectively enrolled 192 patients who underwent resections for NSCLC. We used tissue microarrays (TMA) with multiplex immunofluorescence and immunohistochemistry staining to evaluate the expression of CD8, FOXP3, cytokeratin, DAPI and PD-L1. We then used Wilcoxon test, Kaplan-Meier method, and Cox hazard proportion model to analyze their relationships with clinicopathologic factors and prognosis. Results: Density of tumor CD8+ FOXP3+ T cells was significant by univariate analysis, and positively associated with tumor CD8+ T cells and FOXP3+ T cells. Density of tumor CD8+ T cells was higher in lung adenocarcinoma (LUAD) than squamous cell carcinoma (LUSC), and was an independent prognostic factor for NSCLC. The density of tumor FOXP3+ T cells decreased with tumor size. Tumor PD-L1 expression was higher in LUSC than LUAD. Cox hazard proportion model analysis correlated being younger than 65 years, early TNM stage, early T stage, high tumor CD8+ T cell density, and adjuvant chemotherapy with longer overall survival. Conclusion: Infiltration of CD8+ FOXP3+ T cells, CD8+ T cells, and FOXP3+ T cells is important in non-small cell lung cancer microenvironment, and needs to be investigated more.

Keywords: Lung cancer, CD8+ FOXP3+ T cells, PD-L1, multiplex immunofluorescence staining

Introduction

Lung cancer accounts for almost one-fifth of all cancer deaths worldwide [1]. In the past decade, conventional treatments, such as resection, platinum-based chemotherapy, radiotherapy, and targeted therapy have significantly improved the prognosis of non-small cell lung cancer (NSCLC), with oncogenic driver mutations, such as EGFR, ALK, and ROS1. More targeted drugs have been developed, including BRAF, MET, RET, HER-2, and NTRK [2]. However, only a portion of patients has these driver mutations, which are inevitably accompanied by acquired resistance. Immunotherapy based on immune checkpoints, such as programmed cell death 1 (PD-1) and programmed cell death ligand 1 (PD-L1), have recently offered a new approach to lung cancer treatment, and have led to longer overall survival (OS) for some NSCLC patients, especially those without sensitive oncogenic driver genes. Immune checkpoint inhibitors (ICIs), including pembrolizumab, nivolumab, atezolizumab, and durvolumab had been approved by the United States FDA to treat lung cancer [3-11]. However, only about 20% of NSCLC patients benefit from ICIs, and new immune biomarkers need to be discovered for the insufficient predictive roles by PD-L1 expression and tumor mutation burden in clinical practice [12].

The PD-1/PD-L1 pathway accounts for less than 40% of immune-escape mechanisms in

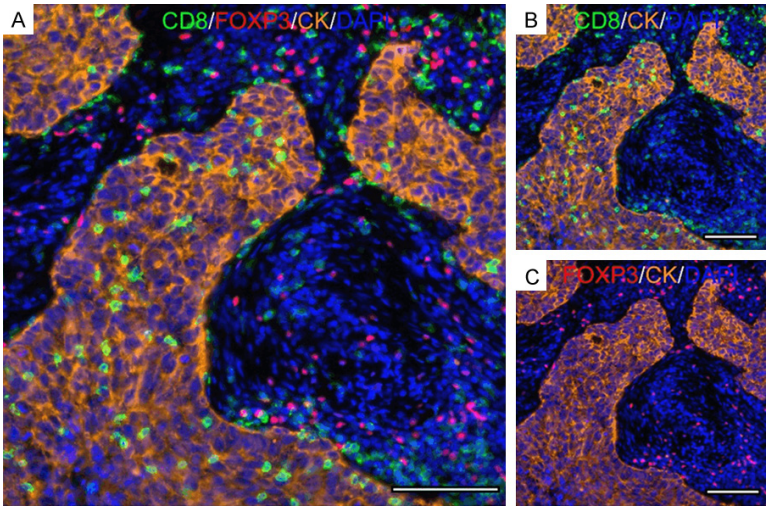

Figure 1. CD8, and FOXP3 expression in non-small cell lung cancer by multiplex immunofluorescence staining. Tumor compartments and stromal compartments divided by CK. CD8 expression (green), FOXP3 expression (red), CK expression (Orange), DAPI (blue). A. A merged picture of expression of CD8, FOXP3, CK and DAPI. B. CD8 expression (green). C. FOXP3 expression (red) (×200).

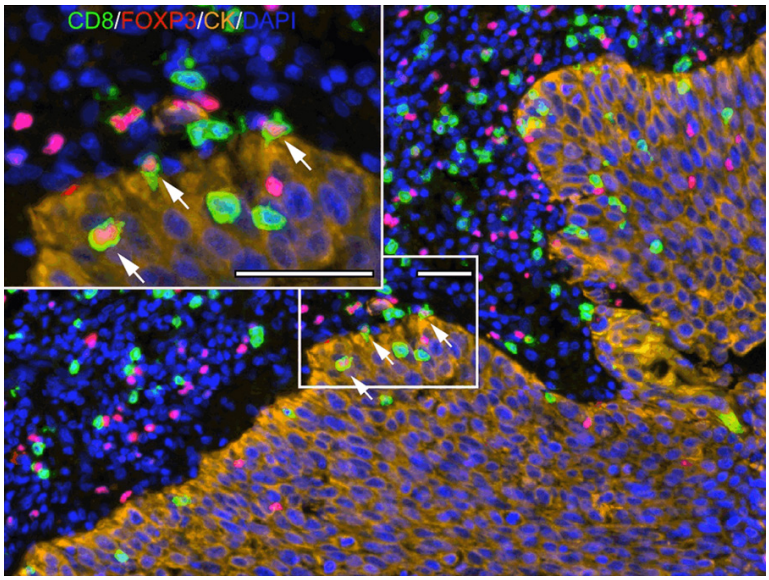

Figure 2. CD8+ FOXP3+ T cells by multiplex immunofluorescence staining. The cell indicated by the arrows is CD8+ FOXP3+ cells (green membrane and red nuclei). CD8+ T cells in green, FOXP3+ T cells in red (×200).

cancers [13, 14]. Moreover, the tumor microenvironment (TME), which includes tumor cells, tumor-infiltrating lymphocytes (TILs), vessel cells, and fibroblasts, has an important role in immunotherapy [15]. FOXP3, as a special transcription factor, is specifically overexpressed in regulatory T cells (Treg cells), and is used to identify Treg cells, which are mainly CD4+ FOXP3+ T cells. Treg cells are generally thought to be negatively immune-regulated cells, but their significance in NSCLC prognosis is unclear [16, 17]. The concept of CD8+ immunosuppressive T cells was proposed by Gershon and Condo in 1971 [18, 19]. The CD8+ FOXP3+ Treg subtype accounts for a small percentage of Treg cells. Chaput et al. further showed that freshly isolated CD8+ Treg cells from colorectal cancers have strong regulatory cell functions [20]. Although CD8+ FOXP3+ T cells from NSCLC are less widely studied, their significance should not be ignored [21-23]. Reportedly, CD8+ T cells, FOXP3+ T cells are important TILs with variable anti-tumor properties. Although lung cancers with high CD8+ T cell density are thought to have a better prognosis [15], there are still reports with no statistical significance.

Associations among CD8+ FOXP3+ T cells, CD8+ T cells and FOXP3+ T cells with NSCLC clinicopathologic factors and PD-L1 expression remain unclear, and their impact on prognosis is controversial. Our study investigated the significance of these TILs and PD-L1 expression on clinicopathologic factors and prognosis in patients with NSCLC.

Materials and methods

Patient cohort

We retrospectively included patients who were treated at Beijing Chest Hospital from 2013 to 2016, with (a) newly diagnosed primary NSCLC histology, (b) no therapy before surgery, and (c) adequate follow-up information. We excluded patients (a) for whom we did not have enough tissue enough tissue for testing, (b) who had other malignant tumors. Staging was per-

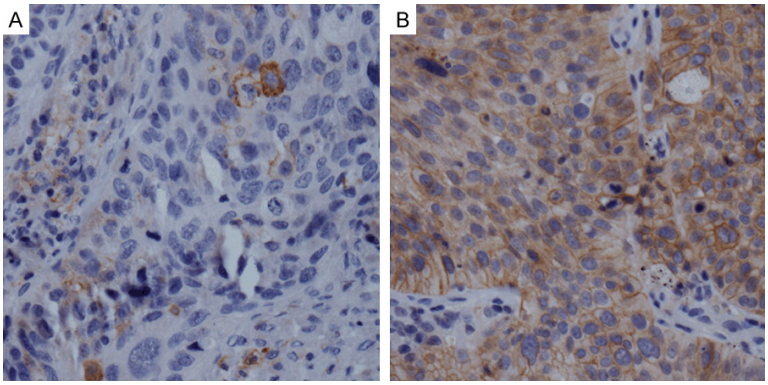

Figure 3. PD-L1 expression in tumors. A. Negative PD-L1 expression in non-small cell lung cancer (< 50%). B. Positive PD-L1 expression in non-small cell lung cancer (≥ 50%) (×200).

formed according to the AJCC guidelines (8th edition) [24]. NSCLC was classified according to subtype using the WHO Guidelines [25]. This study was approved by the Ethics Committee of Beijing Chest Hospital.

Multiplex immunofluorescence staining

Antibodies, including those against CD8, FOXP3, CK, and DAPI were stained using Opal 7-color reagents (PerkinElmer, USA) on 4-μm-thick tumor TMA sections from continual cuts of 2-mm cores in each tumor, using a method described in our previous work [26]. As the immunohistochemistry (IHC) assay, TMA sections were dewaxed and rehydrated. Antigen retrieval was performed with citrate antigen retrieval solution, which was brought to a boiling point at 100% power first and 15 min at 20% power in addition in a microwave oven. After being blocked with blocking buffer for 25 min, slides were firstly incubated with the primary antibody for 2 hours at room temperature (RT), and then incubated with the secondary antibody (Opal Polymer HRP Ms + Rb, USA) for another 30 min at RT, and washed. To generate the Opal signal, the slides were incubated with the Opal working solution for 10 min at RT. Before the next marker staining, sections were treated by microwave oven, and the above steps repeated. Finally, slides were stained with DAPI for 5 min, washed once by TBST for 10 min, and cover-slipped [27]. Primary antibodies were CK (1st cycle; clone PAN-CK (Cocktail); dilution 1:1000; Abcam, Science Park in Cambridge, UK), CD8 (2nd cycle; clone EP334; dilution 1:600; ZSBIO, Beijing, China), and FOXP3 (3rd cycle; clone 236A/E7; dilution 1:600; Abcam).

Imaging and data analysis of multiplex immunofluorescence staining

The stained slides were scanned by Vectra multispectral microscope (Perkin Elmer). Fluorescence spectroscopy of continuous, single-color-stained tissue sections under the same shooting conditions was processed by Inform software 2.2.0 (Perkin Elmer) to remove autofluorescence and analyze fluorescence for the multi-color immunofluorescence staining slides. The images were analyzed in two compartments (defined by CK staining)-tumor and stromal-which were differentiated by applying the Inform software tissue segmentation tool. Trained pathologists identified the cells, and the tumor and stromal compartments. Individual cells (defined by DAPI staining) were identified by the cell segmentation tool. Data of $CD8^+$ $FOXP3^+$ T cells, $CD8^+$ T cells, and $FOXP3^+$ T cells in each TMA core (including tumor/stroma data) were collected. In each tumor, every marker expression was analyzed by density, which was calculated by percentage of positive cells among total tumor or stromal cells (**Figures 1**, **2**).

PD-L1 immunohistochemistry (IHC)

Expression of PD-L1 (clone 22C3, Dako North America, Inc.) was tested on consecutive TMA sections, using an AutostainerLink-48 machine (Dako North America, Inc.) combined with Dako En Vision™ FLEX+ and DAB Enhancer solution, according to the manufacturers' instructions. PD-L1 expression in ≥ 50% of tumor cells was regarded as $PD\text{-}L1^+$ and expression in < 50% of tumor cells was PD-L1. PD-L1 expression was confirmed by two trained pathologists (**Figure 3**).

Statistical analysis

We used the Wilcoxon test for non-normal distributions of measured data and categorical variables; Spearman's rank correlation test for quantitative data; Kaplan-Meier method for OS;

Table 1. Patients' characteristics

	Number	%
Age (years)		
Range	35-84	
Median	62	
< 65	118	61.5
≥ 65	74	38.5
Gender		
Male	135	70.3
Female	57	29.7
Smoking status		
Current smoking	115	59.9
Non-smoking	77	40.1
TNM Stage		
I	46	28.0
II	14	8.5
III	87	53.0
IV	17	10.4
T staging		
T1	59	20.7
T2	78	40.6
T3	33	17.2
T4	22	11.5
N staging		
N0	101	52.6
N1	22	11.5
N2	69	35.9
Histology		
Squamous	83	43.2
Adenocarcinoma	109	56.8
EGFR status		
EGFR mutation	27	44.3
EGFR wild type	34	55.7
Adjuvant chemotherapy		
Yes	111	57.8
No	81	42.2

log-rank test for intergroup comparison; and Cox hazard proportion model for multivariate analyses. $P < 0.05$ (two-sided) was considered significant. Data were analyzed in SPSS software v21.0, R software v3.6.0, and GraphPad Prism v8.0.

Results

Patients

We enrolled 192 patients with NSCLC. Their median age was 62 years old (range: 35-84 years); 74 (38.5%) were ≥ 65 years old; 135 (70.3%) were men and 57 (29.7%) were women. Of the 192 patients, 115 (59.9%) were smokers. Their stages were stage I: n = 46 (28%), stage II: n = 14 (8.5%), stage III: n = 87 (53%), and stage IV: n = 17 (10.4%). Other tumor characteristics were T1: n = 59 (20.7%), T2: n = 78 (40.6%), T3: n = 33 (17.2%) and T4: n = 22 (11.5%); N0: n = 101 (52.6%), N1: n = 22 (11.5%), and N2: n = 69 (35.9%); lung squamous cell carcinoma (LUSC): n = 83 (43.2%) and lung adenocarcinoma (LUAD): n = 109 (56.80%); EGFR mutation: n = 27 (44.3%), and EGFR wild type: n = 34 (55.7%). Of the 192 patients, 111 (57.8%) patients received postoperative chemotherapy and 81 (42.2%) did not (**Table 1**).

Significance of CD8+ FOXP3+ T cells, CD8+ T cells, FOXP3+ T cells and PD-L1 expression with clinicopathologic characteristics

Density of CD8+ FOXP3+ T cells increased with age ($P = 0.001$). Density of tumor CD8+ T cells was significantly higher in LUAD than that in LUCC ($P = 0.006$), and increased with age ($P = 0.030$). Density of tumor FOXP3+ T cells increased with age ($P = 0.012$), and decreased significantly with T stage ($P = 0.018$). Tumor PD-L1 expression was significantly higher in LUSC than LUAD ($P = 0.002$) (**Figure 4**; Supplementary Table 1).

The correlation of density of CD8+ FOXP3+ T cells, CD8+ T cells, FOXP3+ T cells, and PD-L1 expression

Density of tumor CD8+ FOXP3+ T cells was positively correlated with tumor CD8+ T cells ($r = 0.606$, $P < 0.001$) and tumor FOXP3+ T cells ($r = 0.604$, $P < 0.001$). Density of tumor CD8+ T cells also positively correlated with tumor FOXP3+ T cells ($r = 0.509$, $P < 0.001$) (Supplementary Table 2).

Prognosis

The last follow-up date for all patients was June 30st, 2019. At that time, 120 patients had died and 72 patients were alive. We calculated OS from the date of surgery to the date of death. We analyzed associations between patients' outcomes and clinicopathological characteristics (age, sex, smoking, TNM stage, T stage, N stage, histology, EGFR status),

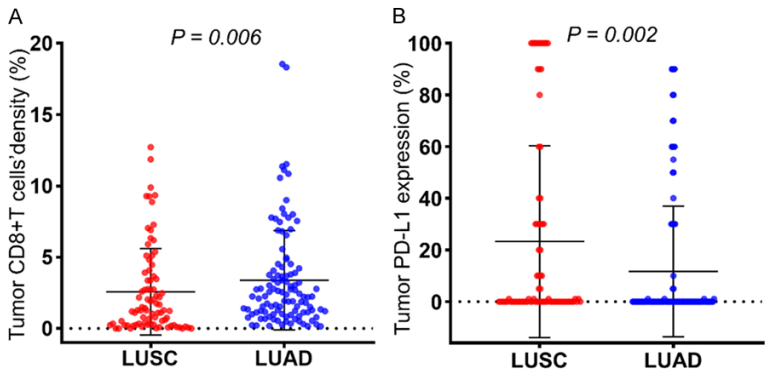

Figure 4. Expression of CD8 and PD-L1 with histologic characteristics and their correlation. A. Tumor CD8+ T cells' density between lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC). B. Tumor expression of PD-L1 between LUAD and LUSC (×200).

immune markers (tumor CD8+ FOXP3+, CD8+, and FOXP3+ T cell density and PD-L1 expression), and adjuvant chemotherapy. We used 50% for the tumor PD-L1 expression cut-off, and minimum *P*-value for the cut-offs of other immune markers. Univariate analysis showed that age < 65 years old ($P = 0.046$), early TNM stage ($P < 0.001$), early T stage ($P < 0.001$), early N stage ($P < 0.001$), LUAD ($P = 0.001$), low tumor expression of PD-L1 ($P = 0.015$), low tumor density of CD8+ FOXP3+ T cells ($P = 0.034$), high tumor density of CD8+ T cells ($P = 0.010$), and receiving adjuvant chemotherapy ($P = 0.014$) were associated with better OS compared with the absence of these factors. Factors that were significant in univariate analysis were entered into multivariate analyses (Cox hazard model), which showed that age < 65 years old (HR: 0.549, 95% CI: 0.367-0.822, $P = 0.004$), early TNM stage (HR: 0.452, 95% CI: 0.225-0.907, $P = 0.025$), early T stage (HR: 0.387, 95% CI: 0.248-0.605, $P < 0.001$), high tumor density of CD8+ T cells (HR: 0.565, 95% CI: 0.355-0.900, $P = 0.016$), and adjuvant chemotherapy (HR: 0.447, 95% CI: 0.299-0.668, $P < 0.001$) were independent predictors of better OS (**Figures 5**, **6**; Supplementary Table 3).

Discussion

Whereas previous studies had not considered CD8+ FOXP3+ T cells separately from CD8+ and FOXP3+ T cells in this setting, this study evaluated numbers of CD8+ FOXP3+ T cells apart from other Treg cells. As CD8+ T cells include only CD8+ FOXP3- T cells, and FOXP3+ T cells include only FOXP3+ CD8- T cells, this method is more reliable.

Some studies have indicated that CD8+ FOXP3+ T cells may contribute to immune escape by tumors, and disease progression in colorectal and prostate cancer [20, 21]. However, few studies have addressed infiltration by CD8+ Treg cells in NSCLC. In our study, longer OS was associated with low tumor CD8+ FOXP3+ T cell density in univariate analysis ($P = 0.034$), and tended to be associated, but not significantly so, in multivariate analysis ($P = 0.074$). This suggests that CD8+ FOXP3+ T cells may have a critical role in NSCLC.

CD8+ T cells play an anti-tumor role in many cancer types, and have been associated with better prognosis in colorectal cancer, breast cancer, head and neck cancer, and NSCLC [15]. In our study, density of tumor CD8+ T cells was significantly higher in LUAD specimens than LUSC, which corresponds with our finding that associated LUAD with better OS in univariate analysis ($P = 0.001$), and marginally so in multivariate analysis ($P = 0.052$).

FOXP3+ T cells are also a very important component among TILs, and which represents a majority of regulatory T cells that inhibit the function of CD8+ T cells [15]. However, the prognostic and clinicopathologic value of FOXP3+ T cell infiltration in NSCLC remains undetermined [28, 29]. In our study, we found that tumor FOXP3+ T cell density was associated with early T stage, but saw no association between FOXP3+ T cell tumor density and OS.

PD-L1 expression is an important predictor of response to ICIs [7, 30]. In our study, PD-L1 expression was significantly higher in LUSC than LUAD, that was similar to some studies [31, 32]. Patients with lung squamous cell carcinoma responded well to immunotherapy [10], and could make up for the limits of targeted therapy in LUSC with few oncogenic driver mutations. We found that low tumor PD-L1 expression was associated with better OS in univariate analysis ($P = 0.015$), but only margin-

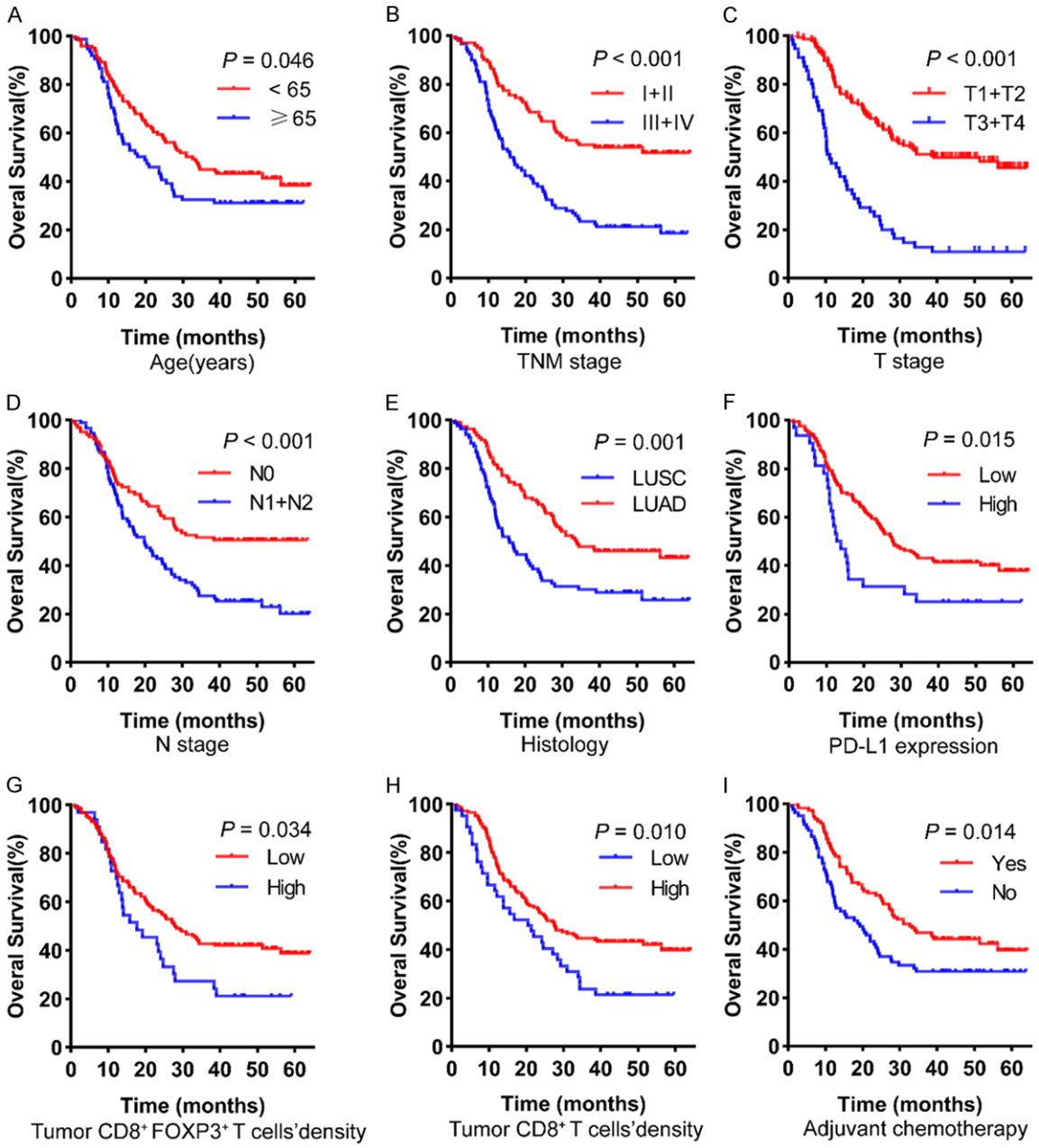

Figure 5. Overall survival curves of patients using Kaplan-Meier. A. According to age. B. According to TNM stage. C. According to T stage. D. According to N stage. E. According to histology. F. According to PD-L1 expression level. G. According to the density of $CD8^+$ $FOXP3^+$ T cells in tumor. H. According to the density of $CD8^+$ T cells in tumor. I. According to adjuvant chemotherapy.

ally significant in multivariate analysis (P = 0.062), which was similar to other studies [33].

Our study had some limitations. First, this was a retrospective study that focuses on resected NSCLC with follow-up data, with some possible bias. Second, patients in this study had no history of immunotherapy, so predictive roles of these markers for ICIs cannot be confirmed. Third, TMA sections might not be representative of whole tumors. In addition, relatively few of the patients were tested for oncogenic driver mutations. Tumors harboring EGFR and ALK alterations are regarded as resistant to ICIs and TME of different oncogenic alterations are needed further investigated.

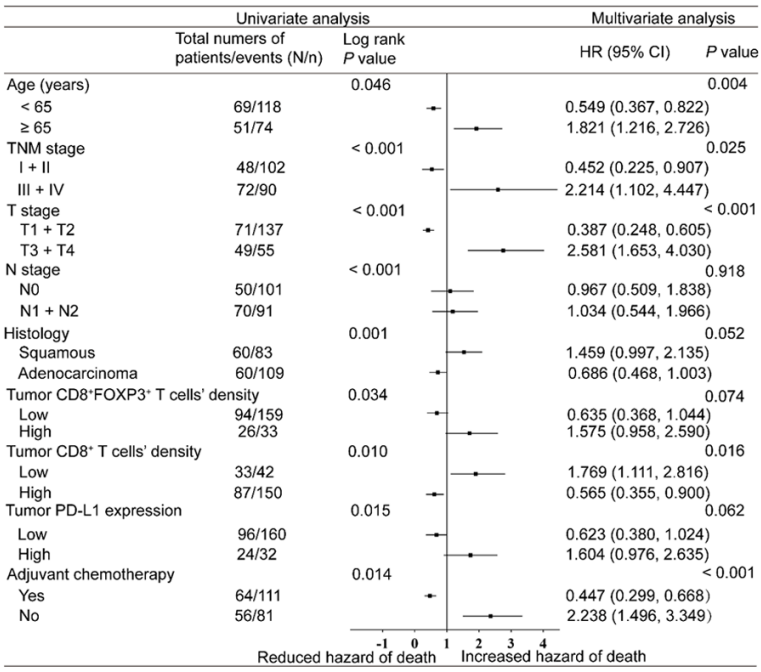

Figure 6. Subgroup analyses of overall survival among 192 patients with resected non-small cell lung cancer by univariate and multivariate analysis.

In conclusion, tumor CD8+ FOXP3+ T cells are probably a negative prognostic factor in NSCLC. Further large studies regarding CD8+ FOXP3+ T cells, CD8+ T cells, and FOXP3+ T cells in NSCLC should be performed to validate our findings. Multiplex immunofluorescence staining is a powerful tool for studying the TME. Cancer immune escape mechanisms clearly merit further study.

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Disclosure of conflict of interest

None.

Address correspondence to: Jinghui Wang, Department of Medical Oncology, Beijing Chest Hospital, Capital Medical University, Beijing Tuberculosis and Thoracic Tumor Research Institute, Tongzhou District, Beijing 101149, China. Tel: +86-136-8312-8239; E-mail: jinghuiwang2006@163.com; Hongtao Zhang, Department of Central Laboratory, Beijing Chest Hospital, Capital Medical University, Beijing Tuberculosis and Thoracic Tumor Research Institute, Tongzhou District, Beijing 101149, China. Tel: +86-186-1275-1900; E-mail: zhtbeijing@163.com

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Supplementary Table 1. The association of density of CD8+ FOXP3+ T cells, CD8+ T cells, FOXP3+ T cells and the rate of PD-L1 expression in tumor with Clinicopathological characteristics

	N	CD8+ FOXP3+ T cells' density in tumor		CD8+ cells' density in tumor		FOXP3+ cells' density in tumor		The rate of PD-L1 expression in tumor	
		Mean ± SD (%)	*P* value	Mean ± SD (%)	*P* value	Mean ± SD (%)	*P* value	Mean ± SD (%)	*P* value
Age (year)									
< 65	118	0.06 ± 0.14	0.001	2.72 ± 3.17	0.030	0.63 ± 0.97	0.012	16.62 ± 30.88	0.964
≥ 65	74	0.11 ± 0.20		3.52 ± 3.50		0.86 ± 1.30		16.68 ± 32.41	
Gender									
Male	135	0.09 ± 0.18	0.484	2.93 ± 3.34	0.145	0.72 ± 1.08	0.672	18.15 ± 32.97	0.532
Female	57	0.06 ± 0.13		3.28 ± 3.28		0.71 ± 1.19		13.07 ± 27.26	
Smoking									
Yes	115	0.10 ± 0.20	0.263	2.97 ± 3.40	0.172	0.75 ± 1.15	0.744	17.82 ± 32.38	0.815
No	77	0.05 ± 0.11		3.12 ± 3.22		0.67 ± 1.06		14.88 ± 29.99	
TNM Stage									
I+II	102	0.07 ± 0.14	0.733	2.82 ± 2.77	0.925	0.80 ± 1.37	0.926	13.98 ± 28.88	0.216
III+IV	90	0.09 ± 0.20		3.27 ± 3.85		0.62 ± 0.72		19.66 ± 33.94	
T stage									
T1+T2	137	0.08 ± 0.14	0.806	3.16 ± 3.38	0.203	0.81 ± 1.24	0.018	14.98 ± 29.74	0.391
T3+T4	55	0.10 ± 0.22		2.71 ± 3.16		0.50 ± 0.67		20.78 ± 35.15	
N stage									
N0	101	0.07 ± 0.14	0.594	2.80 ± 2.72	0.713	0.78 ± 1.37	0.734	13.99 ± 29.17	0.234
N1+N2	91	0.09 ± 0.20		3.29 ± 3.88		0.65 ± 0.73		19.58 ± 33.62	
Histology									
LUSC	83	0.09 ± 0.18	0.643	2.57 ± 3.04	0.006	0.66 ± 0.90	0.449	23.20 ± 37.13	0.002
LUAD	109	0.08 ± 0.16		3.39 ± 3.49		0.76 ± 1.25		11.64 ± 25.26	
EGFR status									
EGFR mutation	27	0.04 ± 0.04	0.845	2.96 ± 2.96	0.983	0.56 ± 0.52	0.744	8.41 ± 21.77	0.743
EGFR wild type	34	0.06 ± 0.10		3.36 ± 3.90		0.62 ± 0.72		15.29 ± 28.20	

Supplementary Table 2. The correlation of density of CD8+ FOXP3+ cells, CD8+ T cells, FOXP3+ T cells and the rate of PD-L1 expression in tumor

	CD8+ FOXP3+ cells' density in tumor		CD8+ cells' density in tumor		FOXP3+ cells' density in tumor		The rate of PD-L1 expression in tumor	
	r	*P* value	r	*P* value	r	*P* value	r	*P* value
CD8+ FOXP3+ cells' density in tumor	-	-	0.606	< 0.001	0.604	< 0.001	0.053	0.463
CD8+ cells' density in tumor	0.606	< 0.001	-	-	0.509	< 0.001	0.108	0.136
FOXP3+ cells' density in tumor	0.604	< 0.001	0.509	< 0.001	-	-	0.123	0.089
Tumor PD-L1 expression	0.053	0.463	0.108	0.136	0.123	0.089	-	-

Supplementary Table 3. Univariate and multivariate analysis of overall survival

Variables	Univariate analysis			Multivariate analysis	
	Event/Number (N/n)	Median survival, 95% CI (month)	Log rank, *P* value	HR, 95% CI	*P* value
Age (years)					
< 65	69/118	30.9 (24.956, 36.844)	0.046	0.549 (0.367, 0.822)	0.004
≥ 65	51/74	17.7 (8.801, 26.599)		1.821 (1.216, 2.726)	
Gender					
Male	89/135	23.5 (18.415, 28.585)	0.096		
Female	31/57	33.7 (-, -)			
Smoking status					
Current smoking	77/115	23.1 (16.390, 29.810)	0.071		
Non-smoking	43/77	30.9 (22.397, 39.403)			
TNM Stage					
I+II	48/102	—	< 0.001	0.452 (0.225, 0.907)	0.025
III+IV	72/90	15.8 (11.405, 20.195)		2.214 (1.102, 4.447)	
T stage					
T1+T2	71/137	39.0 (-, -)	< 0.001	0.387 (0.248, 0.605)	< 0.001
T3+T4	49/55	11.2 (7.930, 14.470)		2.581 (1.653, 4.030)	
N stage					
N0	50/101	—	< 0.001	0.967 (0.509, 1.838)	0.918
N1+N2	70/91	19.8 (14.531, 25.069)		1.034 (0.544, 1.966)	
Histology					
LUSC	60/83	15.8 (11.039, 20.561)	0.001	1.459 (0.997, 2.135)	0.052
LUAD	60/109	33.7 (12.354, 55.046)		0.686 (0.468, 1.003)	
EGFR status					
EGFR mutation	16/27	33.3 (25.328, 41.272)	0.157		
EGFR wild type	24/34	25.1 (16.386, 33.814)			
Tumor CD8+ FOXP3+ T cells' density					
Low	94/159	28.0 (21.095, 34.905)	0.034	0.635 (0.386, 1.044)	0.074
High	26/33	17.7 (7.234, 28.166)		1.575 (0.958, 2.590)	
Tumor CD8+ T cells' density					
Low	33/42	20.4 (9.497, 31.303)	0.010	1.769 (1.111, 2.816)	0.016
High	87/150	27.6 (19.948, 35.252)		0.565 (0.355, 0.900)	
Tumor FOXP3+ T cells' density					
Low	93/140	21.9 (15.870, 27.930)	0.063		
High	27/52	38.4 (-, -)			
PD-L1 expression in tumor					
Low	96/160	27.7 (21.037, 34.363)	0.015	0.623 (0.380, 1.024)	0.062
High	24/32	12.7 (8.126, 17.274)		1.604 (0.976, 2.635)	
Adjuvant chemotherapy					
Yes	64/111	32.6 (23.467, 41.733)	0.014	0.447 (0.299, 0.668)	< 0.001
No	56/81	19.2 (11.459, 26.941)		2.238 (1.496, 3.349)	