Original Article

Tumor-infiltrating CD45RO$^+$ memory cells correlate with favorable prognosis in patients with lung adenocarcinoma

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Background: The present study aimed to investigate the association of CD45RO$^+$, CD8$^+$, CCR7$^+$ and FOXP3$^+$ tumor-infiltrating lymphocytes (TILs) with the clinicopathological features as well as survival of patients with lung adenocarcinoma.

Methods: Ninety patients with lung adenocarcinoma who underwent surgery were recruited in the present study. Lung adenocarcinoma tissues and paired adjacent lung tissues were obtained from all participants, and immunohistochemistry was performed to detect the expression of CD45RO, CD8, CCR7 and FOXP3. After multiplying the staining intensity score by the labeling frequency score, the immunohistochemical results were divided into three groups: TILs low, TILs intermediate and TILs high.

Results: CD45RO$^+$, CD8$^+$ and CCR7$^+$ infiltrating lymphocytes were markedly increased in lung adenocarcinoma (all P<0.001) while FOXP3$^+$ infiltrating lymphocytes were reduced (P<0.001) compared with than in adjacent tissues. CD45RO$^+$ TILs were negatively associated with tumor size (P=0.002), lymph node metastasis (P<0.001) and TNM stage (P<0.001). CD8$^+$ TILs were also negatively correlated with lymph node metastasis (P=0.016). Kaplan-Meier curve analysis revealed that CD45RO$^+$ TILs were positively associated with longer disease-free survival (DFS) (P<0.001) and overall survival (OS) (P<0.001). Univariate and multivariate Cox's proportional hazards regression confirmed that CD45RO$^+$ TILs (high) independently predicted longer DFS (P=0.002) and OS (P=0.009).

Conclusions: The present study demonstrates that CD45RO$^+$ TILs are negatively correlated with tumor size, lymph node metastasis and TNM stage and that CD45RO$^+$ TILs (high) can be regarded as a novel and promising biomarker for prolonged DFS and OS in lung adenocarcinoma patients.

Keywords: Lung adenocarcinoma; CD45RO; CD8; overall survival; pathological grade

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Introduction

Lung cancer, the leading cause of cancer-related death worldwide, has a high incidence and mortality rate (1). The main classification of lung cancer includes small cell lung carcinoma (SCLC) and non-small cell lung cancer (NSCLC) (2). NSCLC, which accounts for 85% of all lung cancers, primarily comprises adenocarcinoma, squamous cell carcinoma, large cell carcinoma, sarcomatoid carcinoma, and adenosquamous cell carcinoma, among which lung adenocarcinoma is one of the most aggressive histological types in lung cancer and is the main subtype of NSCLC (3). Despite the improvement in lung adenocarcinoma treatments, surgical resection is still the primary option for patients with lung adenocarcinoma; however, most clinically

diagnosed cases are inoperable because of metastasis or present poor prognosis after surgery (4). Therefore, additional novel and convincing biological markers, which are helpful to indicate the diagnosis and prognosis for lung cancer, are urgently needed.

Although the pathogenesis of lung adenocarcinoma is complicated and obscure, genetic features, environmental factors and dysregulated living habits greatly contribute to the development and progression of this disease (5,6). Among these factors, the development of lung adenocarcinoma is controlled by a biological system that depends on genetic factors as well as the interplay between tumor cells, stromal cells, and host immune cells (7). Immune system invasion and/or escape is essential in the etiology of lung adenocarcinoma, and the presence of tumor-infiltrating lymphocytes (TILs) in the tumor microenvironment is an indication of the host immune response to tumor antigens (8). TILs comprise a variety of immune cells, including $CD4^+$ T lymphocytes, $CD8^+$ lymphocytes, $FOXP3^+$ regulatory T cells, natural killer (NK) cells, dendritic cells and macrophages (9). TILs are attracting attention for their use in monitoring the immune response in the tumor microenvironment and predicting the prognosis and treatment response in several cancers, such as colorectal and breast cancers (8,9).

Therefore, in the present study, we proposed that the infiltration of lymphocytes in lung adenocarcinoma and expression of chemokine receptors might be correlated with tumor differentiation, TNM stage, clinical stage, disease-free survival (DFS) or overall survival (OS). We aimed to evaluate the infiltration of $CD45RO^+$, $CD8^+$, $CCR7^+$ and $FOXP3^+$ cells in paraffin-embedded lung adenocarcinoma tissues and examine their correlation with clinicopathological parameters as well as prognosis.

Methods

Patients

Ninety patients with lung adenocarcinoma who underwent the surgery at Shanghai Chest Hospital (Shanghai, China) between Jan. 2007 and Jun. 2011 were consecutively recruited in the present cohort study. All patients were diagnosed with lung adenocarcinoma based on clinical manifestations, radiological and histological findings (*Table 1*), and no patients had received new adjuvant therapies, such as radiotherapy or chemotherapy, prior to surgery. Lung adenocarcinoma and paired adjacent lung tissues were obtained from all participants, and clinical as well as pathological data were collected. In the present study, the number of patients with lobectomy, local resection and pneumonectomy were 75 (83%), 10 (11%) and 5 (6%), respectively. In addition, among the 78 patients with stage IB–IV, 63 (80.8%) patients received adjuvant chemotherapy and 15 (19.2%) patients did not. The present study was approved by the Institutional Review Board for Clinical Research of the Shanghai Chest Hospital. Written informed consent was obtained from all subjects prior to the study. The number/ID of the Ethic Approval was ILU02.

Follow-ups

All patients were followed up by clinic visits or telephone calls, once a month in the first half year, once every 3 months from 0.5 to 2 years, and then once every 6 months from 2 years to the last date of follow up (2016/08/20). OS and DFS were calculated from the surgery to the time of death from any cause, and the time of documented local or distant recurrence of the initial cancer, respectively. Five patients were lost to follow-up during the entire study, with follow-up durations of 8, 40, 48, 52 and 64 months, respectively. The median follow-up duration was 46.0 months (ranging from 1 to 121 months).

Immunohistochemistry

Prior to immunohistochemistry analysis, all the sections were stained with hematoxylin and eosin and reviewed to confirm the histopathological diagnosis. As previously described (10), paraffin-embedded lung adenocarcinoma and paired adjacent lung tissue specimens were cut into 4-μm sections and mounted on poly-L-lysine-coated glass slides. Following deparaffinization and rehydration, antigen retrieval was performed by autoclaving the sections in 10 mmol/L Tris-EDTA buffer (pH 9.0) at 121 ℃, subsequently endogenous peroxidase was blocked using 3% H_2O_2, and the sections were immersed in 4% bovine serum albumin. The sections were then incubated with a 1:100 dilution of rabbit or mouse monoclonal antibody against human CD45RO, CD8, CCR7 and FOXP3 (All from Abcam, USA) at 4 ℃ overnight according to the manufacturer's instructions. For negative controls, sections were treated with phosphate-buffered saline (PBS) instead of primary antibodies. After washing with PBS, the sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at 37 ℃ for 1 hour. Subsequently, the signal was detected using DAB

Table 1 Demographic, clinical and pathological characteristics of lung adenocarcinoma patients

Parameters	Cases (n=90)	CD45RO$^+$ TILs expressions				CD8$^+$ TILs				CCR7$^+$ TILs				FOXP3$^+$ TILs			
		L	I	H	P value	L	I	H	P value	L	I	H	P value	L	I	H	P value
Age (years)																	
≤60	41	8	16	17	0.608	4	19	18	0.713	2	11	28	0.715	12	22	7	0.968
>60	49	7	18	24		3	21	25		1	12	36		15	25	9	
Gender																	
Female	41	9	11	21	0.124	5	19	17	0.275	0	13	28	0.156	15	21	5	0.298
Male	49	6	23	20		2	21	26		3	10	36		12	26	11	
Smoking history																	
Never	37	4	13	20	0.292	1	15	21	0.161	2	7	28	0.350	10	20	7	0.872
Ever	53	11	21	21		6	25	22		1	16	36		17	27	9	
Pathologic type																	
Adenocarcinoma	69	14	23	32	0.430	7	30	32	0.723	2	18	49	0.626	22	34	13	0.426
BAC	14	0	7	7		0	6	8		0	4	10		3	10	1	
Mucinous	6	1	3	2		0	3	3		1	1	4		1	3	2	
Papillary	1	0	1	0		0	1	0		0	0	1		1	0	0	
Differentiation																	
Well	12	0	3	9	0.225	0	8	4	0.119	0	2	10	0.322	5	7	0	0.327
Moderate	57	11	22	24		3	24	30		1	17	39		16	31	10	
Poor	21	4	9	8		4	8	9		2	4	15		6	9	6	
Tumor size (cm)																	
≤5	52	4	17	31	0.002	2	27	23	0.115	2	14	36	0.883	17	25	10	0.654
>5	38	11	17	10		5	13	20		1	9	28		10	22	6	
Lymph node metastasis																	
Negative	54	6	14	34	<0.001	2	20	32	0.016	2	14	38	0.964	14	31	9	0.464
Positive	36	9	20	7		5	20	11		1	9	26		13	16	7	
Distant metastasis																	
Negative	89	14	34	41	0.080	7	39	43	0.546	3	23	63	0.814	27	46	16	0.630
Positive	1	1	0	0		0	1	0		0	0	1		0	1	0	
TNM stage																	
I	29	2	8	19	<0.001	1	14	14	0.139	2	7	20	0.829	8	15	6	0.965
II	32	3	10	19		0	15	17		0	8	24		10	16	6	
III	28	9	16	3		5	11	12		1	8	19		9	15	4	
IV	1	1	0	0		0	1	0		0	0	1		0	1	0	

Data were presented as counts. Comparison among groups was determined by Chi-square test. P<0.05 was considered significant. BAC, bronchioloalveolar carcinoma; TILs, tumor-infiltrating lymphocyte; H, high; I, intermediate; L, low.

and hematoxylin was used for counterstaining. One hundred cells from five selected representative high-power fields (HPF, ×400) of each section were independently counted by pathologists without prior knowledge of the clinicopathological data for the determination of the immunostaining intensity. The staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong), while the grading scale for labeling frequency ranged from 0 (0%) to 1 (1–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%) on the basis of the percentage of positively stained cells. After multiplying the staining intensity score by the labeling frequency score, the sections were divided into three groups: TILs low (final score ≤3), TILs intermediate (3< final score ≤6) and TILs high (final score >6).

Statistical analysis

The data were presented as the means ± standard deviation (SD), count (percentage). Chi-square test was used to compare CD45RO$^+$, CD8$^+$, CCR7$^+$ and FOXP3$^+$ infiltrating lymphocytes among the different groups. DFS and OS were analyzed by the Kaplan-Meier method. In addition, univariate Cox's proportional hazards regression model was performed to determine predictors of DFS as well as OS in lung adenocarcinoma patients, while all factors with a P value less than 0.1 were subsequently included in the multivariate Cox's proportional hazards regression model to investigate the independent predictive factors. Statistical analysis was performed using the SSPS 21.0 program and a P value <0.05 was considered significant.

Results

Patient characteristics

Among the 90 patients with a median age of 61.5 [55–71] years, 49 (54%) patients were males. Twelve cases (13%) were well differentiated, while 57 (63%) and 21 (24%) cases were moderately and poorly differentiated, respectively. A total of 29 (32%), 32 (36%), 28 (31%), and 1 (1%) cases were TNM stages I, II, III, and IV, respectively. The other baseline characteristics of the lung adenocarcinoma patients are summarized in *Table 1*.

CD45RO$^+$, CD8$^+$, CCR7$^+$ and FOXP3$^+$ infiltrating lymphocytes in lung adenocarcinoma and adjacent lung tissues

To explore the role of immune cells in the tumor microenvironment, we examined the expression of different immune cell proteins by immunohistochemistry. As shown in *Figure 1A*, CD45RO was mainly expressed in immune cells infiltrated in adenocarcinoma tissues and predominantly present in the cell membrane. CD45RO$^+$ infiltrating lymphocytes were markedly increased in lung adenocarcinoma than those in adjacent lung tissues (P<0.001) (*Table 2*). Consistent with the increased CD45RO$^+$ infiltrating lymphocytes, CD8 and CCR7 were also expressed on the membranes of immune cells and presented a high level of expression in lung adenocarcinoma compared with that in adjacent lung tissues (both P<0.001) (*Figure 1B*,*C*, *Table 2*). However, FOXP3 was primarily expressed in the nucleus of immune cells and presented a decreasing tendency in lung adenocarcinoma (P<0.001) (*Figure 1D*, *Table 2*).

Correlation of CD45RO$^+$, CD8$^+$, CCR7$^+$ and FOXP3$^+$ TILs with clinicopathological features

CD45RO$^+$ TILs were negatively associated with tumor size (P=0.002), lymph node metastasis (P<0.001) and TNM stage (P<0.001), as presented in *Table 1*. CD8$^+$ TILs were also negatively correlated with lymph node metastasis (P=0.016) (*Table 1*). While no other correlations of CD45RO$^+$ and CD8$^+$ TILs with clinicopathological features were observed in the present study. CCR7$^+$ and FOXP3$^+$ TILs showed no association with the clinical and pathological parameters (*Table 1*).

Association of CD45RO$^+$, CD8$^+$, CCR7$^+$ and FOXP3$^+$ TILs with DFS and OS

Kaplan-Meier curve analysis was performed to evaluate the effects of CD45RO$^+$, CD8$^+$, CCR7$^+$ and FOXP3$^+$ TILs on DFS and OS. As shown in *Figure 2*, CD45RO$^+$ TILs were positively associated with longer DFS (P<0.001), while no association of CD8$^+$ (P=0.188), CCR7$^+$ (P=0.988) and FOXP3$^+$ (P=0.487) TILs with DFS was observed. As shown in *Figure 3*, CD45RO$^+$ TILs were also positively associated with better OS (P<0.001), and CD8$^+$ (P=0.247), CCR7$^+$ (P=0.928) and FOXP3+ (P=0.399) TILs were not correlated with OS.

Analysis of baseline factors affecting DFS

In the present study, a total of 82 (91%) patients had local (n=71) or distant (n=11) recurrence of the initial cancer,

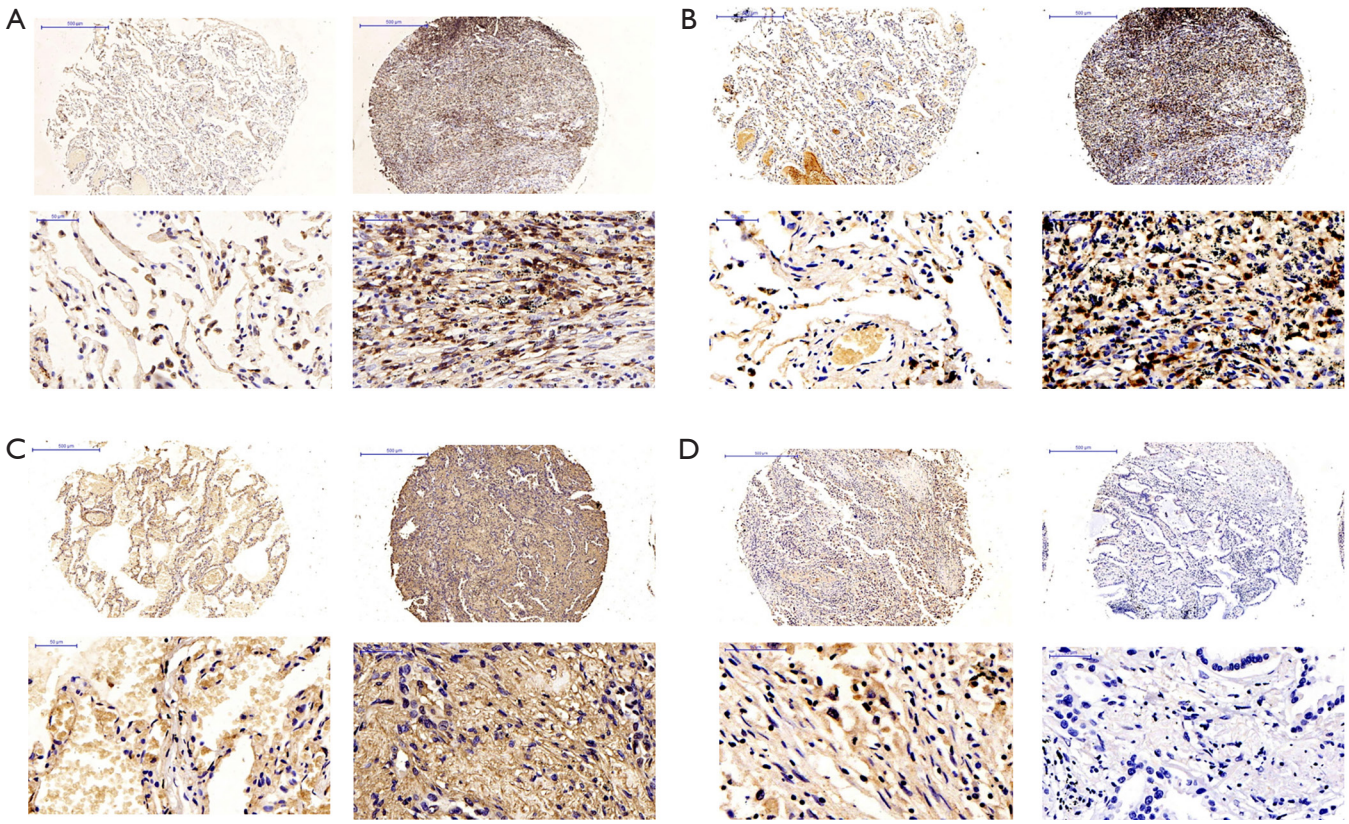

Figure 1 CD45RO$^+$, CD8$^+$, CCR7$^+$ and FOXP3$^+$ infiltrating lymphocytes in lung adenocarcinoma and adjacent lung tissue. Representative protein expression of CD45RO (A), CD8 (B), CCR7 (C) and FOXP3 (D) were examined by immunohistochemistry in lung adenocarcinoma tissues (right, n=90) and adjacent lung adenocarcinoma tissues (left, n=90). Original magnification, ×200 (up), ×400 (down).

Table 2 CD45RO$^+$, CD8$^+$, CCR7$^+$ and FOXP3$^+$ infiltrating lymphocytes in lung adenocarcinoma and adjacent lung tissue

Parameters	CD45RO$^+$ (P<0.001)			CD8$^+$ (P<0.001)			CCR7$^+$ (P<0.001)			FOXP3$^+$ (P<0.001)		
	H	I	L	H	I	L	H	I	L	H	I	L
Lung adenocarcinoma	41	34	15	43	40	7	64	23	3	16	47	27
Adjacent lung tissue	4	55	31	8	57	25	32	52	6	32	52	6

Data were presented as counts. Comparison among groups was determined by Chi-square test. P<0.05 was considered significant. H, high; I, intermediate; L, low.

whereas 8 (9%) patients were free of disease at the end of the follow-up. To investigate the correlation of the baseline factors with DFS, a univariate Cox's proportional hazards regression model was generated, and CD45RO$^+$ TILs (high) (P<0.001) were determined to be predictors for longer DFS, while poor differentiation (P=0.020) and lymph node metastasis (P=0.003) were associated with shorter DFS (*Table 3*). All factors with P<0.1 were further analyzed by multivariate Cox's proportional hazards regression model, and CD45RO$^+$ TILs (high) were revealed as an independent factor for prolonged DFS (P=0.002), and on the contrary, lymph node metastasis could independently predict worse DFS (P=0.005) (*Table 3*).

Analysis of baseline factors affecting OS

In the present study, a total of 67 (74.4%) patients died for the high mortality of lung adenocarcinoma, whereas

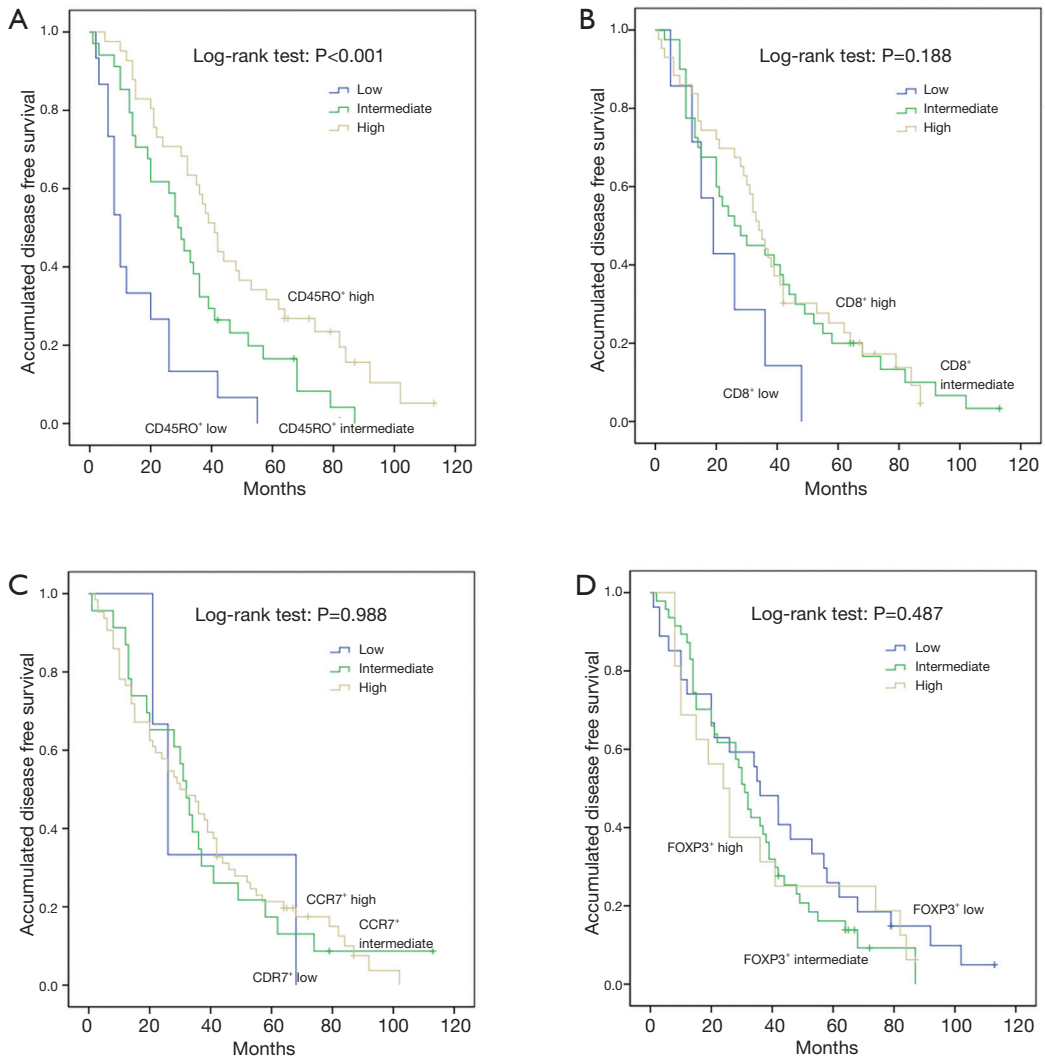

Figure 2 The association of CD45RO$^+$, CD8$^+$, CCR7$^+$ and FOXP3$^+$ TILs with DFS. CD45RO$^+$ TILs (high) were correlated with prolonged DFS compared with CD45RO$^+$ TILs (intermediate) and CD45RO$^+$ TILs (low) (A). While no associations were observed in CD8$^+$ (B), CCR7$^+$ (C) and FOXP3$^+$ (D) TILs with DFS. TILs, tumor-infiltrating lymphocytes; DFS, disease-free survival.

23 (25.6%) patients were alive at the end of the follow-up. Univariate Cox's proportional hazards regression model showed that CD45RO$^+$ TILs (high) was a predictive factor for longer OS (P<0.001), whereas poor differentiation (P=0.016), tumor size (P=0.024), lymph node metastasis (P=0.001) and TNM stage (P=0.022) were correlated with short OS (*Table 4*). Subsequent multivariate Cox's proportional hazards regression model showed that CD45RO$^+$ TILs (high) independently predicted better OS (P<0.001), while poor differentiation (P=0.002), positive lymph node metastasis (P=0.005) and positive distant metastasis (P=0.024) were independent factors for worse OS (*Table 4*).

Discussion

In the present study, (I) CD45RO$^+$, CD8$^+$ and CCR7$^+$ infiltrating lymphocytes were markedly increased in lung adenocarcinoma than in adjacent lung tissues, while FOXP3$^+$ infiltrating lymphocytes were reduced; (II) CD45RO+ TILs were negatively correlated with tumor size, lymph node metastasis and TNM stage, and CD8+ TILs were only negatively associated with lymph node metastasis; (III) CD45RO$^+$ TILs were associated with

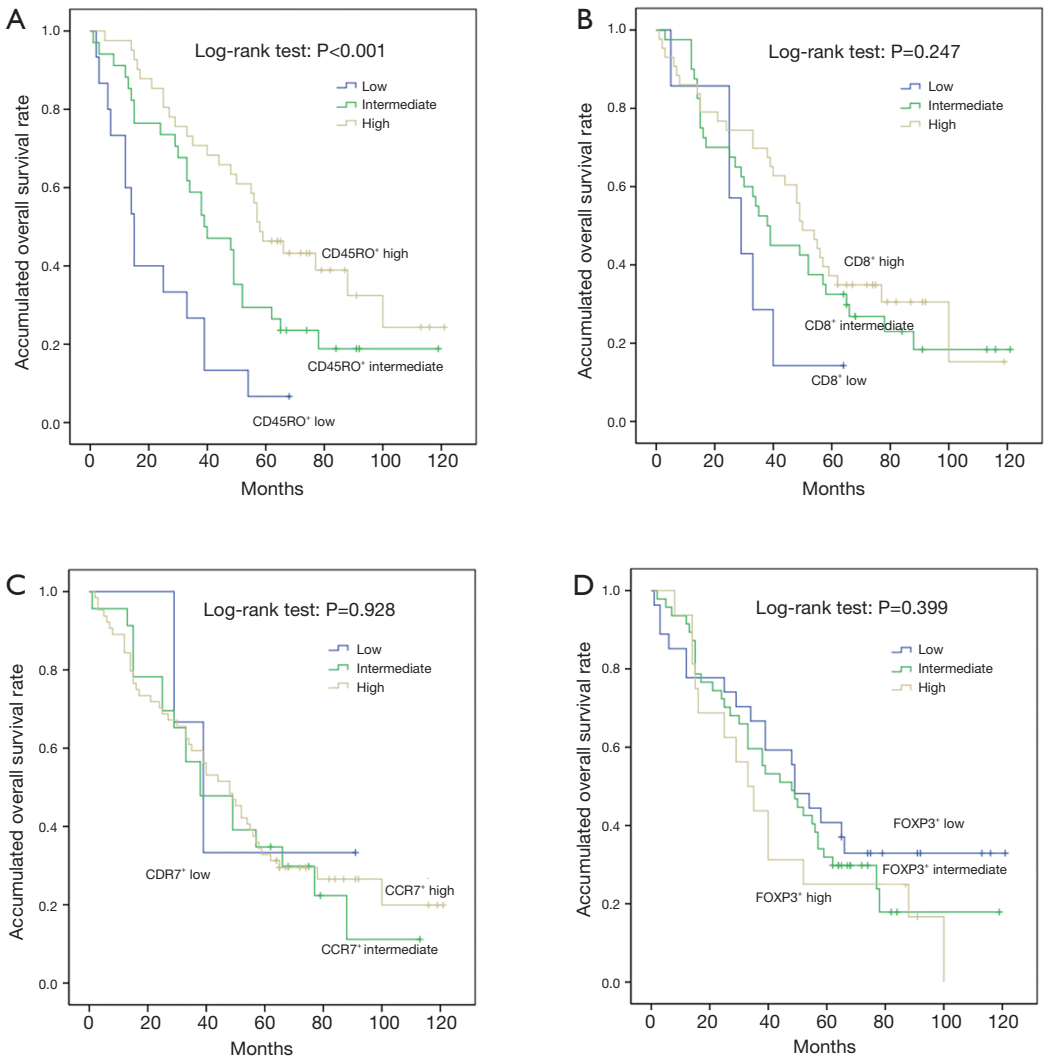

Figure 3 The correlation of CD45RO$^+$, CD8$^+$, CCR7$^+$ and FOXP3$^+$ TILs with OS. CD45RO$^+$ TILs (high) were correlated with longer OS compared with CD45RO$^+$ TILs (intermediate) and CD45RO$^+$ TILs (low) (A). While no associations were observed in CD8$^+$ (B), CCR7+ (C) and FOXP3$^+$ (D) TILs with OS. TILs, tumor-infiltrating lymphocytes; OS, overall survival.

prolonged DFS and OS, as independent predictive factors for better DFS as well as OS.

The microenvironment, comprising endothelial cells and fibroblasts, structural components and infiltrating immune cells, plays a critical role in the development and progression of various cancers (11-14). Among these factors, accumulating evidence has revealed that immune cells cross-interact with tumors, leading to immune responses (15). Additionally, the immune context, defined by the type, density, location as well as organization of immune cells, is a crucial determinant of the clinicopathological features of tumors and patient prognosis (11,15). Accumulating evidence has proven that in general, tumor stroma immune cells mainly consist of TIL and tumor associated macrophages (TAM), but almost no NK cells, and a few dendritic cells. As to TIL cells, T-cells occupy nearly 80%, and approximately 30% T-cells are TIA-1 positive CD8 activated cytotoxic lymphocytes (16). TILs, as a group of heterogeneous immune cells, infiltrate tumors and reflect the immune response status between host immune cells and tumors. Previous studies have shown that the strong infiltration of TILs correlates with tumor development, individual properties, clinical response and long-term survival in several cancers, including lung cancer (17,18).

Table 3 Analysis of baseline factors affecting disease free survival in lung adenocarcinoma patients

Parameters	Univariate Cox's			Multivariate Cox's		
	P value	HR	95% CI	P value	HR	95% CI
CD45RO$^+$ (TILs high)	<0.001	0.490	0.356–0.675	0.002	0.609	0.449–0.827
CD8$^+$ (TILs high)	0.263	0.811	0.562–1.170	–	–	–
CCR7$^+$ (TILs high)	0.880	0.970	0.649–1.448	–	–	–
FOXP3$^+$ (TILs high)	0.332	1.166	0.855–1.590	–	–	–
Age (>60 years)	0.757	1.072	0.692–1.660	–	–	–
Gender (male)	0.798	1.059	0.685–1.637	–	–	–
Smoking history (ever)	0.108	1.442	0.923–2.253	–	–	–
Pathologic type (adenocarcinoma)	0.292	1.328	0.784–2.250	–	–	–
Differentiation (poor)	0.020	1.523	1.070–2.167	0.069	1.420	0.973–2.074
Tumor size (>5 cm)	0.071	1.521	0.965–2.398	0.426	1.264	0.710–2.249
Lymph node metastasis (positive)	0.003	1.964	1.254–3.078	0.005	2.426	1.311–4.490
Distant metastasis (positive)	0.232	3.394	0.458–25.135	–	–	–
TNM stage (high)	0.057	1.299	0.993–1.701	0.563	0.887	0.590–1.333

Significance was determined by univariate Cox's regression model, while all factors with a P value less than 0.1 were subsequently included into multivariate Cox's proportional hazards regression model to investigate the independent predictive factors. The expressions of CD45RO, CD8, CCR7 and FOXP3 were scored as 0 (low), 1 (intermediate) and 2 (high) for the Cox's analysis; differentiations were scored as 0 (well), 1 (moderate) and 2 (poor) for the Cox's analysis; TNF stage were scored as 0 (stage I), 1 (stage II), 2 (stage III), 3 (stage IV) for the Cox's analysis. P<0.05 was considered significant. TNF, tumor necrosis factor.

Thus, in the present study, we examined the CD45RO$^+$, CD8$^+$, CCR7$^+$ and FOXP3+ infiltrating lymphocytes by immunohistochemistry in lung adenocarcinoma tissues and paired adjacent lung tissues to explore their association with the occurrence, clinicopathological properties and prognosis of lung adenocarcinoma.

Some tumors, including lung adenocarcinoma, acquire the ability to damage and exploit inflammatory responses to promote the proliferation, survival, and invasiveness of tumor cells (19,20). Thus, the presence of leukocytes within tumor microenvironment may be a consequence of an inflammatory response that favors tumor development (21). CD45, also known as leukocyte common antigen, is encoded by the PRPRC gene and serves as a pan-inflammatory marker widely used in routine diagnostic pathology laboratories (22). According to the differences in the expressional features on T cells, CD45$^+$ T cells were divided into CD45RA$^+$ naive T cells and CD45RO$^+$ memory T cells, and the latter could kill tumor cells by the immediate activation of immune responses (23,24). A previous study revealed that CD45RO$^+$ T cells inhibit the proliferation and migration of cervical cancer cells by regulating interleukin (IL)-2, interferon (IFN)-γ and tumor necrosis factor (TNF)-α (25). Additionally, CD45RO+ T cells could decrease the production of high-mobility group box 1 (HMGB1), and subsequently suppress the proliferation and migration of colorectal tumor cells (26). These studies indicate the anti-tumor effect of CD45RO$^+$ T cells. In addition, CD45RO$^+$ TILs are negatively correlated with tumor size, lymph nodes metastasis in breast cancer patients (27), associated with lesser advanced T stage and TNM stage in rectal cancer (28), and correlated with well differentiation and lower TNM stage in renal cell carcinoma (29). Consistent with previous studies, we found that CD45RO expression was increased in lung adenocarcinoma tissues compared to paired adjacent lung tissues, and CD45RO$^+$ TILs were negatively correlated with tumor size, lymph node metastasis and TNM stage in lung adenocarcinoma patients. These findings might result from the effect of CD45RO$^+$ TILs on inhibiting the proliferation and migration of tumor cells through multiple mechanisms, such as excreting various cytokines, activating shrewd

Table 4 Analysis of baseline factors affecting overall survival in lung adenocarcinoma patients

Parameters	Univariate Cox's			Multivariate Cox's		
	P value	HR	95% CI	P value	HR	95% CI
CD45RO$^+$ (TILs high)	<0.001	0.507	0.361–0.714	<0.001	0.425	0.297–0.608
CD8$^+$ (TILs high)	0.141	0.746	0.505–1.102	–	–	–
CCR7$^+$ (TILs high)	0.989	0.997	0.641–1.552	–	–	–
FOXP3$^+$ (TILs high)	0.182	1.269	0.895–1.799	–	–	–
Age (>60 years)	0.790	0.937	0.579–1.515	–	–	–
Gender (male)	0.670	1.110	0.686–1.798	–	–	–
Smoking history (ever)	0.041	1.694	1.021–2.811	0.425	1.433	0.592–3.467
Pathologic type (adenocarcinoma)	0.098	1.699	0.908–3.182	0.988	1.007	0.412–2.459
Differentiation (Poor)	0.016	1.620	1.095–2.397	0.002	2.092	1.309–3.344
Tumor size (>5 cm)	0.024	1.753	1.077–2.853	0.569	1.273	0.555–2.923
Lymph node metastasis (positive)	0.001	2.330	1.433–3.787	0.005	2.665	1.349–5.264
Distant metastasis (positive)	0.087	5.878	0.773–44.703	0.024	14.750	1.437–151.401
TNM stage (high)	0.022	1.421	1.053–1.917	0.238	0.745	0.457–1.215

Significance was determined by univariate Cox's regression model, while all factors with a P value less than 0.1 were subsequently included into multivariate Cox's proportional hazards regression model to investigate the independent predictive factors. The expressions of CD45RO, CD8, CCR7 and FOXP3 were scored as 0 (low), 1 (intermediate) and 2 (high) for the Cox's analysis; differentiations were scored as 0 (well), 1 (moderate) and 2 (poor) for the Cox's analysis; TNF stage were scored as 0 (stage I), 1 (stage II), 2 (stage III), 3 (stage IV) for the Cox's analysis. P<0.05 was considered significant. TNF, tumor necrosis factor.

immune responses, etc.

Apart from the correlation with clinicopathological properties, CD45RO$^+$ TILs (high) are associated with favorable outcomes in several cancers. In breast cancer patients, CD45RO$^+$ TILs (high) correlates with prolonged recurrence-free survival (27), and in stage II colorectal cancer patients, CD45RO$^+$ TILs (high) could predict better clinical response and cancer-specific survival (9). Moreover, CD45RO$^+$ TILs are positively associated with DFS and OS in patients with renal cell carcinoma, gastric cancer and ovarian carcinoma (24,29,30). As for NSCLC patients, a previous study indicated that CD45RO$^+$ TILs are an independent positive prognostic factor for disease-specific survival (DSS) (31). Another interesting study revealed that CD45RO$^+$ T-cells could independently predict better DSS in NSCLC patients with subtype of squamous cell carcinoma. Consistent with previous studies of other cancers, CD45RO$^+$ TILs (high) were associated with prolonged DFS and OS in lung adenocarcinoma patients, serving as an independent predictive factor for better DFS as well as OS. These results might be explained by (I) the direct killing effect of CD45RO$^+$ TILs on tumor cells by repressing the proliferation and migration through activating immune responses (23-26); and (II) CD45RO$^+$ TILs increase the efficacy of chemotherapy and/or radiotherapy, thus improving the prognosis of cancer patients (28,31).

In the present study, we also investigated the roles of CD8$^+$, CCR7$^+$ and FOXP3$^+$ TILs in the development and prognosis of lung adenocarcinoma and showed that CD8 and CCR7 expression was elevated in lung adenocarcinoma tissues compared with paired adjacent lung tissues, while FOXP expression was decreased. However, only CD8$^+$ TILs were negatively correlated with lymph node metastasis, while no correlations of these three-marker TILs with other clinicopathological features and prognosis were detected in the present study. These results might be due to relatively small samples, which resulted a lack of sufficient patients with specific TILs (high) or TILs (low), thus the statistics results showed no significance.

The relapse rate was relatively high, and the possible reasons were as follows: Firstly, the follow-up duration was

relatively long in this study with the longest follow-up of 121 months (nearly 10 years). Secondly, more than half patients were with age >60 years, and the median age of these 90 patients was 61.5 [55–71] years old, which might contribute to the relatively high relapse rate. There were some limitations in the present study. Among these, the major limitation was that this was a retrospective study, we did not identify the major findings in an independent cohort of surgically resected tumors; thus, additional studies are needed. Moreover, the treatments after surgery were not analyzed, which might lead to bias during the study. However, the therapeutic options did not differ much in lung adenocarcinoma patients after surgery. In addition, the sample size was relatively small; thus, some differences or correlations might not have been observed. Few patients with $CD8^+$ TILs (low) and $CCR7^+$ TILs (low) were recruited, leading to a lack of efficiency in the statistical analysis for survival. Furthermore, only one distant metastasis patient was included in the present study; thus, the influence of specific TILs on prognosis in lung adenocarcinoma patients with distant metastasis was not analyzed. Finally, we performed immunohistochemistry to detect expressions of CD45RO, CD8, CCR7 and FOXP3, and during the processes of immunohistochemistry, we only marked CD45RO, CD8, CCR7 and FOXP3, without marked total T-cells, which caused that we could not know the detailed ratio of $CD45RO^+$, $CD8^+$, $CCR7^+$ and $FOXP3^+$ positive cells *vs*. total lymphocytes or total T-cells in this study. Thus, further study is great needed.

In conclusion, the present study demonstrates that $CD45RO^+$ TILs are negatively correlated with tumor size, lymph node metastasis and TNM stage and that CD45RO+ TILs (high) can be regarded as a novel and promising biomarker for prolonged DFS and OS in lung adenocarcinoma patients.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The present study was approved by the Institutional Review Board for Clinical Research of the Shanghai Chest Hospital (No. ILU02). Written informed consent was obtained from all subjects prior to initiating the present study.

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