The impact of the molecular profile of the tumor microenvironment on the prognosis of NSCLC

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Abstract

Purpose The present study was performed to clarify the correlation between macrophages, tumor neo-vessels and programmed cell death-ligand 1 (PD-L1) in the tumor microenvironment (TME) and the clinicopathological features of non-small cell lung cancer (NSCLC) and to explore the prognostic factors of stromal features in NSCLC.

Methods Tissue microarrays containing 92 NSCLC patients were studied with immunohistochemistry (IHC). The distribution and quantitative data of CD68- and CD206-positive tumor-associated macrophages (TAMs) in tumor islets and tumor stroma, and the expression of tumor neo-vessels and PD-L1, were analyzed by inverted microscopy and Image-Pro Plus 6.0 software. Prognostic analyses with the clinicopathological characteristics and tumor microenvironment features were performed.

Results The number of CD68-positive macrophages in each location of the tumor islets and tumor stroma was significantly higher than that of CD206-positive macrophages, and they were significantly correlated ($P < 0.0001$). Survival analysis revealed that CD68- and CD206-positive TAMs in the tumor stroma and tumor islets were significant prognostic factors ($P < 0.05$, respectively). Comprehensive analysis of CD206-positive stromal TAMs showed that CD105 and PD-L1 were significant prognostic factors ($P=0.045$). Moreover, CD68-positive TAMs in tumor islets and the expression of PD-L1 were independent predictors of poor prognosis for NSCLC.

Conclusion Thus, the key elements in the tumor microenvironment, including tumor neo-vessels, macrophages and PD-L1, were heterogenic in NSCLC tissues and had significant roles in cancer invasion and metastasis. The combined analysis of key components in the tumor microenvironment was an important prognostic factor.

Introduction

Lung cancer is the most common cause of cancer-related deaths, of which non-small cell lung cancer (NSCLC) comprises approximately 85-90%(Siegel et al. 2020). Despite the great progress of comprehensive treatment strategies based on surgery for resectable NSCLC in recent years, approximately 50% of early-stage NSCLC patients will relapse or develop distant metastases within 5 years after radical surgery(Asamura et al. 2008).

Paget's "seed-soil" theory makes people realize that the tumor microenvironment plays a vital role in tumor metastasis(Paget 1989)· The tumor microenvironment (TME) is a complex and dynamic community that consists of extracellular matrix (ECM), tumor cells, inflammatory cells, immune infiltrating cells, vascular endothelial cells, fat cells, and fibroblasts(Catalano et al. 2013). Among them, immune infiltrating cells and vascular endothelial cells are the most representative factors in the tumor microenvironment. Macrophages, important representatives of immune infiltrating cells, act as vital components of the host's defense and antigen-preserving cells and effector cells(Mosser and Edwards 2008; Schmieder et al. 2012). They can be classified into two types: the classic M1 type and the alternative M2 type. M1 type macrophages are a tumor suppressor type that participate in the inflammatory response, pathogen removal and antitumor immunity, while M2 type macrophages promote the occurrence and development of tumors by inducing angiogenesis and anti-inflammation(Pollard 2004). Tumor-associated macrophages (TAMs) are thought to be more similar to M2 macrophages(Petty et al. 2019). Angiogenesis occurs in the normal and vital processes of growth and development, as well as in the tumor transition between benign and malignant states. In addition, studies have shown that TAMs can promote tumor angiogenesis and metastasis(Murdoch et al. 2008). TAMs and cancer cells can secrete various growth factors, angiogenic factors and enzymes, which play important roles in angiogenesis(Allavena et al. 2008; Ohba et al. 2005; Wu et al. 2014; Yeo et al. 2014). Thus, TAMs, as representatives of immune cell infiltration, can interact with cancer cells to promote cancer invasion and metastasis(Han et al. 2018). Immature and structural disorders of vascular endothelial cells are the main reason for tumor hypoxia, and their discontinuities are also the morphological basis for tumor cells entering the vasculature to metastasize(Kerbel 2008). However, many conflicting results have been reported regarding the prognostic significance of TAMs and tumor neo-vessels in NSCLC(Becker et al. 2014; Du et al. 2015; Keskin et al. 2019; Zhang et al. 2012).

Immune checkpoint inhibitors targeting the programmed cell death protein 1 (PD-1)/programmed cell death-ligand 1 (PD-L1) pathway have shown impressive clinical benefit in several cancers, including NSCLC(Anagnostou and Brahmer 2015). However, the relationship between PD-L1/PD-1 expression and patient prognosis is still unclear, although some studies have reported that high PD-L1 expression is related to worse prognosis(Mu et al. 2011; Zhang et al. 2014). Thus, it has not been proven to be adequately reliable as a single biomarker to evaluate the prognosis of NSCLC patients(Brahmer et al. 2015; Patel and Kurzrock 2015).

To confirm whether the molecular profile of the TME has a great effect on the prognosis of NSCLC, the distribution and quantitative expression of TAMs, tumor neo-vessels and PD-L1 were analyzed, as well as the relationship between the clinicopathological and prognostic impact of the above three components in 92 NSCLC cases. In addition, the relationship between the differential expression and distribution of CD68-positive and CD206-positive TAMs in different intratumoral infiltration sites (tumor islets and tumor stroma) was explored, and the correlation between TAMs, tumor neo-vessels and PD-L1 was analyzed.

Material And Methods

Case collection

A total of 92 paraffin-embedded NSCLC samples, including tumor and peritumor tissues, were collected from Zhejiang Cancer Hospital between April 2008 and January 2014. All patients underwent radical surgery and were diagnosed with NSCLC by histology. Patients did not receive any treatment before surgery, while most patients received chemotherapy after surgery. The clinical parameters evaluated for each patient included sex, age, tumor size, smoking status, differentiation, pathological typing, and tumor staging. The OS time was measured from the date of surgery to death due to NSCLC or the date when the patient was last recorded. The study protocol was approved by the Ethics Committee of Zhejiang Cancer Hospital.

Immunohistochemistry (IHC) and Multiplexed Immunofluorescence

Tissue microarrays containing 92 NSCLC patients were studied. Briefly, tissue microarrays were treated by deparaffinization in xylene, hydration with graded alcohol and subjected to antigen retrieval. Then, the tissue microarrays were placed in 3% hydrogen peroxide (H_2O_2) for 10 min at room temperature to inactivate endogenous peroxidases. After washing three times in PBS, the slides were blocked with 2% bovine serum albumin (BSA, B2064, Sigma, USA) for 30 min at room temperature, followed by incubation with primary antibodies against PD-L1 (1:100, ab205921, Abcam, UK), CD105 (1:500, ab28364, Abcam, UK) and CD68 (1:200, ab34710, Abcam, UK) at 4 °C overnight. After washing with PBS, the slides were incubated with secondary antibody (1:200, ab150077, Abcam, UK) for 60 min at 37 °C. The slides were then washed in PBS three times, followed by Dako REALTM EnVisionTM (DAB, PW017, Sangon Biotech, China) detection and counterstaining with hematoxylin. Similar to IHC, after antibody incubation, all sections were covered using Fluoroshield containing 4',6-diamidino-2-phenylindole (DAPI, Abcam) for 10 min at room temperature to identify nuclei. Normal lung tissue was used as a positive control.

Quantification of immunohistochemical staining

All slides were scanned with an Olympus BX51 microscope equipped with an Olympus DP72 camera (Olympus Optical Co., Ltd., Tokyo, Japan) and a CRi Nuance multispectral imaging system (Cambridge Research & Instrumentation, Inc., Woburn, MA, USA). Positive staining was indicated by brownish granules. Then, after obtaining the images of signal unmixing, TAMs were analyzed based on the expression of CD68 and CD206. For each slide, 3 high-power fields of the tumor islets and tumor stroma per tissue section were separately selected. Tumor islets were defined as areas where tumor cells accounted for more than 70% of the total cells and tumor stroma as areas where tumor stromal cells accounted for more than 70% of the total cells(Li et al. 2018). The average number of these three fields represented the CD68- or CD206-positive cell number for each component of the tumor (Figure. 1A). Tumor neo-vessels marked by CD105 and tumor cells expressing PD-L1 were counted in six high-power fields selected at the tumor site, and the mean cell counts were documented. Image-Pro Plus 6.0 software was used to count positive cells. According to the HIS color selection scheme (H=0-30; I=0-255; S=0-255), define the area range and filter the below 50 pixels, which is nonspecific positive color noise. To determine the density of infiltrating macrophages, tumor neo-vessel density and PD-L1 expression, the cut-off value to classify subgroups was according to the median of each value.

For the combined group, according to the expression levels of the three components, taking the median value of each component as the cutoff value, the expression of tumor neo-vessels, macrophages and PD-L1 could be divided into low- and high-density groups. For the combined group, according to the expression levels of the three key stromal components, patients were divided into three subgroups according to the density of TAMs, tumor neo-vessels and PD-L1: group 1, all components were expressed at a low level; group 2, one of components was expressed at a high level; and group 3, all components were expressed at a high level. The represented digital images were independently collected by two pathology investigators who were blinded to the clinicopathological characteristics of all tissue specimens.

Statistical analysis

Statistical analyses were performed with SPSS 25.0 (SPSS Inc., Chicago, IL, USA). For categorical data, $\chi 2$ test was performed. Spearman rank correlation analysis was used to analyze the correlation between macrophages, tumor neo-vessels and PD-L1 expression. Differences in the CD68-positive TAMs and CD206–positive TAMs among the groups were analyzed by the Mann-Whitney test. The Kaplan-Meier method was used to estimate the survival curve for OS, and the log-rank test was used to assess the difference in survival between groups. The Cox regression model was used to perform univariate and multivariate analyses. A two-tailed $P<0.05$ was considered statistically significant.

Results

Distribution and expression of CD68-positive TAMs, CD206-positive TAMs, CD105 and PD-L1 in NSCLC

A series of 92 NSCLC specimens were examined for CD68- and CD206-positive TAMs in tumor islets and tumor stroma (Figure. 1B). The mean number of CD68-positive TAMs was significantly higher than that of CD206-positive TAMs in each area: in tumor islets, the mean numbers of CD68-positive TAMs and CD206-positive TAMs were 146 (median: 131, range: 8-348) and 70 (median 52, range 2-220), respectively ($P< 0.001$); in tumor stroma, the mean numbers of CD68-positive TAMs and CD206-positive TAMs were 186 (median: 169, range: 23-412) and 103 (median: 81, range: 7-358), respectively ($P< 0.001$). The distributions of CD68- and CD206-positive TAMs were significantly different in each location, with a higher distribution in the tumor stroma ($P<0.0001$). In addition, significant correlations were found between the distributions of CD68-positive TAMs and CD206-positive TAMs in each area (tumor islets: $r = 0.5179$; tumor stroma: $r = 0.5081$, $P<0.0001$, respectively) (Figure. 1C).

Coexpression analysis of CD105 and CD68 in NSCLC patients with multiplexed immunofluorescence

CD68 and CD105 were clearly expressed in the cytoplasm, and macrophages preferred to distribute along with tumor neovascularization (Figure. 3A). TAMs were positively correlated with blood vessel formation, and there was no obvious correlation between CD206 and CD105 in IHC (Spearman's rho=0.109, $P>0.05$), but the two (CD68 and CD105) showed a certain positive correlation (Spearman's rho= 0.241, $P=0.021$, Figure. 3B).

CD105 and PD-L1 staining were mainly located in the cytoplasm or on the cell membrane of the tumor stroma. PD-L1 expression is low in most tumor tissues; however, there are also cases with high expression. The quantitative density of CD105-positive cells in tumor tissues was 19-368 (median: 156). The number of PD-L1-positive cells in tumor tissues was 9-493 (median: 103). The distributions of CD68-positive TAMs, CD206-positive TAMs, CD105-positive cells and PD-L1-positive cells were significantly different in each location ($P<0.05$). However, all of the above components, including tumor neo-vessels, TAMs, and PD-L1, are heterogeneously expressed in the tumor microenvironment, in some cases with low expression and in other cases with high expression (Figure 1B-2A).

Correlations between CD68- and CD206-positive TAMs, tumor neo-vessels, PD-L1 expression and clinicopathological features

In the low and high CD68-positive TAM subgroups, the high tumor neo-vessel density cases were 20 (43.5%) and 26 (56.5%), respectively. In the low and high PD-L1 expression subgroups, high densities of CD68-positive TAMs were observed in 18 cases (40.9%) and 26 cases (59.1%), respectively. In the low and high CD206-positive TAM subgroups, 21 (45.7%) and 25 (54.3%) patients had high tumor neo-vessel density, respectively. In the low and high PD-L1 expression subgroups, there were 15 (33.3%) and 30 (66.7%) cases with high CD68-positive TAM density, respectively. Of note, tumor neo-vessels, CD68-positive TAMs and PD-L1 expression were not significantly correlated with any of the clinicopathological characteristics, which indicated that these key components of the tumor microenvironment were independent of clinical features, including tumor size, tumor histological type, degree of differentiation, lymph node metastasis and tumor staging (Table. 1a). Overall, CD206-positive TAMs in tumor islets and stroma were significantly correlated with lymph node metastasis. However, there was no significant correlation between TAMs, which were positive for both indicators in any part, and the other clinicopathological factors of NSCLC (Table. 1b).

Table 1a. Relationship between CD68-positive TAMs, CD206-positive TAMs, CD105, PD-L1 and the clinical features of NSCLC

		CD68-positive TAMs						CD206-positive TAMs			
		Tumor islets			Tumor stroma			Tumor islets			Tu
		Low	High	P-value	Low	High	P-value	Low	High	P-value	Low
Gender	F	8(38.1%)	13(61.9%)	0.193	10(47.6%)	11(52.4%)	0.759	9(42.9%)	12(57.1%)	0.390	11(52.4%)
	M	38(54.3%)	32(45.7%)		36(51.4%)	34(48.6%)		38(53.5%)	33(46.5%)		35(49.3%)
Age (years	60	21(51.2%)	20(48.8%)	0.908	20(48.8%)	21(51.2%)	0.760	21(51.2%)	20(48.8%)	0.982	23(56.1%)
	60	25(50.0%)	25(50.0%)		26(52.0%)	24(48.0%)		26(51.0%)	25(49.0%)		23(45.1%)
Smoking status	Never smoker	11(42.3%)	15(57.7%)	0.320	14(53.8%)	12(46.2%)	0.691	12(46.2%)	14(53.8%)	0.552	13(50.0%)
	Smoker	35(53.8%)	30(46.2%)		32(49.2%)	33(50.8%)		35(53.0%)	31(47.0%)		33(50.0%)
Histology	Adenocarcinoma	28(52.8%)	25(47.2%)	0.607	23(43.4%)	30(56.6%)	0.107	29(53.7%)	25(46.3%)	0.549	27(50.0%)
	Non-adenocarcinoma	18(50.5%)	20(52.6%)		23(60.5%)	15(39.5%)		18(47.4%)	20(52.6%)		19(50.0%)
Tumor size (cm)	5	28(47.5%)	31(52.5%)	0.423	31(52.5%)	28(47.5%)	0.606	31(51.7%)	29(48.3%)	0.879	32(53.3%)
	5	18(56.3%)	14(43.8%)		15(46.9%)	17(53.1%)		16(50.0%)	16(50.0%)		14(43.8%)
Differentiation	Low	5(33.3%)	10(66.7%)	0.413	6(40.0%)	9(60.0%)	0.144	11(68.8%)	5(31.3%)	0.371	10(62.5%)
	Moderate	18(52.9%)	16(47.1%)		13(38.2%)	21(61.8%)		17(50.0%)	17(50.0%)		17(50.0%)
	High	16(51.6%)	15(48.4%)		19(61.3%)	12(38.7%)		15(48.4%)	16(51.6%)		15(48.4%)
Lymph node metastasis	Negative	14(46.7%)	16(53.3%)	0.603	15(50.0%)	15(50.0%)	0.941	20(66.7%)	10(33.3%)	0.038	20(66.7%)
	Positive	32(52.5%)	29(47.5%)		31(50.8%)	30(49.2%)		27(43.5%)	35(56.5%)		10(33.3%)
Stage	I, II	25(61.0%)	16(39.0%)	0.072	24(58.5%)	17(41.5%)	0.168	22(52.4%)	20(47.6%)	0.820	24(57.1%)
	III	21(42.0%)	29(58.0%)		22(44.0%)	28(56.0%)		25(50.0%)	25(50.0%)		22(44.0%)

Table 1b (follow Table 1a)

		CD105-positive cells			PD-L1-positive cells		
		Low	High	P-value	Low	High	P-value
Gender	F	11(52.4%)	10(47.6%)	0.804	14(66.7%)	7 (33.3%)	0.082
	M	35(49.3%)	36(50.7%)		32(45.1%)	39(54.9%)	
Age (years	60	18(43.9%)	23(56.1%)	0.294	19(46.3%)	22(53.7%)	0.529
	60	28(54.9%)	23(45.1%)		27(52.9%)	24(47.1%)	
Smoking status	Never smoker	12(46.2%)	14(53.8%)	0.643	16(61.5%)	10(38.5%)	0.552
	Smoker	34(51.5%)	32(48.5%)		30(45.5%)	36(47.0%)	
Histology	Adenocarcinoma	26(48.1%)	28(51.9%)	0.672	25(46.3%)	29(54.5%)	0.165
	Non-adenocarcinoma	20(52.6%)	18(47.4%)		21(55.3%)	17(44.7%)	
Tumor size (cm)	5	28(46.7%)	32(53.5%)	0.381	31(51.7%)	29(48.3%)	0.662
	5	18(56.3%)	14(43.8%)		15(46.9%)	17(53.1%)	
Differentiation	Low	9(56.3%)	7(43.8%)	0.691	7(43.8%)	9(56.3%)	0.399
	Moderate	15(44.1%)	19(55.9%)		16(47.1%)	18(52.9%)	
	High	16(51.6%)	15(48.4%)		19(61.3%)	12(38.7%)	
Lymph node metastasis	Negative	14 (46.7%)	16(53.3%)	0.656	14(46.7%)	16(53.3%)	0.656
	Positive	32(51.6%)	30(48.4%)		32(51.6%)	30(48.4%)	
Stage	I, II	20(47.6%)	22(52.4%)	0.675	24(57.1%)	18(42.9%)	0.209
	III	26(52.0%)	24(48.0%)		22(44.0%)	28(56.0%)	

Prognostic significance of tumor stromal features in NSCLC

Among 92 NSCLC cases, the median OS was 22.5 months. A Kaplan-Meier analysis revealed that the degree of differentiation, the different parts of the CD68-positive, CD206-positive TAMs, and the expression of PD-L1 and combined features in tumor tissues were associated with the OS of NSCLC patients (P<0.05) (Figure. 1B, 2D). Furthermore, CD68-positive TAMs and PD-L1 expression were negatively related to OS in tumor tissue (Spearman's rho= -0.342, P= 0.001 and Spearman's rho= -0.246, P = 0.018, respectively). In peritumor tissues, except for the expression of PD-L1 (Spearman's rho= -0.207, P = 0.05), the expression of the other three components was negatively correlated with OS, but the difference was not statistically significant (P>0.05). The combined analysis indicated that the OS of the third group was worse than that of the first and second groups of patients (P=0.016).

Cox proportional hazard models were used to test the prognostic significance of macrophage infiltration, tumor neo-vessels and the expression of PD-L1 when adjusted for known prognostic factors. In univariate analysis, differentiation degree, CD68-positive TAMs in tumor islets, CD68-positive TAMs and CD206-positive TAMs in tumor stroma and PD-L1-positive cells were related to OS. In addition, analysis of the combined key components of tumor neo-vessels, macrophages and PD-L1 indicated that the mortality risk in combined group 4 was significantly increased (P = 0.045, Figure 4D). As presented in Table 2, the factors with $P \leq 0.20$ in the univariate analyses and the components of interest were entered into the multivariate analyses. Smoking status, differentiation degree, CD68-positive TAMs in tumor islets and PD-L1-positive cells were independent prognostic factors for OS (P<0.05 for all) (Table 2).

Table 2. Univariate and multivariate analyses of the clinicopathological factors for OS in non-small cell lung carcinoma

	Univariate analysis			multivariate analyses		
	HR	(95%CI)	P-value	HR	(95%CI)	P-value
Gender (Male vs. Female)	0.980	0.572-1.678	0.941		/	
Age (60 vs. 60)	1.269	0.804-2.005	0.306		/	
Smoking status (Smoker vs. Never smoker)	1.162	0.698-1.932	0.564	0.505	0.160-1.593	0.244
Histological type (Adenocarcinoma vs. Non-adenocarcinoma)	0.734	0.462-1.168	0.192	0.444	0.167-1.183	0.104
Tumor size (5vs. 5)	1.373	0.850-2.218	0.195	1.945	1.089-3.475	0.025
Differentiation			0.045			0.033
(low vs. Moderate)	2.118	1.087-4.127	0.028	0.595	0.252-1.406	0.237
(low vs. High)	1.752	1.013-3.030	0.045	0.336	0.146-0.774	0.010
Lymph node metastasis (Positive vs. Negative)	0.909	0.694-1.190	0.486	0.810	0.384-1.710	0.581
Stage status (III vs. I, II)	1.123	0.706-1.785	0.624	0.785	0.374-1.645	0.521
CD105 expression (high vs. low)	1.106	0.698-1.753	0.667	1.002	0.998-1.005	0.301
PD-L1 expression (high vs. low)	1.685	1.066-2.663	0.025	1.003	1.001-1.011	0.010
CD68-positive TAMs in tumor islets (high vs. low)	1.666	1.051-2.641	0.030	1.006	1.001-1.011	0.031
CD206-positive TAMs in tumor islets (high vs. low)	1.580	0.996-2.506	0.052	0.999	0.991-1.007	0.750
CD68-positive TAMs in tumor stroma (high vs. low)	1.916	1.202-3.055	0.006	1.000	0.995-1.005	0.941

Discussion

It is generally believed that cancer invasion, including NSCLC, is not merely a local problem but a multifactor and multistep continuum, with a variety of molecular dysfunctions and cell signaling dysregulations. Genetic or epigenetic changes in cancer cells are the initial factors driving carcinogenesis, and the responses of stromal cells in the tumor microenvironment may promote or regulate cancer invasion and metastasis, which ultimately results in an altered tumor microenvironment favoring cancer invasion and progression. In this study, CD68 was selected as the TAM marker. CD68 is commonly considered to be a pan-macrophage marker but cannot distinguish between M1 and M2 subtypes(Falini et al. 1993). M2 macrophages have a variety of surface markers, including CD163, CD204 and CD206. Among them, CD206 is known to be expressed on the surface of most classes of macrophages and dendritic cell subpopulations and is routinely used to identify the M2 phenotype(Gordon 2003). Recent studies on M2 macrophage markers (such as CD163 and CD204) have also shown that M2 macrophage density is more closely related to poor prognosis than CD68-positive TAMs[(Komohara et al. 2014)]. TAMs have different effects on the prognosis of different types of cancer. In NSCLC, the prognostic relevance of TAMs is still under debate. The reasons for the inconsistent reports may be related to the choice of markers, different statistical powers, and differences in evaluation modes. CD105 is an endoglin used to evaluate blood vessels such as CD31 and CD34 (total endothelial markers), while CD105 is considered to recognize only abnormal blood vessels induced by tumors(Weidner et al. 1992). To our knowledge, the present study is the first to compare the TAM distribution using CD68 and CD206 in two intratumoral areas and to compare the distribution of TAMs, tumor neo-vessels and PD-L1 in NSCLC.

The analyzed results showed that the number of CD68-positive TAMs and CD206-positive TAMs was higher in the tumor stroma but lower in tumor islets, which was consistent with several previous reports(Dai et al. 2010; Li et al. 2018). We also found that there was a strong correlation between the distribution of CD68-positive TAMs and CD206-positive TAMs in tumor islets and tumor stroma. Furthermore, the mean numbers of CD68-positive TAMs in each location of the tumor islets and tumor stroma were significantly higher than those of CD206-positive TAMs. Univariate analysis showed that a large number of CD68-positive TAMs and CD206-positive TAMs in the tumor stroma were associated with shorter OS, which was consistent with the results of Li Z et al(Li et al. 2018) and showed that the tumor stroma is the most suitable intratumoral area for evaluating TAMs. This study also found that the comprehensive analysis of CD206-positive stromal TAMs, CD105 and PD-L1 had a certain relationship with prognosis. The number of positive cells in each part (tumor islets and tumor stroma) was summed, and CD68-positive TAMs or CD206-positive TAMs were combined with the other two key components to analyze the results, and no interesting results were obtained. The discrepant role and regulatory mechanism of TAMs in different interspaces of the tumor microenvironment merit further study.

A high density of macrophages is associated with poor prognosis and immune failure in cancer patients, suggesting that macrophages play an important role in assisting tumors in escaping immune surveillance. During tumorigenesis, macrophages produce a mutagenic inflammatory environment and promote the growth of tumors. As tumors develop into malignant tumors, macrophages stimulate angiogenesis, enhance tumor cell migration, invade and inhibit antitumor immunity(Qian and Pollard 2010). In tumors, new blood vessels are usually abnormal, immature, and leaky and show insufficient or excessive expression in tumor tissues depending on the tumor type. New blood vessels support rapid tumor tissue growth, providing nutrients and oxygen to thriving tumor cells(Muz et al. 2015). However, in this study, tumor neo-vessel density was not significantly correlated with the prognosis of patients with NSCLC, and macrophages have a certain correlation with the prognosis of patients with NSCLC. We found that CD68 and CD105 have a certain correlation based on multi-immunofluorescence technology, and the colocalization of proteins can be determined by using different tags for double labeling, which is most suitable for accurate analysis of coexpressed protein markers(Saylor et al. 2018). Our results suggested that macrophages have a significant role in tumor neo-vessels in the process of cancer invasion and metastasis. Macrophages can release a variety of factors, such as cytokines, chemokines, hormones and metabolites, to directly or indirectly promote tumor progression(Qian and Pollard 2010). TAMs are considered to be “angiogenesis switches” and a key factor leading to a proangiogenic environment(Murdoch et al. 2008) (Mazzieri et al. 2011). We confirmed that PD-L1 expression was highly correlated with the prognosis of NSCLC and could be used as an independent prognostic factor for patients with NSCLC. PD-L1 mediates immunosuppressive signals. The results also

showed that patients with low PD-L1 expression in tumor cells may have a longer OS. However, several studies suggest that PD-L1 overexpression achieves longer survival in early NSCLC(Cooper et al. 2015), breast carcinoma(Schalper et al. 2014), gastric cancer(Zheng et al. 2014) and colorectal cancer[Droeser et al. 2013]. Other studies have shown that there is no correlation between PD-L1 expression and OS[Sorensen et al. 2014]. Many recent studies have reported that high PD-L1 is associated with poor prognosis in NSCLC(Igawa et al. 2017; Keller et al. 2018; Li et al. 2019). In different studies, the definition of PD-L1 positivity or high density was different, leading to difficulties in concluding the relationship between PD-L1 expression and NSCLC prognosis. Evidence suggests that PD-L1 expression is actually an adaptive mechanism and may be a response of tumor cells to host immune pressure(Taube et al. 2012). It can also be understood that the expression of PD-L1 is related to the endogenous immune response, such as tumor infiltrating lymphocytes (TILs) in NSCLC and indoleamine 2,3-dioxygenase-1 (IDO-1) expressed by dendritic cells (DCs)(Mandarano et al. 2019). Any possible prognostic significance is not directly related to a single immune signal but to the overall balance between the host's antitumor immune response and tumor-mediated immunosuppression.

It has been reported that tumor cells can induce increased expression of M2 macrophages and PD-L1(Wen et al. 2018). Our study demonstrated that the expression of PD-L1 in cancer cells was correlated with the density of macrophages. A Japanese study suggested that M2 macrophages were associated with PD-L1 expression on NSCLC cytotoxic T cells[Sumitomo et al. 2019]. There are also correlations in other solid tumors, such as gastric adenocarcinoma(Harada et al. 2018).

In conclusion, key components in the tumor microenvironment may interact with cancer cells and together accelerate cancer invasion and metastasis. The present study implied that the different molecular profiles of the tumor microenvironment were intimately linked with the prognosis of NSCLC patients. The combined analysis of key components in the tumor microenvironment was an independent prognostic factor, which showed the importance of comprehensively analyzing the tumor microenvironment.

Declarations

Funding

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Conflict of interest

The authors have stated that they have no conflicts of interest.

Contributions

(I) Conception and design: HJ Ying, M Fan, M Chen XJ Lai ; (II) Administrative support: M Chen, GP Cheng, QX Chen, YH Jiang, Q Zhao ; (III) Provision of study materials or patients: GP Cheng, SF Yang, QX Chen, YH Jiang, Q Zhao; (IV) Collection and assembly of data: M Fan, HJ Ying, QQ Hang; (V) Data analysis and interpretation: HJ Ying , QQ Hang, JN Jin; (VI) Manuscript writing: HJ Ying , QQ Hang; (VII) Final approval of manuscript: All authors.

Ethical approval

All procedures of this study were in accordance with the ethical standards of the Institutional Research Committee and with the 1964 Helsinki declaration and its later amendments.

Informed consent

This retrospective study was approved by the Ethics Committee of Zhejiang Cancer Hospital. Informed consent was waived owing to the retrospective nature of this study.

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Figures

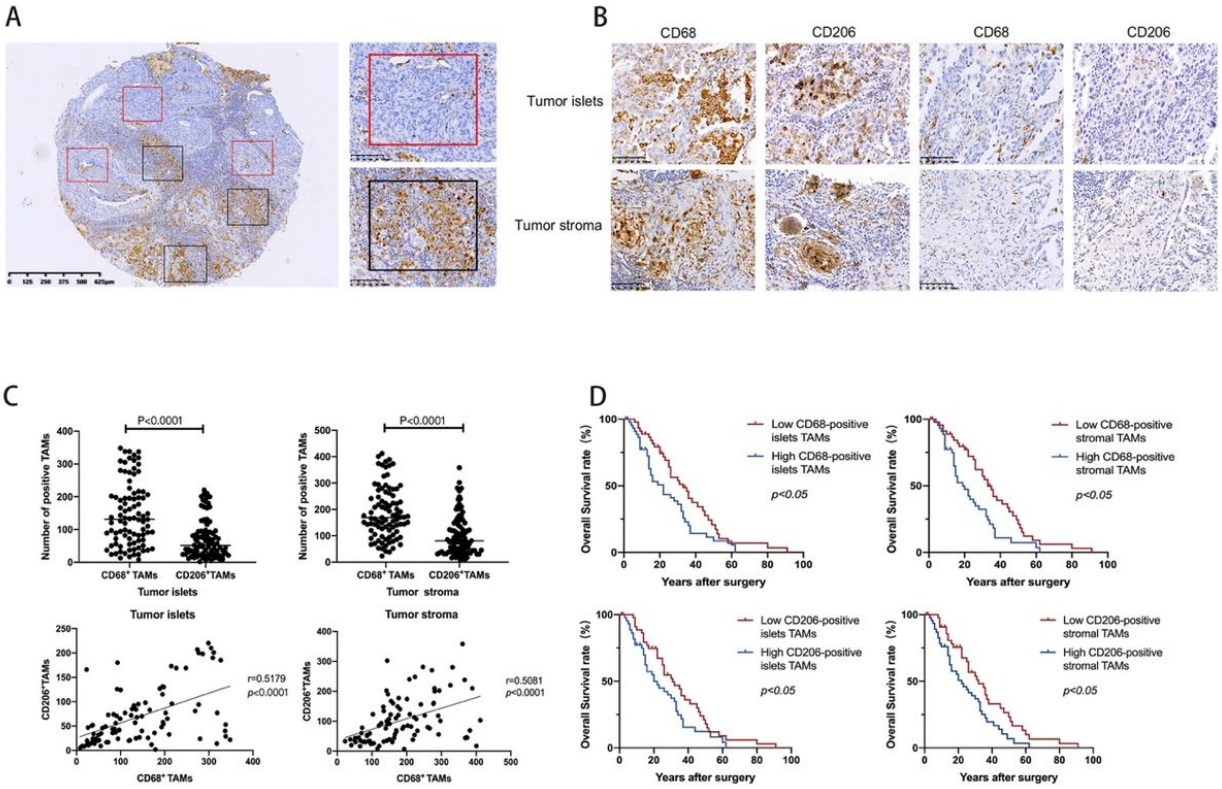

Figure 1

(A) The evaluation of IHC staining. Representative CD68 IHC-stained slides scanned by a pathology digital imaging system at 4× (□). For each slide, 3 representative 0.1 mm2 fields were separately selected for tumor islets marked with a red frame (□) and tumor stroma marked with a black frame (□). 20× (B) Immunostaining of TAMs in NSCLC with CD68 and CD206 antibodies. a-b: Cases with a high number of CD68+ TAMs and CD206+ TAMs in tumor islets. c-d: Cases with a low number of CD68+ TAMs and CD206+ TAMs in tumor islets. e-f: Cases with a high number of CD68+ TAMs and CD206+ TAMs in the tumor stroma. g-h: Case with a low number of CD68+ TAMs and CD206+ TAMs in the tumor stroma. 20× (C) The distributions of CD68+ TAMs and CD206+ TAMs in tumor islets and tumor stroma (above). The correlations between CD68+ TAMs and CD206+ TAMs in tumor islets and tumor stroma (below). (D) CD68+ TAMs and CD206+ TAMs in tumor islets and stroma with patient OS in NSCLC. P<0.05. (A) The evaluation of IHC staining. Representative CD68 IHC-stained slides scanned by a pathology digital imaging system at 4× (□). For each slide, 3 representative 0.1 mm2 fields were separately selected for tumor islets marked with a red frame (□) and tumor stroma marked with a black frame (□). 20× (B) Immunostaining of TAMs in NSCLC with CD68 and CD206 antibodies. a-b: Cases with a high number of CD68+ TAMs and CD206+ TAMs in tumor islets. c-d: Cases with a low number of CD68+ TAMs and CD206+ TAMs in tumor islets. e-f: Cases with a high number of CD68+ TAMs and CD206+ TAMs in the tumor stroma. g-h: Case with a low number of CD68+ TAMs and CD206+ TAMs in the tumor stroma. 20× (C) The distributions of CD68+ TAMs and CD206+ TAMs in tumor islets and tumor stroma (above). The correlations between CD68+ TAMs and CD206+ TAMs in tumor islets and tumor stroma (below). (D) CD68+ TAMs and CD206+ TAMs in tumor islets and stroma with patient OS in NSCLC. P<0.05

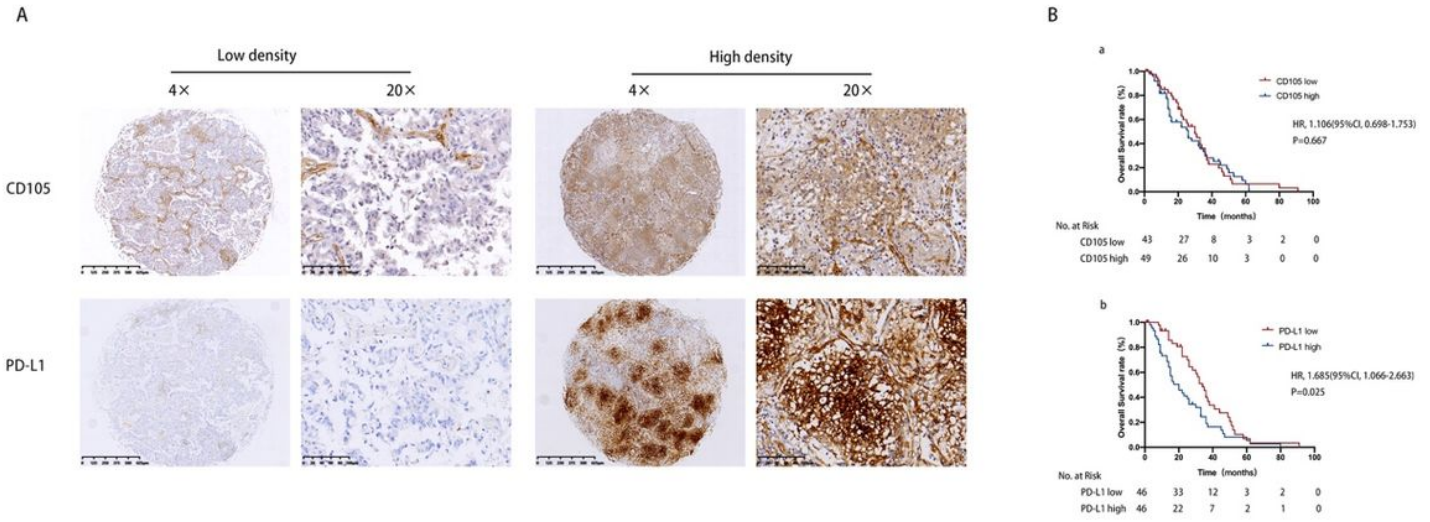

Figure 2

(A) IHC staining for CD105 and PD-L1 expression in NSCLC tissues. From left to right, respectively, low densities and high densities. Every panel shows low tumor neo-vessel density and low expression of PD-L1, magnifications ×4 and ×20, respectively. (B) Cumulative OS of patients with NSCLC. (a) Tumor neo-vessels were not associated with the OS of patients with NSCLC. (c) Patients in the high PD-L1 expression groups had a higher risk of death. HR, Hazard ratio. P<0.05.

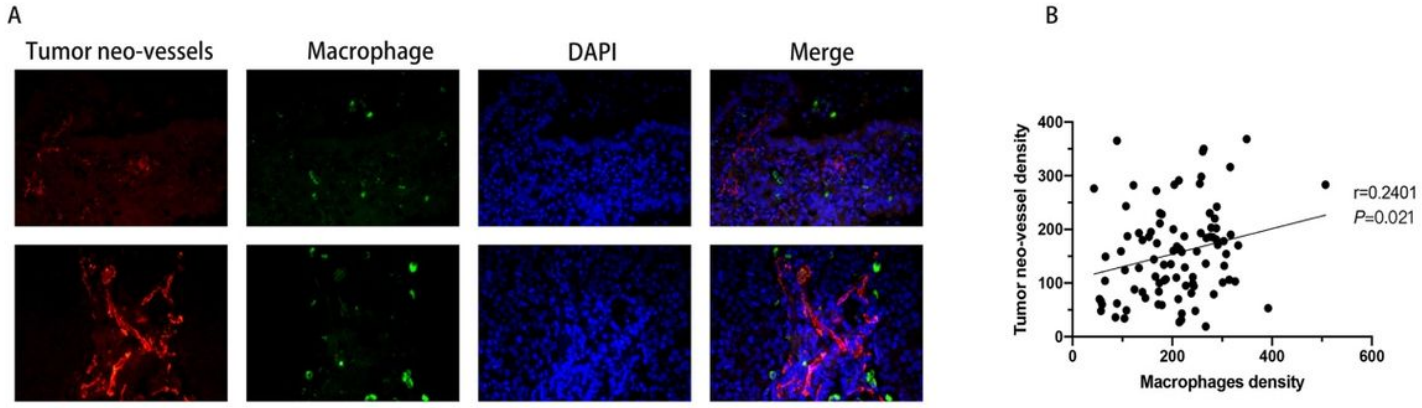

Figure 3

The density of tumor neo-vessel in NSCLC tissue positively correlates with the density of macrophages. (A) Representative fluorescence pictures showing the signal for DAPI (blue), tumor neo-vessel (CD105, red) and macrophage (CD68, green) staining in NSCLC tissue. Bar=100 μm. (B) The density of tumor neo-vessel in 92 NSCLC tissues positively correlated with the density of macrophages. P<0.05

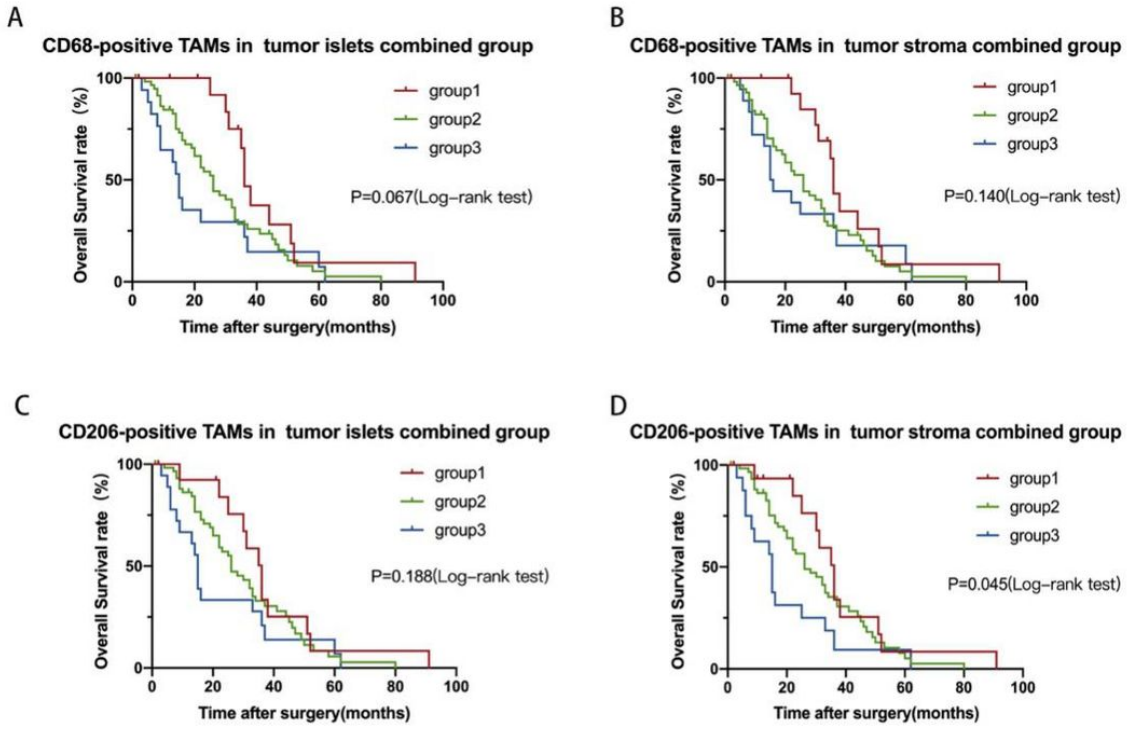

Figure 4

Cumulative OS of patients with NSCLC. group 1, all components were expressed at a low level; group 2, one of components was expressed at a high level; and group 3, all components were expressed at a high level. (A) Combined CD68-positive TAMs in tumor islets, CD105 and PD-L1 comprehensive analysis with OS of patients with NSCLC. (B) Combined CD68-positive TAMs in the tumor stroma, CD105 and PD-L1 comprehensive analysis with OS of patients with NSCLC. (C) Combined CD206-positive TAMs in tumor islets, CD105 and PD-L1 comprehensive analysis with OS of patients with NSCLC. (D) Combined CD206-positive TAMs in the tumor stroma, CD105 and PD-L1 comprehensive analysis with OS of patients with NSCLC. In these combinations, group 3 had a higher risk of death. P<0.05