



CD39: the potential target in small cell lung cancer

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Background: It has been proven that the treatment window of small cell lung cancer (SCLC) is short, so it is vital to find other possible therapeutic targets. CD39 inhibits natural killer (NK) cells and promotes the occurrence and metastasis of tumors. There has been little research about the role of CD39 in SCLC, so we explored the correlation between CD39 and other surface antigens, and its association with survival in SCLC.

Methods: This study included 75 patients with SCLC from Shanghai Pulmonary Hospital. After paraffin embedding and sectioning, immunohistochemistry (IHC) was applied. Then we identify cutoff value for CD39 and other surface antigens based on the analysis of ROC curve in RFS by SPSS. All statistical analyses were based on SPSS and Graphpad Prism8. Chi-square test, Kendall's tau-b correlation analysis, Logistic regression analysis, Kaplan-Meier method, univariate and multivariate Cox regression analysis were conducted. In all analyses, $P = 0.05$ distinguished whether they had statistical significance.

Results: Of the 75 SCLC patients enrolled in this study, 61.33% positively expressed CD39. A correlation between CD39 and programmed cell death-ligand 1 (PD-L1) ($P=0.007$), CD3 ($P<0.001$), CD4 ($P<0.001$), CD8 ($P<0.001$), and forkhead box P3 (FOXP3) ($P<0.001$) on tumor-infiltrating lymphocytes (TILs) was identified by correlation analysis and logistic regression analysis. Based on Kaplan-Meier survival analysis, we found that CD39 affected relapse-free survival (RFS) [negative *vs.* positive, 95% confidence interval (CI): 0.2765–0.9862, $P=0.0390$]. SCLC patients with high-expressed CD39 and low-expressed PD-L1 had poor prognosis ($P<0.001$). Positive expression of CD39 and negative expression of CD3, CD4, CD8, and FOXP3 also indicated shorter RFS ($P=0.0409$). Univariate and multivariate Cox regression analysis was performed to confirm the factors that influenced RFS.

Conclusions: CD39, programmed cell death-1 (PD-1), and PD-L1 expressed on TILs but not on tumor cells. CD39 has a significant association with PD-L1, CD3, CD4, CD8, and FOXP3 on TILs. The positive expression of CD39 predicts poor prognosis. SCLC patients with low expression of CD39 combined with high expression of PD-L1 or CD3, CD4, CD8, and FOXP3 have a more favorable prognosis.

Keywords: CD39; small cell lung cancer (SCLC); programmed cell death-ligand 1 (PD-L1); tumor-infiltrating lymphocyte (TIL); progress-free survival; programmed cell death-1 (PD-1)

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Introduction

Small cell lung cancer (SCLC) is a pathological type of lung cancer, accounting for about 15% of lung cancer cases (1,2). It originates from the precursors of neuroendocrine cells (3). SCLC proliferates, and incidence and mortality rates are high (4). The standard treatment for extensive-stage SCLC (ES-SCLC) is 4 to 6 cycles of platinum-based double chemotherapy (5). Plenty of clinical trials mainly focused on ES-SCLC like CheckMate 032 (6), ECOG-ACRIN 2511 (7), CASPIAN (8) are currently undergoing. The therapeutic schedule in common use is surgical management for limited-stage SCLC (LS-SCLC) and chemotherapy and radiotherapy for ES-SCLC (9). Several studies have suggested that SCLC is sensitive to chemotherapy (10), yet the effect is temporary (11). Most patients are responsive to the initial treatment of platinum doublet therapy, but the majority with this metastatic disease still have rapid tumor progression (12,13). It is of great importance to explore the function of other possible therapeutic targets.

The cluster of differentiation 39 (CD39), also known as ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1), can hydrolyze extracellular ATP and ADP (14). It is one of the key enzymes in adenosine pathway (15) and targeted adenosine can play a role in tumor immunotherapy (16). Tumour-derived exosomes (TDEs) change tumor microenvironment through the expression of CD39, which may be of great significance for immunotherapy as well (17). CD39 pathway inhibits natural killer (NK) cells and promotes the occurrence and metastasis of tumors (18). Evidence shows it may evaluate the immunotherapy effect because it distinguishes tumor-related or unrelated CD8 + tumor-infiltrating lymphocytes (TILs) in human solid tumors and causes the failure of CD8 + TILs (19-22). TGF- β -mTOR-HIF-1 signaling transduction is a pathway of adenosine pathway and up regulates CD39 (23). This demonstrates a possible signal transduction in NSCLC. Notably, it has previously shown that CD39 is highly elevated in intratumoral immune cells in NSCLC (24). Thus, CD39 can be viewed as a novel target for chemotherapy and immunotherapy in non-small cell lung cancer (NSCLC) (25).

Based on potential function and minimal existing research about CD39 in SCLC, we aimed to explore the correlation between CD39 and other surface antigens, including programmed cell death-ligand 1 (PD-L1), programmed cell death-1 (PD-1), CD3, CD4, CD8, and forkhead box P3 (FOXP3). We also researched whether it affected relapse-free survival (RFS) to assess its association with survival in SCLC.

Since Steven Rosenberg and his team found that CD39 may be a possible biomarker for advanced solid tumors (19), CD39 has offered a new strategy to treat advanced cancer patients. Although there were some articles about the relationship between CD39 and NSCLC in recent years, our study is the first to research in SCLC. Compared with NSCLC, malignant degree of SCLC is higher and it is easier to metastasis in early stage. Despite the sensitivity to chemotherapy, drug resistance is the main reason for poor prognosis (26). Considering the bad curative effect, it is much more important to find a potential target in SCLC. Additionally, we discovered the relationship between CD39 and other surface antigens in SCLC. Before this no one had done so. Advance experimental technology and means like immunohistochemistry were applied. Importantly, compared with others, various statistical methods such as univariate and multivariate Cox regression analysis were also used in this research to ensure the reliability of the experimental results. Thus, at current time, we make a breakthrough research on CD39 in SCLC.

Methods

Sample extraction

This research was approved by the ethics committee of the Shanghai Pulmonary Hospital, Tongji University. Tumor samples were surgically collected from 75 patients with SCLC from Shanghai Pulmonary Hospital (from January 2014 to December 2018). Because of the small number of SCLC patients, we have included all SCLC patients in our hospital during this period to increase the amount of cases. Before the surgery, 46 patients had undergone chemotherapy, and 29 had not. The 8th edition of the TNM classification for lung cancer was used to identify the

varying stages of SCLC and prognosis of the patients (27). Written consent was given by all patients, and the experiment confirmed with the tenets of the Declaration of Helsinki (as revised in 2013).

Paraffin embedding and sectioning

Fresh tissues from the patients were fixed in 10% formalin for at least 24 hours (h). After placing them in the dehydration box, they were sequentially immersed in the following solutions for dehydration: 75% alcohol for 4 h, 85% alcohol for 2 h, 90% alcohol for 2 h, 95% alcohol for 1 h, anhydrous ethanol for 30 minutes (min), another anhydrous ethanol box for 30 min, alcohol benzene for 5–10 min, xylene I for 5–10 min, xylene II for 5–10 minutes, wax I for 1 h, wax II for 1 h, and finally wax III for 1 h. Next, we embedded the tissues in paraffin wax and cooled them at -20°C until they became wax blocks. After this entire process, we sliced the wax blocks to a thickness of $4\ \mu\text{m}$ and dried them (28).

Immunohistochemistry (IHC) for CD39 and other surface antigens

The prepared sections were first conventionally dewaxed to water. Antigen thermal remediation was performed by soaking the sections in phosphate buffer saline (PBS) and placing them in a 60°C oven for 1 h. Based on antigen repair, the sections were cooled at room temperature. The cooled sections were immersed in water for 2 min and then placed in 0.3% H_2O_2 (an endogenous peroxidase inhibitor) for 15 min to reduce nonspecific background reactivity caused by endogenous peroxidase. Later, they were washed with water for 2 min and PBS buffer for 2 min. To reduce nonspecific background staining, we next added Ultra V Block and incubated at room temperature for 5 min. Further, primary antibodies like CD39, PD-1 (1:100, Golden bridge Zhongshan, Beijing ZM-0381), PD-L1 (E1L3N 1: 300, CST # 13684S), CD3 (1:100, Dako A0452), CD4 (1:80, Dako M7310), CD8 (1:100, Dako M7103), FOXP3 (1:100, BioLegend 320101) were applied. After incubating at 37°C for 1–2 h and washing in a PBS buffer, we incubated an anti-enhancer at room temperature for 20 min, washed with a PBS buffer, and added horseradish peroxidase (HRP) polymer (an enzyme-labeled polymer) for 30 min. Then, 1 mL 3,3'-diaminobenzidine (DAB) plus substrate and chromogen solution was added to each section. These sections were stained with hematoxylin

and bluing agent. Finally, we started dehydration with 85% ethanol, 95% ethanol, and anhydrous ethanol. Xylene was used for transparency, and neutral gum was used as a sealant.

The identification of cut off value for CD39 and other surface antigens

TILs were composed of plasma cells, macrophages and lymphocytes (29). They were lymphocytes that leave the blood and migrate to the tumor area (30). We observed the lymphocytes in the microscope field of HE staining section mentioned above. 25% was selected as the cut off value of CD39 on TILs considering both sensitivity and specificity; this was decided after analysis of the ROC curve for RFS by IBM SPSS Statistics 22.0 (31). The cut-off value is calculated as the point which makes (sensitivity + specificity-1) maximum. According to the article published, the cut-off values for PD-1 and PD-L1 on tumor cells were 8% and 50%, while PD-1 and PD-L1 on TILs were 1% and 5% (32). Furthermore, CD3 >40%, CD4 >30%, CD8 >30% and FOXP3 >10% in staining were also viewed as positive in the same way.

Statistics analysis

All statistical analyses were based on SPSS (version 22.0) (31) and Graphpad Prism8 (33). We applied the Chi-square test to assess if the expression of CD39 on TILs and other clinicopathologic parameters were cognate. Kendall's tau-b correlation analysis was used for the evaluation of correlation and agreement of CD39 and other surface antigens. Logistic regression analysis was used to evaluate the effect of surface antigens. The Kaplan-Meier method was used to estimate the survival curves.

Additionally, univariate and multivariate Cox regression analysis was conducted to discover possible factors associated with prognosis. The odds ratio (OR) in the model referred to the correlation between surface antigens and RFS. In all analyses, $P=0.05$ distinguished whether there was statistical significance.

Results

Characteristics of patients enrolled in the study

Out of the 75 SCLC patients included in our research, 62 were male and 13 female. The median age was 63 (upper

Table 1 Baseline information of 75 patients diagnosed with SCLC

Variables	Total patients (N=75)
Age, years	
Mean \pm SD	63.28 \pm 9.46
Median (range)	63 (38–81)
Gender	
Female	13 (17.33%)
Male	62 (82.67%)
Smoking status	
Non-smoker	32 (42.67%)
Smoker	43 (57.33%)
SCLC stage	
Stage I	30 (40.00%)
Stage II	10 (13.33%)
Stage III	35 (46.67%)
T	
T1	32 (42.67%)
T2	31 (41.33%)
T3	10 (13.33%)
T4	2 (2.67%)
M	
M0	72 (96.0%)
M1	3 (4.00%)
N	
N0	34 (45.33%)
N1	13 (17.33%)
N2	28 (37.34%)
Chemotherapy	
Yes	46 (61.33%)
No	29 (38.67%)

SCLC, small cell lung cancer.

limit: 81, lower limit: 38). The majority (57.33%) were smokers. By TNM classification from The IASLC Lung Cancer Staging Project 8th Version (27), all patients were sorted into T1–T4, M0–M1, and N0–N2. The baseline information of all patients enrolled is displayed in *Table 1*.

Expression of surface antigens on different cells tested by IHC

After IHC, we performed qualitative and relative quantitative research on the surface antigens. In this study, CD39, PD-1, and PD-L1 have no expression on tumor cells; 61.33% of patients expressed CD39 positively on TILs while 38.67% did not. The positive expression on TILs was 38.67% for PD-1 and 37.33% for PD-L1. The number of SCLC patients who expressed CD3, CD4, CD8, and FOXP3 is displayed in *Table 2*.

Exploration for the relationship of surface antigens and clinical data

We set $P=0.05$ as a threshold. By Chi-square test, the expression of CD39 on TILs had no statistical correlation with clinical data like age ($P=0.428$), gender ($P=0.549$), smoking status ($P=0.477$), SCLC stage ($P=1.000$), and chemotherapy ($P=0.336$) (*Table 3*). Meanwhile, a possible correlation between CD39 expression on TILs and some other surface antigens was proven with $P<0.05$. More specifically, PD-L1 ($P=0.007$), CD3 ($P<0.001$), CD4 ($P<0.001$), CD8 ($P<0.001$), and FOXP3 ($P<0.001$) expression on TILs were statistically correlated with CD39 expression on TILs (*Table 3*).

Correlation analysis and logistic regression analysis

Kendall's tau-b correlation analysis was performed. All possible statistically correlated surface antigens mentioned above were included in the calculation to confirm a significant correlation. The specific Kendall's tau-b and p-value of the targets are shown in *Table 4*. Results showed that the expression of PD-L1, CD3, CD4, CD8, and FOXP3 on TILs had a positive correlation with CD39 expression on TILs. This result had a statistical sense. Among all of them, the CD3 expression had the highest relevancy (Kendall's tau-b =0.552, $P<0.001$).

Based on bivariate logistic regression, we once again supported the statistical correlation mentioned above as $P\leq 0.05$ (*Figure 1, Table S1*). All regression coefficients were more significant than zero, which means a positive correlation coefficient between variables. CD8 expression had the maximum regression coefficient of 3.183 but had no statistical significance ($P=0.998$). Bivariate logistic

Table 2 Relationships between CD39 expression on TILs and other checkpoints (Chi-square test)

Characteristic	CD39 expression on TILs		P value
	Negative	Positive	
PD-1 expression on tumor cells			0.053
Negative	29 (38.67%)	46 (61.33%)	
Positive	0 (0.00%)	0 (0.00%)	
PD-1 expression on TILs			0.519
Negative	22 (29.33%)	24 (32.00%)	
Positive	7 (9.33%)	22 (29.34%)	
PD-L1 expression on tumor cells			0.007
Negative	29 (38.67%)	44 (58.67%)	
Positive	0 (0.00%)	2 (2.66%)	
PD-L1 expression on TILs			<0.001
Negative	24 (32.00%)	23 (30.67%)	
Positive	5 (6.66%)	23 (30.67%)	
CD3 expression on TILs			<0.001
Negative	24 (32.00%)	12 (16.00%)	
Positive	5 (6.66%)	34 (45.34%)	
CD4 expression on TILs			<0.001
Negative	28 (37.33%)	20 (26.67%)	
Positive	1 (1.33%)	26 (34.67%)	
CD8 expression on TILs			<0.001
Negative	29 (38.67%)	25 (33.33%)	
Positive	0 (1.00%)	21 (28.00%)	
FOXP3 expression on TILs			–
Negative	28 (37.33%)	23 (30.67%)	
Positive	1 (1.33%)	23 (30.67%)	
CD39 expression on tumor cells			–
Negative	29 (38.67%)	46 (61.33%)	
Positive	0 (0.00%)	0 (0.00%)	

TIL, tumor-infiltrating lymphocyte.

regression of PD-L1, CD3, CD4, and FOXP3 on TILs showed that CD39 impacted them greatly.

In summary, the significant association between CD39 expression on TILs, and PD-L1, CD3, CD4, and FOXP3 was identified based on correlation analysis and logistic regression analysis performed after the Chi-square test.

Predictive factors of RFS in SCLC

By Kaplan-Meier survival analysis, relationships between RFS status and clinical data were evaluated (*Figure 2A,B,C,D,E, Table S2*). It was confirmed that age (<70 *vs.* >70, 95% CI: 0.1964–0.9172, P=0.0119) and SCLC stage (Stage I–II *vs.* Stage III–IV, 95% CI: 0.2186–

Table 3 Relationships between CD39 and clinical data

Characteristic	CD39 expression on TILs		P
	≤25	>25	
Age			0.428
<70	23 (30.67%)	32 (42.67%)	
≥70	6 (8.00%)	14 (18.66%)	
Gender			0.549
Female	6 (8.00%)	7 (9.33%)	
Male	23 (30.67%)	39 (52.00%)	
Smoking status			0.477
Non-smoker	19 (25.33%)	26 (34.67%)	
Smoker	10 (13.33%)	20 (26.67%)	
SCLC stage			1.000
Stage I-II	15 (20.00%)	25 (33.33%)	
Stage III-IV	14 (18.67%)	21 (28.00%)	
Chemotherapy			0.336
No	9 (12.00%)	20 (26.67%)	
Yes	20 (26.67%)	26 (34.66%)	

SCLC, small cell lung cancer.

Table 4 Relationships between CD39 expression on TILs and other checkpoints (Kendall's tau-b correlation analysis)

Characteristic	CD39 expression on TILs		Kendall's tau-b value	P
	Negative	Positive		
PD-L1 expression on TILs			0.330	0.002
Negative	24 (32.00%)	23 (30.67%)		
Positive	5 (6.66%)	23 (30.67%)		
CD3 expression on TILs			0.552	<0.001
Negative	24 (32.00%)	12 (16.00%)		
Positive	5 (6.66%)	34 (45.34%)		
CD4 expression on TILs			0.538	<0.001
Negative	28 (37.33%)	20 (26.67%)		
Positive	1 (1.33%)	26 (34.67%)		
CD8 expression on TILs			0.496	<0.001
Negative	29 (38.67%)	25 (33.33%)		
Positive	0 (1.00%)	21 (28.00%)		
FOXP3 expression on TILs			0.486	<0.001
Negative	28 (37.33%)	23 (30.67%)		
Positive	1 (1.33%)	23 (30.67%)		

PD-L1, programmed cell death-ligand 1; FOXP3: forkhead box P3; TIL, tumor-infiltrating lymphocyte.

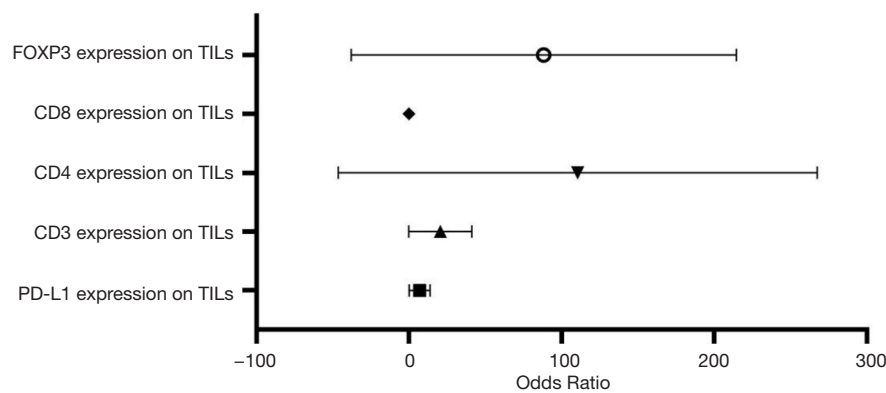


Figure 1 Logistic regression analysis. Bivariate logistic regression of PD-L1, CD3, CD4, CD8, and FOXP3. FOXP3, forkhead box P3; TIL, tumor-infiltrating lymphocyte; PD-L1, programmed cell death-ligand 1.

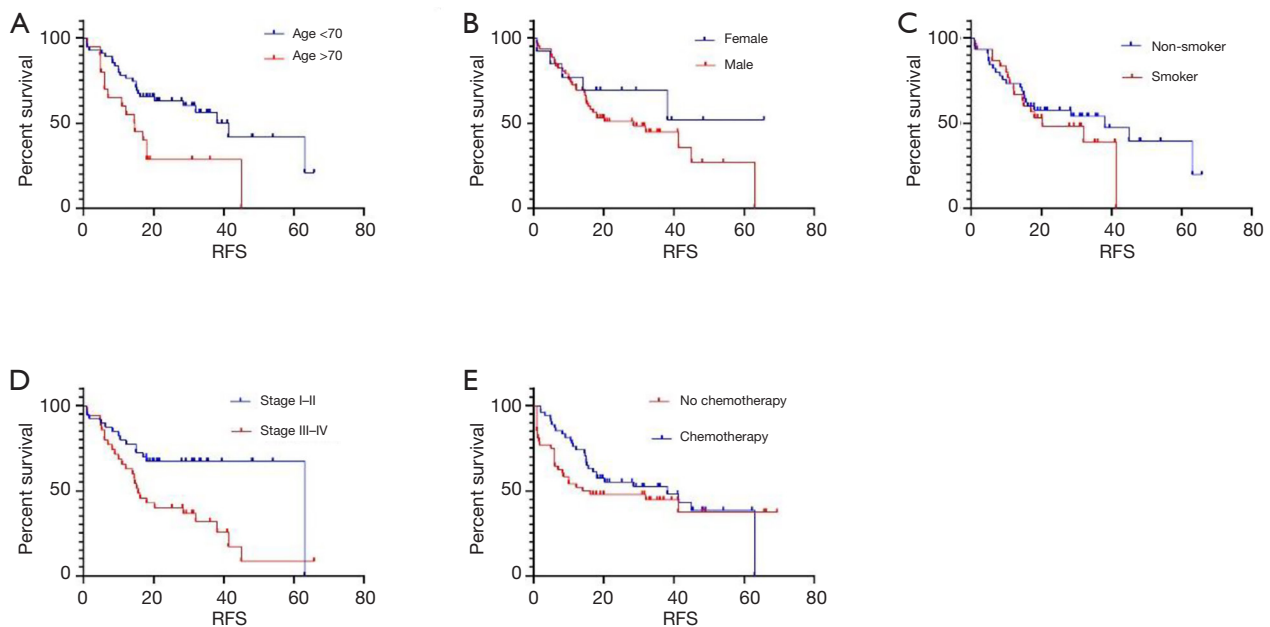


Figure 2 Kaplan-Meier curve for clinical factors related to prognosis. Kaplan-Meier curve for age (A), gender (B), smoking (C), SCLC stage (D), chemotherapy (E). RFS, relapse-free survival; SCLC, small cell lung cancer.

0.7640, (P=0.0050) were significantly associated with RFS, while gender (P=0.2318), smoker status (P=0.4255) and chemotherapy (P=0.3256) had no statistical associations. Age and SCLC stages were considered as risk factors, as hazard ratios (HR) were less than one (<70 vs. >70, Stage I–II vs. Stage III–IV).

The association between RFS and surface antigens was also explored using the Kaplan-Meier method (Figure 3A,B,C,D,E,F, Table S2) and whether CD39 expressed on TILs or not significantly impacted RFS

[negative vs. positive (neg vs. pos), 95% CI: 0.2765–0.9862, P=0.0390]. The same was found to be true for PD-L1 (neg vs. pos, 95% CI: 1.177–4.190, P=0.0256), CD3 (neg vs. pos, 95% CI: 1.037–3.868, P=0.0342), CD4 (neg vs. pos, 95% CI: 1.125–4.169, P=0.0367), CD8 (neg vs. pos, 95% CI: 1.136–4.597, P= 0.0497) and FOXP3 (neg vs. pos, 95% CI: 1.137–4.374, P=0.0440) expression on TILs. In summary, CD39 was the risk factor, while PD-L1, CD3, CD4, CD8, and FOXP3 on TILs had a protective effect for SCLC patients.

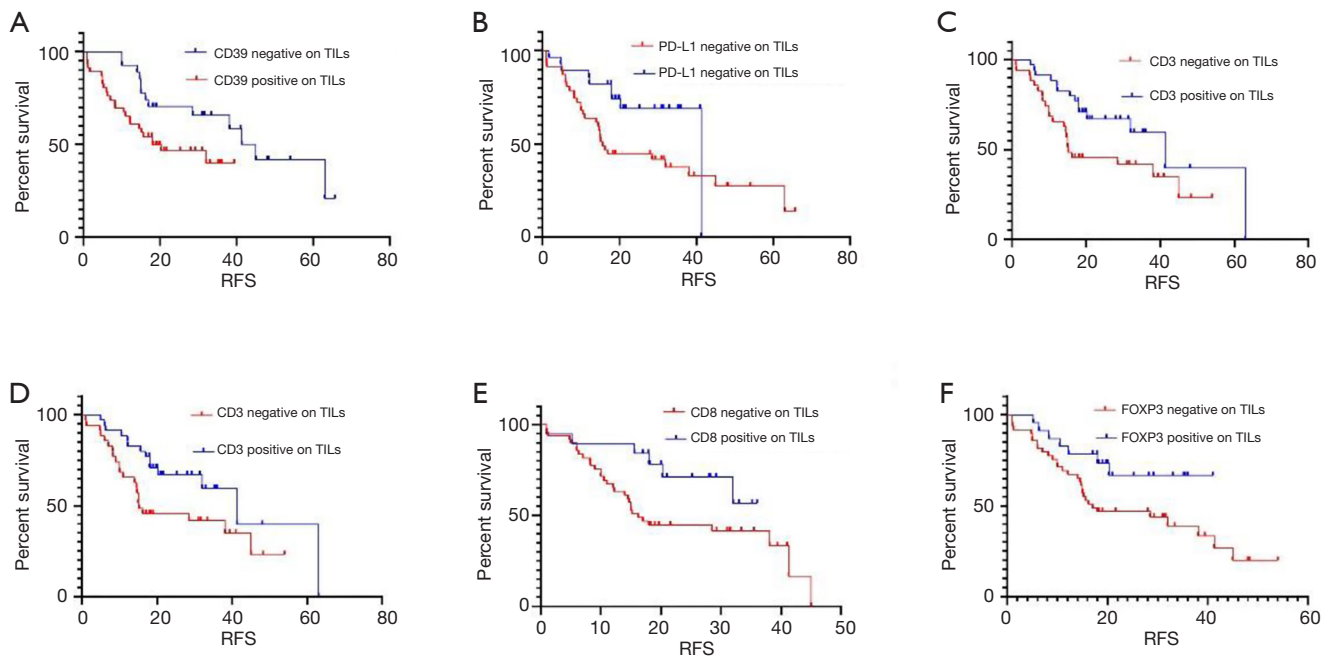


Figure 3 Kaplan-Meier curve for surface antigens. Kaplan-Meier curve for CD39 (A), PD-L1 (B), CD3 (C), CD4 (D), CD8 (E), FOXP3 (F). TIL, tumor-infiltrating lymphocyte; RFS, relapse-free survival; PD-L1, programmed cell death-ligand 1; FOXP3, forkhead box P3.

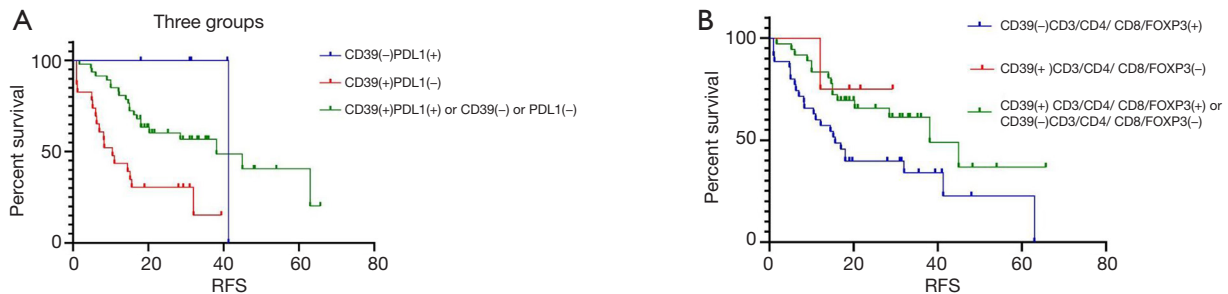


Figure 4 Kaplan-Meier curve for subgroups. (A) The expression of CD39 combined with PD-L1. (B) CD39 combined with CD3, CD4, CD8, and FOXP3. RFS, relapse-free survival; PD-L1, programmed cell death-ligand 1; FOXP3, forkhead box P3.

After evaluating each surface antigen separately, we created subgroups and combined them for analysis. Because CD3, CD4, CD8, and FOXP3 were the primary markers of lymphocytes, we finally divided all surface antigens mentioned above into three groups: CD39, PD-L1 on TILs, and primary markers of lymphocytes (CD3, CD4, CD8, and FOXP3). The result of Kaplan-Meier analysis was that SCLC patients with low expression of CD39 and high expression of PD-L1 had the best RFS, while the positive expression of CD39 and negative expression of PD-L1 suggested poor prognosis ($P=0.0007$) (Figure 4A). Positive expression of CD39 and negative expression of

CD3, CD4, CD8, and FOXP3 also indicated shorter RFS and poorer prognosis ($P=0.0409$) (Figure 4B).

It was proven that CD39, PD-L1, CD3, CD4, CD8, and FOXP3 are all involved in SCLC prognosis and improve patients' prognosis.

Univariate and multivariate Cox regression analyses of RFS

Univariate and multivariate Cox regression analyses were performed to confirm the possible factors that influenced RFS (Table S3). By using univariate Cox regression analysis,

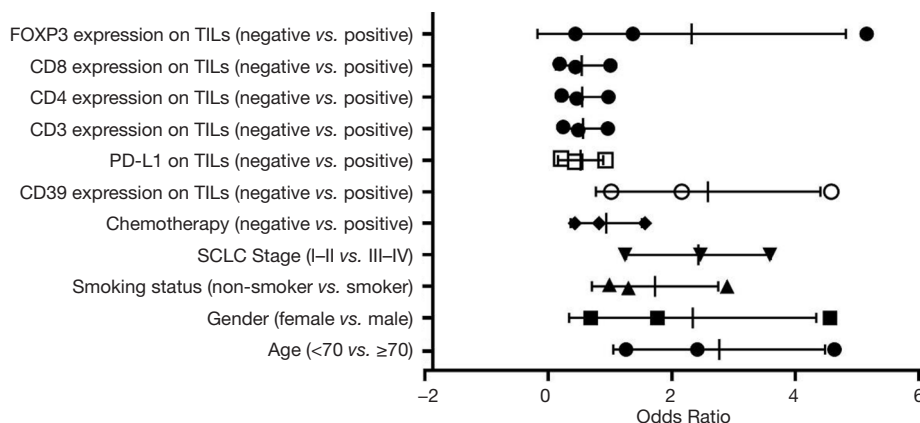


Figure 5 Univariate Cox regression analysis. Univariate Cox regression analysis of clinical factors and surface antigens with RFS. FOXP3, forkhead box P3; TIL, tumor-infiltrating lymphocyte; PD-L1, programmed cell death-ligand 1; SCLC, small cell lung cancer; RFS, relapse-free survival.

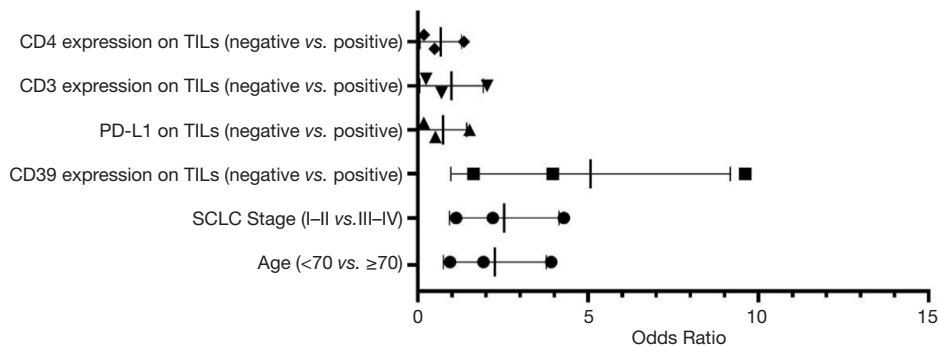


Figure 6 Multivariate Cox regression analysis. Multivariate Cox regression analysis of clinical factors and surface antigens with RFS. TIL, tumor-infiltrating lymphocyte; PD-L1, programmed cell death-ligand 1; SCLC, small cell lung cancer; RFS, relapse-free survival.

we found age (P=0.008), SCLC stage (P=0.007), CD39 (P=0.044), PD-L1 (P=0.030), CD3 (P=0.039), and CD4 (P=0.042) expression on TILs had a significant association with RFS (Figure 5). Cox regression analysis was performed to eliminate confounding effects (Figure 6), and it demonstrated a significant correlation between the SCLC stage (P=0.021), CD39 expression on TILs (P=0.002), and RFS.

Discussion

SCLC progresses rapidly and has a poor prognosis (34-36). However, immunotherapy offers new prospects for SCLC patients, and findings of trials from recent years have supported this. The trial CheckMate-032 (37,38) finds nivolumab monotherapy to be an effective third-

line therapy (37). KEYNOTE-028 (39) reveals good treatment results using pembrolizumab for PD-L1-positive SCLC (39). IMpower133 shows that atezolizumab combined with etoposide and carboplatin could prolong overall survival (OS) and progression-free survival (PFS) for SCLC patients (40), and CASPIAN proves the role of PD-L1 inhibitors combined with chemotherapy in improving OS (41). CD39 may have a significant role in the evaluation of the effect of immunotherapy because the differential expression of CD39 is key to distinguishing tumor-related or unrelated CD8 + TILs (19-22). It may be a potential target for immunotherapy in glioblastoma because of its function in tumor-related macrophages and T cells (42). CD39 blocking may work in the eATP-P2X7-inflammasome-IL18 axis and decrease the number of macrophages in the tumor as a result (43). Little research

has been done regarding the role of CD39 in NSCLC. Evidence shows that anti-CD39 combined with anti-PD-1 can inhibit the metastasis of NSCLC (44), and this may be related to inhibiting the CD39/CD73-adenosine pathway, an essential mechanism of tumor immunosuppression (25). However, until now, no other study about the impact of CD39 on SCLC had been conducted. We are the first to explore the possible relationship between CD39 and SCLC and its connection with other surface antigens like PD-1, PD-L1, CD3, CD4, CD8, and FOXP3.

We obtained 75 clinical samples upon which IHC was performed, and the expression of surface antigens on different cells was expressed. There were 38.67% of patients expressed CD39 positively on TILs. Methods including the Chi-square test, Kendall's tau-b correlation analysis, and logistic regression analysis were used to explore and confirm the possible relationship between CD39 and other surface antigens. In SCLC, PD-L1 expression on TILs, CD3, CD4, and FOXP3 expression on cells was significantly correlated with CD39 expression on TILs. Ahlmanner *et al.* found the same situation in colon tumors: tumor-infiltrating CD39+ regulatory T cells (Tregs) high expressed immunosuppressive molecules like PD-L1 and cytotoxic t-lymphocyte associated protein 4 (CTLA-4) (45). A study by Syed Khaja also proved the co-expression of PD-1/CTLA-4 and PD-1/CD39, adding that the expression of CD4 in T cells constitutes the CD3+ T cells in the tumor microenvironment (TME) and promotes tumor metastasis (46).

Using Kaplan-Meier survival analysis, we looked for factors that may be related to prognosis. Age and SCLC stage appear to be risk factors of SCLC, with HR <1 (<70 vs. >70, Stage I-II vs. Stage III-IV). CD39 expression on TILs is another identified risk factor. This is consistent with Canale's study, where CD39 was viewed as an immunosuppressive molecule that induces CD8+ T cell exhaustion (47). This has also been proven in tumors of oral and gastric cancer (48), colorectal cancer (49), NSCLC (25,50,51), and others. In our findings, PD-L1, CD3, CD4, CD8, and FOXP3 expression on TILs had a protective effect in SCLC patients. Among them, CD3 promotes a good prognosis for many cancers, including SCLC (52-54).

Because of the significant correlation between CD39 and other surface antigens, we divided the samples into subgroups and found SCLC patients with low expression of CD39 and high expression of PD-L1 had the best RFS while the positive expression of CD39 and negative expression of PD-L1 suggested poor prognosis. Positive

expression of CD39 combined with negative expression of CD3, CD4, CD8, and FOXP3 also indicated shorter RFS and poorer prognosis.

In summary, we discovered the relationship between CD39 and other surface antigens in SCLC, along with the related risk and protective factors for RFS. Admittedly, our study had some limitations; most notable was the small sample size; all patients were recruited from the Shanghai Pulmonary Hospital. In order to reduce the error caused by small sample size, we divided subgroups to better understand the relationship between CD39 and SCLC, moreover, the significant correlation between CD39 and other surface antigens. We also used various statistical methods, for example, Chi-square test, Kendall's tau-b correlation analysis, Logistic regression analysis, Kaplan-Meier method, univariate and multivariate Cox regression analysis to reduce errors as much as possible. In spite of the limitations, our study was the first to analyze CD39 in SCLC and its relationship with PD-L1, CD3, CD4, CD8, and FOXP3. According to our findings, CD39 is a potential target for therapy. This needs to be verified through further animal and clinical research.

Conclusions

CD39 expresses on TILs and has a significant association with PD-L1 on TILs, CD3, CD4, CD8, and FOXP3. The positive expression of CD39 leads to short RFS and poor prognosis. Low expression of CD39 combined with high expression of PD-L1 or CD3, CD4, CD8, and FOXP3 indicates a good prognosis for SCLC patients.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tlcr-20-798>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This research was approved by the ethics committee of the Shanghai Pulmonary Hospital, Tongji University (No. K20-022). Written consent was given by all patients, and the experiment confirmed with the tenets of the Declaration of Helsinki (as revised in 2013).

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Supplementary**Table S1** Relationships between CD39 expression on TILs and other checkpoints (logistic regression analysis)

Characteristic	Regression coefficient	Standard error	Odds ratio	95% CI	P value
PD-L1 expression on TILs	1.569	0.573	4.800	1.561–14.764	0.006
CD3 expression on TILs	2.610	0.595	13.600	4.234–43.680	<0.001
CD4 expression on TILs	3.595	1.060	36.400	4.556–290.808	0.001
CD8 expression on TILs	21.351	8,770.825	1.9×10 ⁹	–	0.998
FOXP3 expression on TILs	3.332	1.060	28.000	3.510–233.387	0.002

PD-L1, programmed cell death-ligand 1; FOXP3: forkhead box P3; TIL, tumor-infiltrating lymphocyte.

Table S2 Predictive factors of RFS in SCLC

Characteristic	Log-rank (Mantel-Cox) test		Median survival			Hazard ratio (log-rank) (negative vs. positive)	
	Chi square	P value	Value	Ratio	95% CI	Ratio	95% CI
Age, n (%)							
<70	6.328	0.0119	38.10	2.610	1.376–4.950	0.4245	0.1964–0.9172
≥70			14.60				
Gender, n (%)							
Female	1.430	0.2318	Undefined	Undefined	Undefined	0.5742	0.2643–1.247
Male			28.50				
Smoking status, n (%)							
Smoker	0.6350	0.4255	38.10	1.877	1.003–3.513	0.7806	0.4102–1.485
Non-smoker			20.30				
SCLC Stage, n (%)							
Stage I–II	7.890	0.0050	63.00	4.038	2.109–7.734	0.4087	0.2186–0.7640
Stage III–IV			15.60				
Chemotherapy							
No	0.9663	0.3256	15.10	0.3963	0.2346–0.6694	1.295	0.7613–2.202
Yes			38.10				
CD39 expression on TILs							
Negative	4.259	0.0390	45.00	2.500	1.279–4.887	0.5222	0.2765–0.9862
Positive			18.00				
PD-L1 expression on TILs							
Negative	4.983	0.0256	15.60	0.3777	0.1798–0.7934	2.221	1.177–4.190
Positive			41.30				
CD3 expression on TILs							
Negative	4.487	0.0342	15.20	0.3680	0.1883–0.7193	2.003	1.037–3.868
Positive			41.30				
CD4 expression on TILs							
Negative	4.364	0.0367	16.20	Undefined	Undefined	2.166	1.125–4.169
Positive			Undefined				
CD8 expression on TILs							
Negative	3.851	0.0497	16.20	Undefined	Undefined	2.286	1.136–4.597
Positive			Undefined				
FOXP3 expression on TILs							
Negative	4.058	0.0440	17.00	Undefined	Undefined	2.230	1.137–4.374
Positive			Undefined				

RFS, relapse-free survival; CI, confidence interval; SCLC, small cell lung cancer; TIL, tumor-infiltrating lymphocyte; PD-L1, programmed cell death-ligand 1; FOXP3, forkhead box P3.

Table S3 Univariate and multivariate COX regression analysis of RFS

Variables	Univariate			Multivariate		
	Odds ratio	95% CI	P value	Odds ratio	95% CI	P value
Age (<70 vs. ≥70)	2.413	1.257–4.632	0.008	1.922	0.944–3.913	0.072
Gender (female vs. male)	1.767	0.685–4.562	0.239			
Smoking status (Non-smoker vs. smoker)	1.297	0.990–2.896	0.428			
SCLC stage (I–II vs. III–IV)	2.461	1.243–3.586	0.007	2.195	1.124–4.287	0.021
Chemotherapy (negative vs. positive)	0.821	0.431–1.566	0.550			
CD39 expression on TILs (negative vs. positive)	2.161	1.019–4.580	0.044	3.962	1.632–9.617	0.002
PD-L1 on TILs (negative vs. positive)	0.436	0.205–0.925	0.030	0.510	0.172–1.516	0.226
CD3 expression on TILs (negative vs. positive)	0.483	0.242–0.963	0.039	0.692	0.236–2.028	0.503
CD4 expression on TILs (negative vs. positive)	0.457	0.215–0.973	0.042	0.487	0.176–1.348	0.166
CD8 expression on TILs (negative vs. positive)	0.440	0.182–1.006	0.069			
FOXP3 expression on TILs (negative vs. positive)	0.439	1.370–5.153	0.051			

RFS, relapse-free survival; CI, confidence interval; SCLC, small cell lung cancer; TIL, tumor-infiltrating lymphocyte; PD-L1, programmed cell death-ligand 1; FOXP3, forkhead box P3.