LAG-3 combined with PD-1/PD-L1 inhibitors might become a promising treatment for small cell lung cancer

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Background: Immune therapy has achieved notable success in cancer treatment. A novel immune checkpoint, lymphocyte activation gene-3 (LAG-3), has shown promising therapeutic efficacy in non-small cell lung cancer (NSCLC). We statistically analyzed how its expression correlated with programmed death-ligand 1 (PD-L1) and programmed cell death protein 1 (PD-1), its survival prediction, and explored the possibility of it becoming a treatment alternative of small cell lung cancer (SCLC).

Methods: In this study, we included 102 patients diagnosed with SCLC. Protein expression was evaluated by immunohistochemistry (IHC) staining. We performed correlation analysis and survival analysis with the statistical software SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA).

Results: In SCLC, LAG-3 was not found to be expressed by tumor cells. The expression of LAG-3 on tumor infiltrating lymphocytes (TILs) was remarkably associated with PD-1 and PD-L1 expression on TILs (P=0.006, P=0.001, respectively). The presence of LAG-3 was the predictive factor for the expression of PD-L1 by TILs [odds ratio (OR) =0.161, 95% confidence interval (CI): 0.063 to 0.412, P<0.01]. Although LAG-3-positive patients had relatively longer recurrence-free survival (RFS), LAG-3 expression had no statistically significant difference in predicting prognosis (P=0.088).

Conclusions: The function of T cells can be affected by LAG-3, an important immune checkpoint closely linked to PD-1/PD-L1 and a promising novel alternative of immune therapy for cancer. In this study, we determined the significant association of LAG-3, PD-1, and PD-L1.

Keywords: Lymphocyte activation gene-3 (LAG-3); small cell lung cancer (SCLC); immunotherapy

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Introduction

Among all cancer types, lung cancer ranks first in both morbidity and mortality and poses an increasingly serious threat to human health (1,2). Approximately 10–15% of cases can be categorized as small-cell lung cancer (SCLC), a cancer type with high growth fraction and high recurrence rate, which lead to poor prognosis (3-5). Although chemotherapy is the standard first-line treatment for SCLC (6), resistance to chemotherapy hinders survival prolongation. Therefore, it is urgently important to explore more effective therapeutic strategies for patients with SCLC.

Some tumor cells with less immunogenicity can escape immune elimination and develop into cancers, which reportedly can be reversed by suppressing certain immune checkpoints (7-10). The notable success of some immune checkpoint inhibitors in treating cancers has been previously reported (11,12). The programmed cell death protein 1/programmed death-ligand 1 (PD-1/PD-L1) pathway inhibitor has been shown as effective in treating non-small-cell lung cancer (NSCLC). It could also lead to significantly longer survival in SCLC patients when combined with first-line chemotherapy (13-16).

Whereas the insensitivity to PD-1/PD-L1 blockade hinders its extended use (17,18), some other alternatives are now at the forefront of research, such as lymphocyte activation gene-3 (LAG-3) (19), a novel immune inhibitory checkpoint (20).

The surface molecule LAG-3, also known as cluster of differentiation 223 (CD 223), was first identified in 1990 (21). It is expressed on the membrane of various immunocytes, including tumor-infiltrating lymphocytes (TILs), dendritic cells (DCs), T regulatory cells (Treg), natural killer cells, B cells, and so on (22,23). As a member of immunoglobulin superfamily, LAG-3 is structurally similar to CD4, with approximately 20% homology shared at the DNA sequence (24). Likewise, LAG-3 shows a stronger affinity to human leukocyte antigen II (HLA-II) expressed on antigen presenting cells (APCs) compared to CD4, and thereby prohibits the binding of HLA-II with TILs, hindering the anti-tumor response (25,26). In HLA-II-positive melanoma tumors, this might pave the way for immune escape with bidirectional function (23).

The presence of LAG-3 serves as an essential marker of T cell exhaustion, promoting T-cell apoptosis, inhibiting proliferation, suppressing activation, decreasing cytokines secretion, and increasing tolerance (27,28). Elevated LAG-3-expression was observed on TILs of patients with various solid tumors, such as hepatocellular carcinoma and gastric carcinoma, as well as hematologic malignancies (29).

It has been thought that LAG-3 co-functions with PD-L1 and PD-1 (11). *In vivo* research has suggested that T cells would be activated if one of the pathways was blocked. The strategic blocking of both pathway resulted in an even more notable effect (30). It is possible that LAG-3 might become a promising new immune checkpoint in cancer treatment. And current study has suggested that blocking LAG-3 can activate the professional antigen presenting cells (APCs), inhibit the immunosuppression of regulatory T cells (Tregs) and promoting the proliferation and cytokine production of $CD8^+$T cells (31). Additionally, combining the LAG-3 and PD-1 pathways could be a more promising therapeutic strategy (11). Moreover, soluble LAG-3 might have promising potential as an anti-cancer vaccine (32).

Similar to other types of cancer, our recent study has found that some NSCLC patients had LAG-3-positive TILs. The expression of LAG-3 can be predicted by PD-1 expression and is related to worse prognosis (23). Current research mainly focused on the function of LAG-3 in NSCLC. However, until now, the role of LAG-3 in immune evasion of SCLC has not been revealed. Further analyzing the expression of LAG-3 in SCLC and its impact on survival of SCLC will be indispensable for expand the therapeutics for SCLC. In this study, we enrolled 102 patients with SCLC, investigated LAG-3 expression in TILs, conducted survival analysis, and performed correlation analysis of clinical pathological traits and PD-L1, PD-1, LAG-3 expression. And we present the following article in accordance with the REMARK reporting checklist (available at http://dx.doi.org/10.21037/atm-21-1919).

Methods

Patients

We included 102 patients aged 38–81 years who were diagnosed as SCLC in Shanghai Pulmonary Hospital from March 2017 to January 2019 and collected their formalin fixed paraffin-embedded (FFPE) samples. We reviewed the surgical histology reports and categorized lung cancer stages according to the 8th edition International Association for the Study of Lung Cancer (IASLC) TNM staging system. A total of 54 participants received chemotherapy after diagnosis. And we chose the relapse free survival (RFS) as endpoint of our study. Once new lesion was found during follow-up of patient after surgery, the patient would be defined as relapsed. This research was approved by the

ethics committee of the Shanghai Pulmonary Hospital, Tongji University (K20-022). Written consent was provided by all participants, and the experiment conformed with the tenets of the Declaration of Helsinki (as revised in 2013).

Immunohistochemistry procedure

Dewaxing Tissue slides were dewaxed with xylene, then alcohol, and were then rinsed with distilled water. After antigen recovery and background staining reduction, we incubated the primary antibody in the slides at room temperature (RT) for 1 h. Then, after rinsing with phosphate-buffered saline (PBS), they were incubated with the horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G detective antibody at RT for 30 min. They were again rinsed with PBS, the antigen was visualized with 3'3-diaminobenzidine (DAB), hematoxylin was used for cell nuclear counterstaining, and the cells were then mounted on slides.

Cutoff value determination of surface markers

We chose a value of at least 5% as the LAG-3 cutoff for the best prediction of recurrence-free survival (RFS). The PD-1 on TILs staining was determined as positive when over 1%. A 5% staining was determined as the lower limit of PD-L1 cutoff. The staining of forkhead box P3 (FOXP3), CD3, CD4, and CD8 was confirmed as positive when there was more than 10%, 40%, 30%, and 30% staining, respectively.

Statistical analysis

We conducted correlation analysis and survival analysis with the statistical software SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA). Spearman's rank correlation was applied to calculate the relativity of PD-L1, PD-1, and LAG-3 expression. The chi-square test was used to evaluate the relativity of clinicopathological traits and PD-L1, PD-1, and LAG-3 expression.

A logistic regression model was applied to determine whether the expression of LAG-3 was meaningful in predicting PD-L1 and PD-1; gender, age, smoking status, lung cancer stage, and history of chemotherapy were all also included.

The Kaplan-Meier method was implemented to estimate survival curves, and the Cox regression model was for correlation analysis on RFS and clinical features, including age, gender, history of chemotherapy, smoking status, staging of lung cancer, PD-L1 on tumor cells, PD-L1 on TILs, PD-1, and LAG-3 on TILs and CD3, CD4, CD8, FOXP3. Statistical significance was defined as $P<0.05$. All statistical tests were 2-sided.

Results

Characterization of LAG-3 expression

A total of 40 participants had LAG-3-positive TILs (39.2%). No tumor cells were LAG-3-positive.

Patient characteristics

A total of 102 participants were enrolled in this study, among them, 84 (82.4%) were female and 18 (17.6%) were male. The median age was 62 years old. A total of 58 (56.9%) participants were non-smokers, and 54 (52.9%) had previously received chemotherapy before. All the patients were diagnosed as SCLC, with 38 patients (37.3%) at stage I, 22 (21.5%) at stage II, and 42 (41.2%) at stage III (*Table 1*).

Correlation of LAG-3, PD-1, and PD-L1 expression

Statistical significance was found between PD-L1 on TILs and LAG-3 expression ($P=0.001$), which was also correlated with PD-1 expression on TILs ($P=0.006$). No significant correlation was detected between PD-L1 expression on tumor cells and LAG-3 ($P=0.365$) (*Table 2*). Based on univariate and multivariate logistic regression analysis, we found that LAG-3 had a certain significance in the prediction of PD-L1 expression on TILs [odds ratio (OR) =0.161, 95% confidence interval (CI): 0.063 to 0.412, $P<0.01$]. It was shown that LAG-3 was not the only important factor in predicting PD-1 expression on TILs, where LAG-3, lung cancer stage, and history of chemotherapy all made meaningful predictions (*Table 3*).

Survival analysis

With Kaplan-Meier analysis, participants who were LAG-3-positive had longer RFS, but there was no statistically significant difference in comparison with LAG-3-negative participants ($P=0.088$) (*Figure 1*).

Cox regression analysis of RFS

Lung cancer stage was revealed to be the only factor

Table 1 Patient characteristics (N=102)

Characteristic	n (%)
Sex	
Male	18 (17.6)
Female	84 (82.4)
Age, median, y	
<70	79 (77.5)
≥70	23 (22.5)
Smoking status	
Nonsmoker	58 (56.9)
Smoker	44 (43.1)
T stage	
1	40 (39.2)
2	47 (46.1)
3	13 (12.7)
4	2 (2.0)
N stage	
0	44 (43.1)
1	23 (22.5)
2	34 (33.3)
3	1 (1.1)
M stage	
0	98 (96.1)
1	4 (3.9)
Lung cancer stage	
I	38 (37.3)
II	22 (21.5)
III	42 (41.2)
Chemotherapy	
Yes	54 (52.9)
No	48 (47.1)

predictive of RFS (P=0.021, OR =1.885, 95% CI: 1.102 to 3.223). The presence of PD-L1, FOXP3, CD3, CD4, and CD8 had significance in Cox regression univariate analysis (P=0.006, 0.004, 0.007, 0.010, and 0.007 respectively), but had no significance in multivariate Cox regression analysis (*Table 4*).

Discussion

As a novel immune checkpoint, literature is scarce on LAG-3 expression in SCLC and its correlation with survival. To our knowledge, this was the first study to reveal the indication of the possible immunotherapeutic effect of LAG-3 for SCLC.

As is stated above, LAG-3 serves as an essential marker of T cell exhaustion (27,28). The TILs are regarded as crucial components in anti-tumor immune response and are directly related to the development of cancer (33). The function of CD4+ and CD8+ T cells, DCs, Tregs, and so on, is regulated by inhibitory and active receptors, remarkably impact cancer immune escape (34).

From a mechanistic standpoint, LAG-3 blockade is equal to the blockade of the binding between LAG-3 and HLA-II molecules, which increases the binding of HLA-II to TILs, thus enhancing anti-tumor responses (35). High LAG-3 expression can be observed on TILs in hematologic malignancies and various solid tumors, including hepatocellular carcinoma, gastric cancer, renal cell carcinomas, and ovarian cancer (29). In our study, LAG-3-positive TILs existed in 39.2% of participants; and no tumor cells expressed LAG-3. Additionally, a research indicated that if the PD-1 or LAG-3 pathway alone was blocked, TILs would be increasingly activated and lead to prolonged survival, either by antibodies or knocking down (36). Based on existing research, LAG-3 was understood to be a novel alternative of immune-based treatment for cancers. In our recent study, we found that NSCLC patients with LAG-3-negative TILs had longer survival (23); however, in SCLC, LAG-3 could not predict survival.

Immune escaping pathways are closely associated with one another (37); LAG-3 is co-expressed with PD-1 on TILs and has remarkable synergy with it, which disrupts immune responses to cancer cells (38). Based on previous research, compared with blocking either LAG-3 or PD-1 alone, blocking both pathways showed much more remarkable therapeutic efficacy for cancers (30,36). Meanwhile, due to the upregulated expression of LAG-3 in patients insensitive to PD-1 blocking treatment, the application of this combined strategy will improve prognosis (29,37). This effect has already been shown in treating melanoma patients with this presentation (39,40).

Given the different impacts of the abovementioned checkpoints on NSCLC and SCLC survival, we considered the immune mechanism. Different from it being over-expressed on variety of tumors including NSCLC, PD-

Table 2 Relationships between different checkpoints

Characteristic	LAG-3 expression on TILs		P value
	Negative	Positive	
PD-1 expression on TILs, n (%)			
Negative	47 (77.1)	14 (22.9)	*0.006*
Positive	15 (36.6)	26 (63.4)	
PD-L1 expression on tumor cells, n (%)			
Negative	60 (61.2)	38 (38.8)	0.365
Positive	2 (50.0)	2 (50.0)	
PD-L1 expression on TILs, n (%)			
Negative	44 (75.9)	14 (24.1)	*0.001*
Positive	18 (40.9)	26 (59.1)	

Italic values indicate statistical significance. LAG-3, lymphocyte activating 3; CI, confidence interval; PD-L1, programmed death ligand 1; PD-1, programmed death 1; TIL, tumor-infiltrating lymphocyte.

Table 3 Univariate and multivariate analysis for prediction of LAG-3 expression in all patients

Variables	Univariate		
	OR	95% CI	P value
Age (<70 *vs.* ≥70 y)	1.256	0.490–3.221	0.635
Sex (female *vs.* male)	0.769	0.275–2.152	0.617
Smoking status (nonsmoker *vs.* smoker)	1.583	0.709–3.535	0.262
Stage (I–II *vs.* III)	0.654	0.288–1.484	0.310
Chemotherapy (yes *vs.* no)	1.897	0.842–4.270	0.122
PD-1 on TILs (negative *vs.* positive)	5.819	2.434–13.914	*<0.001*
PD-L1 on tumor cells (negative *vs.* positive)	1.007	0.977–1.037	0.655

Italic values indicate statistical significance. LAG-3, lymphocyte activating 3; CI, confidence interval; PD-L1, programmed death ligand 1; PD-1, programmed death 1; TIL, tumor-infiltrating lymphocyte.

L1 expression is suppressed in SCLC (41). In our study, there were only 4 participants (3.9%) with PD-L1-positive tumor cells, consistent with previously reported results in an immunohistochemistry (IHC) study of SCLC which found that the presence of PD-L1-positive tumor cells was lower than 20% (42). This might be one of the reasons why PD-1/PD-L1 pathway inhibition therapy alone cannot lead to notably improved prognosis in SCLC (43). Contrastingly, worse outcomes of SCLC were also considered to be linked with higher FOXP3+T cell infiltrates (44). Furthermore, the immune microenvironment of SCLC seems to be distinct from that of other solid tumors, including NSCLC (41).

There were some limitations of this study which should be taken into consideration. Firstly, this study was performed retrospectively, which may have led to some recruitment bias. Secondly, the sample size was small, and more data from larger populations are needed to further verify our findings.

In conclusion, immune checkpoints play a significant role in tumor immune escape and have closely interrelated pathways. In recent years, researchers have been expanding the investigation of immunotherapy, aiming to achieve a more favorable prognosis for patients with SCLC. The checkpoint LAG-3 has a prominent co-function and relevant expression with PD-L1 and PD-1, and might

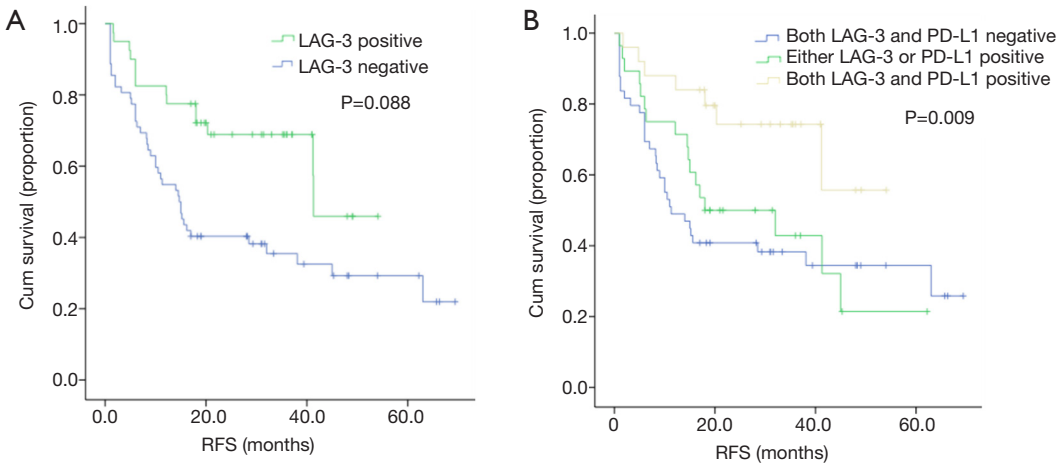

Figure 1 Survival analysis.

Table 4 Univariate and multivariate analysis for RFS in all patients

Variables	Univariate			Multivariate		
	HR	95% CI	P value	HR	95% CI	P value
Age (<70 *vs.* ≥70 y)	1.770	0.997–3.143	0.051			
Sex (female *vs.* male)	1.676	0.757–3.707	0.203			
Smoking status (nonsmoker *vs.* smoker)	1.693	0.990–2.896	0.054			
Stage (I–II *vs.* III–IV)	2.111	1.243–3.586	0.006	1.885	1.102–3.223	0.021
Chemotherapy (yes *vs.* no)	0.770	0.454–1.305	0.331			
PD-1 on TILs (negative *vs.* positive)	0.078	0.342–1.059	0.602			
PD-L1 on tumor cells (negative *vs.* positive)	0.973	0.911–1.039	0.409			
PD-L1 on TILs (negative *vs.* positive)	0.462	0.260–0.819	0.008			
LAG-3 on TILs (negative *vs.* positive)	0.612	0.348–1.076	0.088			
CD3 (negative *vs.* positive)	0.480	0.281–0.820	0.007			
CD4 (negative *vs.* positive)	0.450	0.245–0.825	0.010			
CD8 (negative *vs.* positive)	0.400	0.206–0.776	0.007			
FOXP3 (negative *vs.* positive)	0.376	0.194–0.730	0.004			

become a novel alternative immune therapy marker.

It is noteworthy that the characteristics of the SCLC immune microenvironment remain unclear. Researchers have made progress in their understanding of LAG-3 function and its interaction with other immunomarkers; however, many questions remain: what role does LAG-3 play in the development of SCLC; which immune checkpoint serves as the key regulator in anti-tumor responses in SCLC?; how do immune responses change during the progress of SCLC?; is it possible that different major effector cells are involved in NSCLC and SCLC? Whether anti-LAG-3 will cause the similar immune-related adverse effect as anti-PD-(L)1 and anti-CTLA-4 dose is also unclear. Further studies are needed to explore these

topics and further the base of understanding.

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Footnote

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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 (K20-022). Written consent was provided by all participants, and the experiment conformed with the tenets of the Declaration of Helsinki (as revised in 2013).

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