

Prevalence of DLL3, CTLA-4 and MSTN Expression in Patients with Small Cell Lung Cancer

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Introduction: Immune-based and antibody-drug conjugate therapies have shown promise in the treatment of patients with small cell lung cancer (SCLC). However, better predictive biomarkers are needed for selection of the appropriate SCLC patients for these advanced therapies and also for evaluation of the efficacy of these treatments.

Objective: The aim of this study was to examine the expression of delta-like protein 3 (DLL3), cytotoxic T lymphocyte-associated protein 4 (CTLA-4), and mesothelin (MSTN) in patients with SCLC and compare them with those patients' clinical characteristics.

Methods: Immunohistochemical analyses of DLL3, CTLA-4 and MSTN expression were performed in 38 samples from patients with SCLC.

Results: We found that positive expression in patients of the biomarkers was as follows: for DLL3, 100% (38/38), for CTLA-4, 89.5% (36/38) and for MSTN 81.5% (31/38). The median survival time was 17.9 months in the DLL3 high expression group and 23 months in the DLL3 low expression group. Patients with a high expression of DLL3 showed a poorer prognosis than those with a low expression of DLL3 (HR=3.4; 95% CI, 1.34–8.6; p=0.01).

Conclusion: The expression of DLL3, CTLA-4 and MSTN was not correlated with patients' age, sex, smoking status, stage, and tumor metastasis. The fact that there was a higher expression of DLL3, CTLA-4, and MSTN in SCLC suggested that these molecules could be used as predictive biomarkers for SCLC.

Keywords: small cell lung cancer, DLL3, CTLA-4, MSTN, prognosis

Introduction

Lung cancer has now been confirmed as the most frequently occurring cancer worldwide, being responsible for 2.1 million new cases and 1.8 million deaths in 2018.¹ Lung cancer is generally classified into two distinct types; small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC prevalence is 12–15% of all lung cancers, accounting for over 275,000 of new lung cancer-related cases worldwide annually.^{2,3} Currently the prognosis is poor, with a 5-year survival rate at less than 7%.⁴ Over the last few years however, there has been considerable progress in the treatment of SCLC. In 2018, the US Food and Drug Administration (FDA) approved the use of the immunotherapy checkpoint inhibitor nivolumab (Opdiva) for patients with SCLC who failed to respond to platinum-based chemotherapy with at least one other line of treatment.⁵ The drug Opdiva is a fully human IgG4 monoclonal antibody that primarily suppresses the programmed cell death 1 (PD-1) receptor, thereby effectively blocking the interaction of the PD-1 receptor and its two distinct programmed death ligands PD-L1 and PD-L2. As a result, it can inactivate the negative regulatory mechanisms acting on T-cell

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activation and proliferation.⁶ Assisted by results of the recent IMpower study, accelerated approval was granted by the FDA in March 2019 for the combination of atezolizumab (Tecentriq) with carboplatin and etoposide in the frontline treatment of extensive-stage small cell lung cancer (ES-SCLC).⁷ The study demonstrated an overall survival benefit when the PD-L1 inhibitor atezolizumab was added to platinum/etoposide chemotherapy for the initial treatment of ES-SCLC, with median overall survival (mOS) being 12.3 months in the atezolizumab group and 10.3 months in the placebo group (HR=0.70; 95% CI, 0.54–0.91; $p=0.007$).⁸ Thus, the immunotherapy/chemotherapy combination has now been recognized as an alternative choice for patients with SCLC and has played an increasing role in the treatment of this cancer.

DLL3, a cell surface protein, is abundantly expressed in high-grade neuroendocrine carcinomas of the lung including SCLC, and so can be used to target this cancer with tumor-selective treatment. It has been shown that the cellular NOTCH receptor is mainly downregulated by DLL3, thereby inhibiting the NOTCH signaling pathway within the cell. Therefore, DLL3 can also be used in cancer chemotherapy to target and suppress tumor cells. Rovalpituzumab Tesirine (Rova-T), a DLL3-targeted therapeutic agent containing humanized monoclonal antibody, was the first in the class of antibody-drug conjugates (ADCs). The mechanism of action of Rova-T can be explained as follows. Firstly, ADC becomes integrated with DLL3 on the surface of the tumor cell, forming an ADC-DLL3 complex. Then the ADC-DLL3 complex is internalized into the cell, triggering the release of SC DR002 via proteolytic cleavage in late endosomes. Cross-links between the DNA strands caused by SC DR002 then produce a cytotoxic effect on tumor cells. In a phase I clinical trial, it was confirmed that Rova-T was more effective for SCLC with DLL3 over-expression compared with SCLC with a low level of DLL3 expressed.^{9,10} Hence, DLL3 might be a promising predictive marker suitable for treatment of SCLC. Currently the expression level of DLL3 in SCLC is still unclear however, and more work is required to ascertain the precise DLL3-related mechanisms taking place in SCLC.

CTLA-4, the homologue of the T cell co-stimulatory molecule CD 28, plays an important role in the down-regulation of T cells, which deactivates or blocks the function of T cells.¹¹ It has been established that the CTLA-4 checkpoint pathway is a crucial regulator for the early activation of T-cells in peripheral lymph

tissues.¹² Ipilimumab is a monoclonal antibody which suppresses the interaction between CTLA-4 and its binders, including CD80/CD86. As a consequence of suppressing the interaction of the down-regulator of the T cell, the activation and proliferation of the T cell are continually triggered and then easily diffuse into tumor tissue. A randomized, double-blinded and multi-center phase II trial showed the combination of ipilimumab with paclitaxel and carboplatin in 130 late-stage SCLC patients was free of any patient side effects.¹³ In addition, a meta-analysis of the prognostic value of CTLA-4 in various cancers showed a significant correlation between high expression of CTLA-4 and prolonged overall survival (OS).¹⁴ Clearly, CTLA-4 levels in SCLC should be the subject of further investigation.

MSTN is a glycosyl-phosphatidylinositol-anchored glycoprotein typically expressed on the surface of human solid tumor cells. MSTN was firstly discovered using monoclonal antibody K1¹⁵ and is now in development as a therapeutic target agent for clinical use.¹⁶ It has been demonstrated that increased production of MSTN is detected in 30–70% cases of lung adenocarcinoma.^{17–20} Thomas et al in ClinicalTrials.gov (NCT01306045), have reported that a high expression of MSTN has a significant correlation with poor survival (median OS 18.2 months vs 32.9 months; $p<0.014$). The study provides a strong rationale to intensively investigate anti-MSTN target therapies in advanced lung adenocarcinoma, especially in the KRAS-mutant subgroup.

However, the prevalence of DLL3, CTLA-4 and MSTN expression in SCLC patients, and their associations with patient clinicopathological characteristics, remains unclear. Therefore, in this study, we evaluated the expression of DLL3, CTLA-4, and MSTN in patients with SCLC and correlated these with the clinical characteristics of SCLC.

Materials and Methods

Research Cohort

In this study, we enrolled 38 patients with SCLC who underwent surgical resection of primary lung cancer at the Department of Lung Cancer Surgery, Tianjin Medical University General Hospital during 2009–2014. The inclusion criteria were: (1) primary SCLC; and (2) patients had neither chemotherapy nor radiotherapy administered prior to surgery. Patients' demographics and clinical information were obtained from the hospital database. The clinical and

pathological characteristics of the patients are detailed in Table 1. Lung cancer staging for each patient was performed according to the American Joint Committee on Cancer (AJCC) Staging Manual, Eighth Edition.²¹

Immunohistochemistry

Immunohistochemistry for DLL3, CTLA-4, and MSTN was performed as described previously. Paraffin-embedded lung tissue samples were cut into 4 µm thick sections. The sections were deparaffinized in xylene and rehydrated in a graded ethanol series. Heat-induced antigen retrieval was carried out by microwave pretreatment in 5mM Tris-HCl (pH10.0) for 15 min. After rinsing in PBS, the sections were incubated with rabbit polyclonal anti-DLL3 (1:400, No SAB1302862, Sigma-Aldrich, Shanghai, China), mouse monoclonal antibody anti-CTLA-4 (1:50, (F-8), sc376016, Alexa Fluor, Inc, Oregon USA) and rabbit monoclonal antibody anti-MSTN (1:100, [EPR4509] ab133489, Abcam, Shanghai, China) at 4°C overnight. After washing with phosphate buffered saline (PBS), slides were treated with a ready-to-use peroxidase-labeled polymer conjugated to goat anti-rabbit IgG (catalog no. SM801: EnVision+ kit; Dako; Agilent Technologies, Inc) as the secondary antibody for 30 min at room temperature. The staining was visualized with diaminobenzidine, and followed by counterstaining with hematoxylin. Slides for DLL3, CTLA-4, and MSTN

were evaluated by two independent researchers, including an expert pathologist. The positive cells were counted and assigned with a score as follows: 1 (≤25%), 2 (26%–50%), 3 (51%–75%) and 4 (>75%). The staining intensity was scored as: 1 (none), 2 (weak), 3 (intermediate), and 4 (strong). These scores were then multiplied to evaluate samples individually. The cut-off values were determined by the median value of the expression of each antibody for these three genes.

Statistical Analysis

All analyses were undertaken using SPSS ver. 11 (SPSS Inc., Chicago, IL, USA). The relationship of DLL3, CTLA-4, and MSTN expression with clinicopathologic findings was analyzed with a Chi-Square test. Kaplan-Meier curves and Cox model analyses were used to examine the relationship of DLL3, CTLA-4, and MSTN expression with patients' survival rate. Univariate and multivariate survival analysis were performed with the Cox proportional hazards model. Variables included in the Cox model included the following: age, gender, smoking status, and clinical TNM stages. Hazard ratio (HR) point and interval (95% CI) estimates were computed through the Cox modeling. The two-sided log-rank and likelihood ratio statistics were calculated to test differences between Kaplan-Meier survival probabilities and death rates derived from the Cox regression, respectively. A $p < 0.05$ was considered to be statistically significant.

Table 1 Patient Characteristics

Characteristic	N (%)
Gender	
Male	26(68.4)
Female	12(31.6)
Age	
>58.1	18(47.4)
<58.1	20(52.6)
Smoking history	
Never	12(31.6)
Current >20 years	5(13.2)
<20 years	21(55.3)
Adjuvant chemotherapy	
Yes	14(36.8)
No	24(63.2)
Clinical stage	
I	5(13.2)
II	11(28.9)
III	18(47.4)
IV	4(10.5)

Results

Clinical Characteristics of the Research Cohort

The study cohort included 26 male and 12 female patients, aged from 33 years to 77 years, with a median age of 58 years. Of these, 12 were non-smokers and 26 were current or former smokers. Twenty patients had tumors located in the left lung, and 18 had tumors located in the right lung. For pathological staging, the eighth TNM classification and staging system were used that released by the Union for International Cancer Control (UICC) in 2016. Table 1 shows the clinical characteristics of the cohort in detail.

DLL3 Expression Status in the SCLC Patients

Immunohistochemistry was used to analyze 38 cases of formalin-fixed paraffin-embedded (FFPE) tissue specimens from the SCLC patients. DLL3 was positively expressed in the cytoplasm and the nuclei of tumor cells, but the normal

alveolar epithelial cells were negative. Positively stained tumor cells were found to be arranged in a prominent nesting pattern, and the nests were separated by delicate connective tissues (Figure 1). DLL3 positive expression was found in all 38 cases and, where the DLL3 score of the tumor was 13.5 or higher, were considered to have a high expression (present in 18 of 38 patients). DLL3 with high expression was found in 11 out of 20 (55%) specimens from subjects older than 58 years, and in 9 out of 18 (50%) cases 58 years or younger. DLL3 with high expression was found in 15 specimens out of 26 (57.7%) in men and in 5 out of 12 (41.7%) women. DLL3 with high expression was found in 9 out of 12 (75%) specimens from nonsmokers and in 11 out of 26 (42.3%) from smokers. DLL3 with high expression was found in 6 specimens out of 16 (37.5%) from patients with disease stages I-II and 14 out of 22 (63.6%) patients with disease stages III-IV. DLL3 with high expression was found in specimens from 15 out of 23 (65.2%) patients with metastasis and 5 out of 15 (33.3%) patients without metastasis. Furthermore, the DLL3 staining status was analyzed for correlation with the clinical characteristics of the patients. The expression of DLL3 was not associated with the clinical characteristics of the patients, such as gender, age, smoking, clinical stages, and metastasis (Table 2).

CTLA-4 Expression Status in the SCLC Patients

CTLA-4 positive expression was found in 36 out of 38 (89.5%) specimens and a CTLA-4 score of the tumor

greater than 8.2 was observed in 12 of 36 patients, and these patients were considered to have high expression. CTLA-4 with high expression was found in specimens from 7 out of 20 (35%) patients older than 58 years and 5 out of 18 (27.8%) patients of age 58 years or younger. CTLA-4 with high expression was found in specimens from 7 out of 26 (26.9%) men and in 5 out of 12 (41.7%) women. CTLA-4 with high expression was found in specimens from 3 out of 12 (25%) non-smokers and in 9 out of 26 (34.6%) smokers. CTLA-4 with high expression was found in specimens from 3 out of 16 (18.8%) patients with disease stages I-II and in 9 out of 22 (40.9%) patients with disease stages III-IV. CTLA-4 with high expression was found in specimens from 8 out of 23 (34.8%) patients with metastasis and in 4 of 15 (26.7%) patients without metastasis (Table 2). In addition, the CTLA-4 staining status was analyzed and compared with the clinical characteristics of the patients. The association study showed that CTLA-4 expression was not significantly associated with the clinical characteristics of the patients, such as gender, age, smoking status, clinical stages, and metastasis.

MSTN Expression in the SCLC Patients

MSTN positive expression was found in 31 out of 38 (81.5%) specimens and an MSTN expression score greater than 5.3 was observed in 11 of 31 patients, and these patients were considered to have high expression. High MSTN expression was found in 8 out of 20 (40%) specimens from patients older than 58 years, and in 3 out of 18 (16.7%) patients aged 58 years or younger. High MSTN expression was found in 8 out of 20 (40%) specimens from patients older than 58 years and in 3 out of 18 (16.7%) patients aged 58 years or younger. MSTN with high expression was found in 4 out of 12 (33.3%) specimens from non-smokers and in 7 out of 26 (24.9%) from smokers. MSTN with high expression was found in 4 out of 16 (25%) specimens from patients with disease stages I-II and 7 out of 22 (31.8%) patients with stages III-IV. MSTN with high expression was found in 5 out of 23 (43.5%) specimens from patients with metastasis and in 6 out of 15 (40%) patients without metastasis (Table 2). The MSTN staining status was compared with the clinical characteristics of the patients and the MSTN expression was found not to be significantly associated with the other clinical characteristics of the patients, such as smoking status, clinical stage, and metastasis.

Additionally, from Table 2, it appears that the expression of DLL3 was higher in those patients with stage III-IV (63.6% vs 36.4%), whereas the expression of CTLA-4 and MSTN in

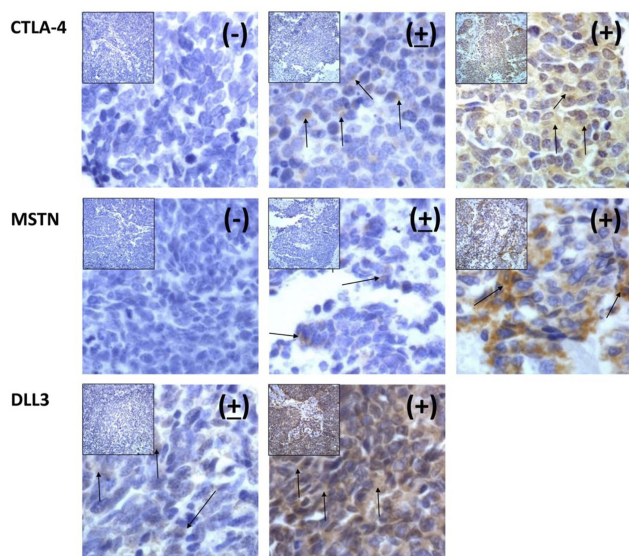


Figure 1 Immunohistochemical staining of CTLA-4, MSTN and DLL3 in SCLC tissues: x400. Positive (+), partially positive (+/-), and negative (-).
Note: the arrows indicate the cells that are positive.

Table 2 Correlation of DLL3, CTLA-4 and MSTN Expression with Clinicopathological Characteristics

Factors	№%	DLL3 Expression			CTLA-4 Expression			Mesothelin Expression		
		Low	High	P Value	Low	High	P Value	Low	High	P Value
Total	N=38	18(47.4)	20(52.6)		26(68.4)	12(31.6)		27(71.1)	11(28.9)	
Gender				0.489			0.460			0.714
Male	26(68.4)	11(42.3)	15(57.7)		19(73.1)	7(26.9)		19(73.1)	7(26.9)	
Female	12(31.6)	7(58.3)	5(41.7)		7(58.3)	5(41.7)		8(66.7)	4(33.3)	
Age				1.000			0.734			0.160
<58	18(47.4)	9(50)	9(50)		13(72.2)	5(27.8)		15(58.3)	3(16.7)	
>58	20(52.6)	9(45)	11(55)		13(65)	7(35)		12(60)	8(40)	
Smoking status				0.086			0.714			0.714
Never	12(31.6)	3(25)	9(75)		9(75)	3(25)		8(66.7)	4(33.3)	
Ever	26(68.4)	15(57.7)	11(42.3)		17(65.4)	9(34.6)		19(73.1)	7(24.9)	
Clinical stage				0.188			0.178			0.729
I-II	16(42.1)	10(62.5)	6(37.5)		13(81.3)	3(18.8)		12(75)	4(25)	
III-IV	22(57.9)	8(36.4)	14(63.6)		13(59.1)	9(40.9)		15(68.2)	7(31.8)	
Metastasis status				0.096			0.728			0.285
Yes	23(60.5)	8(34.8)	15(65.2)		15(65.2)	8(34.8)		18(78.3)	5(21.7)	
No	15(39.5)	10(66.7)	5(33.3)		11(73.3)	4(26.7)		9(60)	6(40)	

patients with stage III-IV was 40.9% vs 50.1%, and 31.8% vs 68.2%, respectively. However, these differences were not significant, which may present difficulty in establishing a negative correlation among these three-gene expressions.

The Expression Status of DLL3, CTLA-4, and MSTN with a Prognosis of SCLC Patients

As of January 2015, complete prognosis data was available for 29 patients, including 4 cases of death and 25 cases of survival. The median period of patient follow-up for the entire series was 39.8 months (range from 7 months to 72

months). As shown in Figure 2 and Table 3, we conducted a survival analysis of these 29 patients with respect to the presence of DLL3, CTLA-4, and MSTN expression. We found that the median survival time of those patients with DLL3 with high expression was 12 months (range from 1 month to 43 months); for those patients with DLL3 low expression, it was 23 months (range from 6 months to 44 months). Furthermore, DLL3 with high expression showed a poorer prognosis than DLL3 with low expression. The difference in median overall survival times between these two groups was statistically significant (HR=3.12; 95% CI, 0.99–9.82; $p=0.000$). We may therefore, infer with 95%

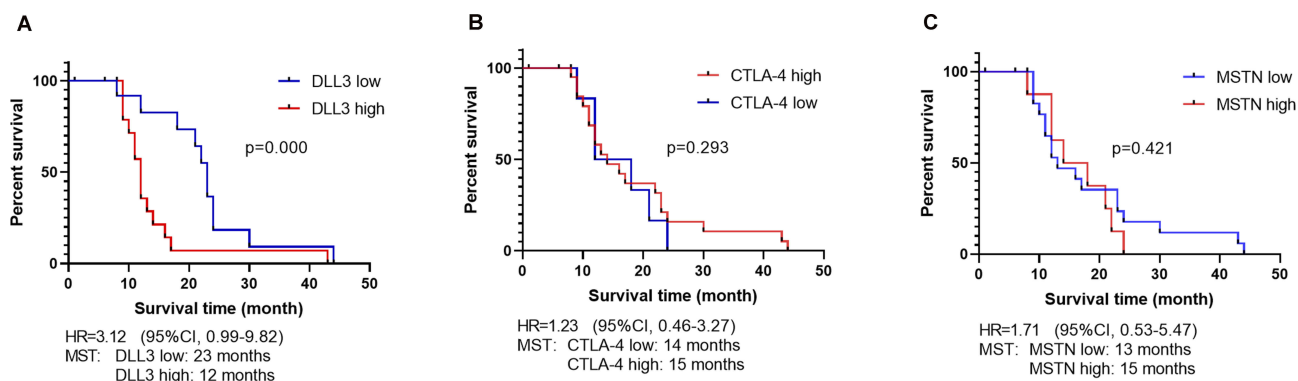


Figure 2 Overall survival study: (A) Kaplan Meier curves for all patients stratified according to the DLL3 expression, (B) Kaplan Meier curves for all patients stratified according to the CTLA-4 expression, (C) Kaplan Meier curves for all patients stratified according to the MSTN expression.

Table 3 Univariate and Multivariate Analysis of the Prognostic Factors on OS in Patients with SCLC

Characteristics	Univariate Analysis			Multivariate Analysis		
	MST(m)	SD	P	HR	95% CI	P
Gender						
Male	12.5	8.34	0.069	0.34	0.11–1.04	0.014
Female	18.5	10.85				
Age						
<58	15.28	11.95	0.593	0.87	0.33–2.3	0.216
>58	13.6	6.7				
Smoking status						
Never	14.77	10.9	0.725	1.16	0.44–3.02	0.000
Ever	13.58	5.4				
Clinical stage						
I-II	16	7.5	0.381	0.81	0.29–2.27	0.016
III-IV	13.23	10.7				
Metastasis status						
Yes	12.13	6.3	0.068	1.31	0.49–3.46	0.698
No	17.87	12.39				
DLL3						
High	12.00	8.16	0.102	3.12	0.99–9.82	0.000
Low	23.01	10.35				
CTLA-4						
High	15.00	6.71	0.146	1.23	0.46–3.27	0.293
Low	13.99	10.2				
MSTN						
High	15.01	6.6	0.786	1.71	0.53–5.47	0.421
Low	13.00	10.5				

confidence that the death rate from DLL3 with high expression is higher than the risk from DLL3 at low expression.

In the case of the patients with CTLA-4 with high expression, their median survival time was 15 months (range from 1 month to 24 months); while those patients with CTLA-4 at low expression showed a median survival of 14 months (range from 6 months to 44 months). The difference in median overall survival times between these two groups was not statistically significant (HR=1.23; 95% CI, 0.46–3.27; $p=0.293$).

In the case of the patients with MSTN with high expression, the median survival time was 15 months (range from 6 months to 24 months); those patients with MSTN at low expression had a median survival time of 13 months (range from 1 month to 44 months). The difference in median overall survival times between these two groups was not statistically significant (HR=1.71; 95% CI, 0.53–5.47; $p=0.421$).

Furthermore, the patients' characteristics were evaluated to determine their prognostic value for OS (Table 3). Multivariate analysis revealed that the gender, smoking status, clinical stage, and expression level of DLL3 were independent favorable OS prognostic factors.

Discussion

SCLC is an aggressive disease characterized by rapid tumor growth and early metastatic dissemination.²² The primary etiologic agent responsible for SCLC, associated with both lung cancer carcinogenesis and the prognosis of lung cancer patients, is considered to be smoking.²³ In general, between 2 and 11.5% of patients diagnosed with SCLC are non-smokers,^{23–25} however in our study, 36.3% of patients had no apparent history of smoking. However, because it was a retrospective study and there were some confounding variables, such as second-hand or third-hand tobacco exposure, they were not able to consider in this

research. The survival rate for SCLC is extremely low and essentially has not changed over the past 30 years.²⁶ However, in recent years, effective strategies have been developed to treat SCLC, such as antibody-drug conjugate or ADC-technology, recognition of appropriate target antigens, novel monoclonal antibodies (mAbs), and immune targets.

In this study, we examined the DLL3 expression in a Chinese SCLC population and confirmed that DLL3 was 100% (38/38) positively expressed in those patients, a similar finding to that of a recent TRINITY trial.²⁷ DLL3 expression was also evaluated in studies of Japanese SCLC patients and DLL3 high expression was found to be 83% (52/62)²⁸ and 90% (18/20).²⁹ We also investigated the correlation between DLL3 expression, patient characteristics and clinical outcomes in SCLC. A significant finding of this study was that DLL3 with high expression is correlated with poorer patient outcomes, a result similar to that of the phase I clinical trials of Rova-T which evaluated DLL3 expression in recurrent SCLC.⁹ However, we found no correlation between DLL3 expression and other patient characteristics. Table 4 shows the review of the studies of DLL3 expression in lung cancer. In a first-in-human, first-in-class, open-label phase I study, Rova-T (rovalpituzumab tesirine), a DLL3 target, has

shown promise.⁹ After Rova T treatment, overall survival was 4.6 months. In an exploratory analysis, in those with DLL3-high expression, median overall survival was 5.8 months and in those with DLL3-low expression, median overall survival was 2.7 months. Median progression survival was 4.3 months and 2.2 months, respectively.⁹ Liu and collaborators further explored correlations between DLL3 expression and clinicopathologic features in high-grade pulmonary neuroendocrine carcinomas, including SCLC and found that 210 of 335 SCLC patients (63%) showed a high DLL3 expression. Low DLL3 expression was also related to a longer OS, although this was not statistically significant ($p=0.077$).³⁰ In a study of 63 Japanese patients, which evaluated the correlation between DLL3 expression and patient characteristics, no significant correlation was found between DLL3 expression and age, sex, smoking history, disease stage or biopsy site. Furthermore, the DLL3 expression status was not found to be correlated to prognosis.²⁸ In the single-arm, phase II TRINITY trial investigating Rova-T in a third-line treatment setting for patients with relapsed/refractory SCLC, the objective response rate (ORR) was 16% in the DLL3-high group and 6% in the DLL3-low group. The clinical benefit rate was 24% and 14%, respectively.²⁷ The phase III TAHOE trial (NCT03061812) has now been evaluated;

Table 4 Studies of DLL3 Immunohistochemistry

Tumor Type	Positive %	N	Correlation With Survival	Reference
Small cell lung cancer	88%	42/48	High expression, mPFS 4.3 months (95% CI, 2.8–5.6); Low expression, mPFS 2.2 months (95% CI, 1.3–2.5) (after Rova-T treatment)	Rudin 2017 ⁹
Small cell lung cancer	82.5%	52/63	No significant association ($p=0.943$)	Tanaka 2018 ²⁸
Small cell lung cancer	90%	18/20	NE	Saito 2017 ²⁹
Small cell lung cancer	72%	120/167	NE	Saunders 2015 ³³
Small cell lung cancer	NA		Low DLL3 expression related to a longer OS ($p=0.077$)	Liu 2017 ³⁰
Small cell lung cancer	100%	339/339	High expression mOS 5.7 months, in all pts mOS 5.6 months (after Rova-T treatment)	Carbone 2018 ²⁷
Small cell lung cancer	100%	38/38	DLL3 high expression associated with poorer outcomes ($p=0.01$)	Current study
Small cell bladder cancer	68%	36/53	DII3 high expression associated with poor outcomes ($p=0.01$)	Koshkin 2017 ³⁴
LCNEC	65%	37/57	NE	Saunders 2015 ³³
Lung adenocarcinoma	3.7%	3/82	NE	Saunders 2015 ³³
Gastrointestinal neuroendocrine tumor	100%	9/9	NE	Zhang 2017 ³⁵

Abbreviations: LCNEC, large cell neuroendocrine carcinoma; mPFS, median progression-free survival; NE, not evaluated; NA, not available.

this compared Rova-T with topotecan in patients with advanced or metastatic SCLC with high levels of DLL3, who experienced their first disease progression during, or following, frontline platinum-based chemotherapy. Disappointingly, in our view, the independent data monitoring committee (in accordance with AbbVie) has stopped further enrollment in the TAHOE trial on basis of the shorter OS reported in the Rova-T arm compared to the control arm.³¹ However, additional Rova-T clinical trials are ongoing and are recruiting participants; including a phase III MERU study (NCT03033511), which is an ongoing trial investigating Rova-T as maintenance therapy to follow first-line platinum-based chemotherapy in patients with advanced stage SCLC. A phase I open-label study, currently active, is also comparing the combination of Rova-T with nivolumab and with or without ipilimumab in adults with advanced stage SCLC who have had first disease progression during at least one platinum-based chemotherapeutic regimen (NCT03026166).³² Several published studies have evaluated the DLL3 expression

occurring in different tumor types, and some of these have investigated the correlation between DLL3 expression and patient characteristics and clinical outcomes.^{33–35} For example, a retrospective study showed that DLL3 high expression had a significant correlation with poorer patient outcomes in small cell bladder cancer (SCBC).³⁴ These studies showed that patients with a higher DLL3 expression in SCLC and SCBC had a poorer prognosis than those with a low expression, suggesting DLL3 as a promising target for tumor ADCs.

In this study, we evaluated CTLA-4 expression in a Chinese SCLC population and confirmed that CTLA-4 is 89.5% (36/38) positively expressed in these patients. To the best of our knowledge, this retrospective study represents the first investigation of CTLA-4 expression in SCLC. In our study cohort, we found no significant correlation of CTLA-4 expression with patient characteristics and clinical outcomes in SCLC. As shown in Table 5, in the past, many researchers have evaluated CTLA-4 expression in different tumor types.^{36–42} For example, a recent study found CTLA-4

Table 5 Studies of CTLA-4 Immunohistochemistry

Tumor Type	Positive %	N	Correlation With Survival	Reference
Small cell lung cancer	89.5%	36/38	Not associated with OS (p=0.656)	Current study
NSCLC	50.6%	41/81	CTLA-4 over-expression tended to have a better prognostic impact than lower expression (5-year probability: 64.8 vs 45.9%; p=0.079)	Salvi 2012 ³⁸
NSCLC (PTs) SCC (s-CTLA4) (t-CTLA-4) ADC (s-CTLA-4) (t-CTLA-4) LN+ SCC (t-CTLA-4) ADC (t-CTLA-4)	47% 37% 44% 42% 24% 38%	536 136/287 107/289 84/196 85/192 18/75 22/58	High expression of t-CTLA-4 associated with poor outcomes in LN+ group. CTLA-4 expression was not associated with poor outcomes in PTs group.	Paulsen 2017 ³⁶
Malignant pleural mesothelioma	56.1%	23/41	Favorable prognostic factor (but not significant) HR=0.57; 95% CI, 0.28–1.20	Roncella 2016 ³⁹
Osteosarcoma	100%	6/6	NE	Contardi 2005 ³⁷
Breast tumor	100%	5/5		
Breast carcinoma	100%	60/60	High CTLA-4 expression tended to have a better prognosis than lower expression	Mao 2010 ⁴⁰
Melanoma	40%	8/20	NE	Laurent 2013 ⁴¹
Nasopharyngeal carcinoma	97.4%	186/191	High expression associated with poorer outcomes (p=0.044)	Huang 2016 ⁴²

Abbreviations: NSCLC, non-small cell lung cancer; OS, overall survival; HR, hazard ratio; SCC, squamous cell carcinoma, ADC, adenocarcinoma; LN+, metastatic lymph nodes; S, stromal; T, tumor epithelial, PT, primary tumor; NE, not evaluated.

Table 6 Studies of MSTN Immunohistochemistry

Tumor Type	Positive %	N*	Correlation With Survival	Reference
Small cell lung cancer	81.5%	31/38	Not associated with OS (p=0.582)	Current study
Lung adenocarcinoma	41%	14/34	NE	Ordenez 2003 ⁴⁵
Lung adenocarcinoma	53%	49/93	High mesothelin expression associated with poor survival (p=0.014)	Thomas 2015 ⁴⁶
Lung adenocarcinoma	69%	834/1209	High mesothelin expression associated with poor survival (p<0.01)	Kachala 2014 ⁴⁷
Lung squamous carcinoma	28%	5/23	NE	Ordenez 2003 ⁴⁵
Ovarian serous carcinoma	100%	14/14		
Ovarian serous adenocarcinoma	55%	109/198	NE	Bauerschlag 2013 ⁶⁴
Ovarian serous carcinoma	55%	92/167	Diffuse MSTN expression is associated with prolonged patient survival (p=0.023)	Yen 2006 ⁴⁸
Ovarian carcinoma	96	24/25	NE	Weekes 2018 ⁶¹
Ovarian cancer	70%	47/67	NE	Frierson 2003 ¹⁸
Ovarian cancer	75%	3/4	NE	Lamberts 2016 ⁵⁷
Breast carcinoma	3%	1/35	NE	Ordenez 2003 ⁴⁵
Breast carcinoma	67%	29/43	NE	Tchou 2012 ⁵⁹
Breast carcinoma	78.6%	22/28	NE	Ordenez 2014 ⁶³
TNBC	39.6%	21/53		
TNBC	34%	37/109	MSTN expression was not independent predictor of survival (p=0.57)	Parinyanitikul 2013 ⁵⁴
TNBC	36%	82/226	Correlated with poor survival (p=0.0003)	Tozbikian 2017 ⁶⁰
Non-TNBC	16%	14/88		
Mesothelioma	60%	60/100	NE	Ordenez 2014 ⁶³
Teratoma	100%	2/2	NE	Ordenez 2003 ⁴⁵
Cholangiocarcinoma	37%	7/19		
Extrahepatic bile duct adenocarcinoma	50%	5/10	NE	Zhao 2007 ⁶²
Extrahepatic bile duct cancer	47.5%	29/61	High level expression was significantly correlated with poorer patient outcomes (p=0.022)	Kawamata 2012 ⁵³
Pancreatico-biliary adenocarcinoma	73%	72/99	NE	Ali 2014 ⁵⁸
Pancreatic ductal adenocarcinoma	86%	12/14		Ordenez 2003 ⁴⁵
Pancreatic ductal adenocarcinoma	39.8%	41/103	Co-expression of MSTN and MUC16 (high levels for both) was associated with poor prognosis (p=0.0006)	Shimizu 2012 ⁵⁵

(Continued)

Table 6 (Continued).

Tumor Type	Positive %	N*	Correlation With Survival	Reference
Pancreatic ductal adenocarcinoma	86.4%	57(66)	Co-expression of MSTN and CA125 were associated with an unfavorable patient outcome(p=0.0062)	Einama 2011 ⁵²
Pancreatic cancer	83%	25/30	NE	Weekes 2016 ⁶¹
Pancreatic cancer	42.8%	3/7	NE	Lamberts 2016 ⁵⁷
Pancreatic cancer	70.8%	97/137	MSTN and MUC1 expression are highly significant predictors of early cancer-specific mortality (p<0.0001)	Winter 2012 ⁵⁶
Esophageal adenocarcinoma	25%	1/4		Ordonez 2003 ⁴⁵
Esophageal squamous carcinoma	75%	3/4		
Gastric cancer	58%	29/50		Ito 2014 ⁵¹
Gastric cancer	44.5%	49/110	Poor prognostic factor (p=0.0073)	Einama 2011 ⁵²
Gastric cancer	59%	73/124	Correlated with prolonged survival (p=0.0628)	Baba 2012 ⁴⁹
Gastric adenocarcinoma	29%	2/7	NE	Ordonez 2003 ⁴⁵
Gastric adenocarcinoma	50.4%	64/127	NE	Illei 2016 ⁵⁰
Cervical squamous carcinoma	25%	1/4	NE	Ordonez 2003 ⁴⁵
Adenomatoid tumor	100%	3/3		
Endometroid uterine adenocarcinoma	64%	7/11		
Endometroid uterine adenocarcinoma	59%	13/22	NE	Frierson 2003 ¹⁸
Adenocarcinoma-ampulla of Vater	100%	3/3	NE	Ordonez 2003 ⁴⁵
Laryngeal squamous carcinoma	100%	1/1		
Desmoplastic small round cell tumor	58%	7/12		
Colorectal adenocarcinoma	49.5%	45/91	High level expression is not correlated with OS compared to low level expression (p=0.26)	Kawamata 2012 ⁵³

Note: *N:number.

Abbreviations: TNBC, triple-negative breast carcinoma; OS, overall survival; NE, not evaluated.

overexpression in 47% of 536 patients with NSCLC.³⁶ Interestingly, CTLA-4 overexpression tended to have a better prognostic impact than lower expression in NSCLC,³⁸ breast cancer, and malignant pleural mesothelioma.³⁹ Targeted immunotherapies involving

CTLA-4 and PD1/PD-L1 checkpoint pathways have shown promise as effective and safe therapies in patients with SCLC, NSCLC and melanoma, as demonstrated by the durable objective responses and overall survival rates achieved with these agents.^{43,44} Additional clinical trials of

ipilimumab are ongoing and are actively recruiting participants, including a phase II study of patients with SCLC treated with combination immunotherapy-ipilimumab-nivolumab-dendritic cell p53 Vac, who have had recurrence of disease following at least one platinum-based chemotherapeutic regimen (NCT03406715).³²

In this study, we examined the MSTN expression in a Chinese SCLC population and confirmed that MSTN is 81.5% (31/38) positively expressed in these patients. To the best of our knowledge, the determination of MSTN expression by IHC staining in SCLC patients has not been previously undertaken. We found no significant correlation between MSTN expression and patient characteristics and clinical outcomes in SCLC. As shown in Table 6, several published studies have evaluated MSTN expression in different tumor types.^{18,20,45–64} For example, a recent retrospective study found MSTN expression in 41–69% cases of lung adenocarcinoma,^{45–47} and high MSTN expression associated with poor survival in these patients.^{46,47} Similarly, in triple-negative breast cancer (TNBC) and in extrahepatic bile duct cancer, high MSTN expression has been shown to be associated with a poor prognosis.^{60,62} But in ovarian serous cancer, diffuse MSTN expression was associated with prolonged patient survival.⁴⁸ The expression of MSTN has been observed and studied in many types of cancer, but particularly in the case of SCLC, further studies are necessary. Currently, several agents that target MSTN expressing tumors are being investigated in clinical trials, including SS1P (anti-MSTN immunotoxin), amatuximab (MORAb-009, a high-affinity chimeric monoclonal immunoglobulin G1/k antibody), anti-MSTN antibody-drug conjugate anetumabravtansine (BAY94-9343), and a MSTN tumor vaccine (CRS-207) as well as a MSTN adoptive T-cell immunotherapy using MSTN-specific chimeric antigen receptors.³⁵ To our knowledge, there are no clinical trials in the field of SCLC currently being undertaken.

Conclusion

We have studied DLL3, CTLA-4, and MSTN expression in SCLC and propose that DLL3 expressions in SCLC might be a promising prognostic markers for patients. In addition, it has been hypothesized that high expressions of DLL3, CTLA-, and MSTN in SCLC play a significant role in tumor pathogenesis. In this study, we found that the levels of expression of DLL3, CTLA-4, and MSTN are were not correlated with clinical characteristics in SLCL, but the precise roles of DLL3, CTLA-4, and MSTN in SCLC development remain unclear and require further investigation.

Ethics Approval and Consent to Participate

This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethical Review Committee of Tianjin Medical University General Hospital. All biological samples and relevant clinical information were obtained with patients' written informed consent.

Availability of Data and Materials

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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Author Contributions

JC and HYL designed and supervised of the study. OR, HYL and JC wrote the manuscript. OR and YL performed the experiments. YWL, HBZ, HG, YY, JW and WTL helped to perform some experiments. All authors contributed to data analysis, drafting and revision of the article, gave approval of the final draft to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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