Journal of Inflammation Research Dovepress

Open Access Full Text Article ORIGINAL RESEARCH

Serpin B9 is Highly Expressed in Lung Adenocarcinoma and is Associated with Progression-Free Survival

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Background: Serpin B9 is highly expressed in breast cancer, melanoma, and various malignant cells and inhibits NK cell killing through the Serpin B9-GrB axle. However, the current studies have only validated the role of Serpin B9 in vivo and vitro, and lack of systematic studies on the expression of Serpin B9 in patients' tumor tissues and its prognostic implications. In this study, we propose to further validate the role of Serpin B9 by comparing its expression level in tissues of lung adenocarcinoma patients and its correlation with the efficacy of immunotherapy.

Methods: This study included 200 patients with LUAD between Feb 2022 and Feb 2023. IHC scoring assessed Serpin B9 expression in the tumor and adjacent tissues, with an H-score of 2 as the cutoff value. Patients were divided into high- and low-expression groups. *T*-tests were used to compare Serpin B9 expression and treatment efficacy between the tumor and adjacent tissues in both groups. Baseline characteristics were compared using X2 tests. Prognostic risk factors were identified using Cox regression and Kaplan-Meier survival curves.

Results: The expression level of Serpin B9 in LUAD tumor tissues are higher than adjacent tissues and positively correlated with the TNM stage and negative correlated with PFS in patients with LUAD. Additionally, immunotherapy efficacy was inversely correlated with Serpin B9 expression.

Conclusion: The increased expression of Serpin B9 in LUAD tumor tissues is negatively linked to prognosis and immunotherapy efficacy. This underscores their potential as prognostic and therapeutic targets.

Keywords: serine proteinase inhibitor B9, lung adenocarcinoma, progression free survival, PD-1/PD-L1

Introduction

Lung cancer remains a pervasive global malignancy, with an estimated 2.0 million new cases reported in 2020.[1] Despite a marginally lower incidence rate compared to breast cancer, its intricate pathology, inconspicuous clinical symptoms in the early stages, and heterogeneity contribute to the highest mortality rate among malignant tumors, resulting in approximately 1.8 million deaths in 2020, constituting 18% of the total tumor-related deaths.[1] LUAD represents the predominant histological subtype, comprising 39% and 57% of cases in males and females, respectively.[2]

Targeted therapy, particularly kinase inhibitors such as EGFR-TKIs, has emerged as a common treatment modality for LUAD, demonstrating efficacy in extending patient survival. However, the emergence of resistance limits their long-term effectiveness.[3,4] Additionally, immunotherapy, notably anti-PD1/PDL1, is an important therapeutic approach. Nevertheless, its efficacy is restricted to approximately 30% of patients with LUAD limiting treatment options and patient survival.[5–8]

Serine proteinase inhibitor B9 (Serpin B9), a member of the serine protease inhibitor family, exhibits increased expression in various malignancies including melanoma, lung cancer, and breast cancer.[9,10] Its interference promotes tumor cell death, and modulation of its expression enhances host-protective immunity.[11] Notably, elevated Serpin B9 expression was observed in NSCLC cells resistant to treatment with anti-PD1, and knockdown of Serpin B9 expression

Received: 4 April 2024
Accepted: 17 October 2024
Published: 14 November 2024

restored sensitivity to anti-PD1.[12] These findings underscore the potential of Serpin B9 as a therapeutic target for LUAD, suggesting its concurrent use with anti-PD1/PDL1 therapy to enhance efficacy.

However, the current research primarily comprises cellular or in vivo studies that lack clinical data from patients with LUAD. To address this gap, we investigated the potential of Serpin B9 as a therapeutic target by analysing its expression in tumors and adjacent tissues, as well as treatment efficacy and prognosis in patients.

Methods

Study Subjects

The study prospectively included 264 patients with LUAD who were diagnosed at the Hefei Cancer Hospital, Chinese Academy of Sciences, First Affiliated Hospital of Anhui Medical University, and Second People's Hospital of Anhui Province 2022.2 to 2023.2. 34 patients were excluded due to missing follow-up information and 30 were excluded due to a lack of adjacent tissues. A total of 200 patients with LUAD were included in this study, and their baseline clinical information was collected. Finally, 142 patients underwent 4 cycles of chemotherapy with platinum-containing regimens, and 80 of them underwent chemotherapy combined with immunotherapy. 58 patients were withdrawn from this study due to intolerance of 4 cycles of chemotherapy or attendance at other hospitals, etc.

The inclusion criteria were as follows: 1. over 18 years of age, and 2. LUAD diagnosed by pathological examination, 3. had a complete medical history, 4. Expected survival time > 6 months or Karnofsky point> 80, 5. not have mutations such as EGFR and ALK and not chance to receive targeted therapy. The exclusion criteria were as follows: 1. combined with other primary malignant tumors and 2. serious systemic infectious diseases or disorders of the immune system; and 3. insufficient pathological tissues were obtained.

The study was approved by the Medical Ethics Committee of Hefei Cancer Hospital, Chinese Academy of Sciences. All the studies described adhered to the principles of the Declaration of Helsinki. All patients signed an informed consent form, the procedure for which is shown in Figure 1.

Study Method

Baseline information of 200 LUAD patients was collected, including gender, age and TNM stage. A total of 200 paired pre-treatment pathological tissues (including tumors and adjacent tissues) were collected in this study, and all of these tissues were subjected to IHC assay for Serpin B9. IHC-stained tissue sections were read and scored by two professional pathologists, with H-values of >2 as high expression (high value group, n=174) and ≤2 as low expression (low value group, n=26).[13] IHC for PDL1 was performed in 180 patients, and PD-L1 expression in the included patients was classified according to the tumor promotion score (TPS): negative (TPS <1%) in 71 (39.4%), low expression (1% ≤ TPS < 50%) in 87 (48.3%), and high expression (TPS ≥ 50%) in 22 (12.3%).

IHC Assay

All paraffin sections were placed in a constant-temperature oven at 60 °C and baked for 120 min to soften the sections, which were sequentially immersed in xylene (10 min, once), xylene (10 min, once), anhydrous ethanol (5 min, twice), 95% ethanol (5 min, twice), 90% (5 min, once), 85% ethanol (5 min, once), 80% ethanol (5 min, once), 75% ethanol (5 min, once), distilled water (5 min, twice), 3% H2O2 incubation (30 min, 37 °C), distilled water wash (5s, twice), PBS wash (5 min, 3 times), and PBS immersion (30 min, once). The specific antibody of anti-Serpin B9 (14,910-1-AP, Proteintech, China) and anti-PDL1 (PA5-20343, Invitrogen, USA) was then added dropwise(1:200) to the tissue at the centre of the section and incubated at 4°C overnight. On the second day, the sections were gently rinsed with PBS (5 min, three times) and anti-rabbit IgG-HRP (Proteintech, China) (30–60 min, incubated at 37 °C), and the sections were gently rinsed with PBS (5 min, 3 times). After the antibody-antigen reaction, 1×DAB chromogenic solution was added dropwise to the dried paraffin tissue (5 min), colour development was observed under a microscope, and staining was promptly terminated with tap water. The moistened paraffin sections were immersed in haematoxylin for 10–20s, then washed with tap water. After staining, sections were sequentially immersed in 70% ethanol (5 min, once), 85% ethanol (5 min, once), 90% ethanol (5 min, once), 95% ethanol (5 min, once), 100% ethanol (10 min, once), 100% ethanol (10 min, once), and

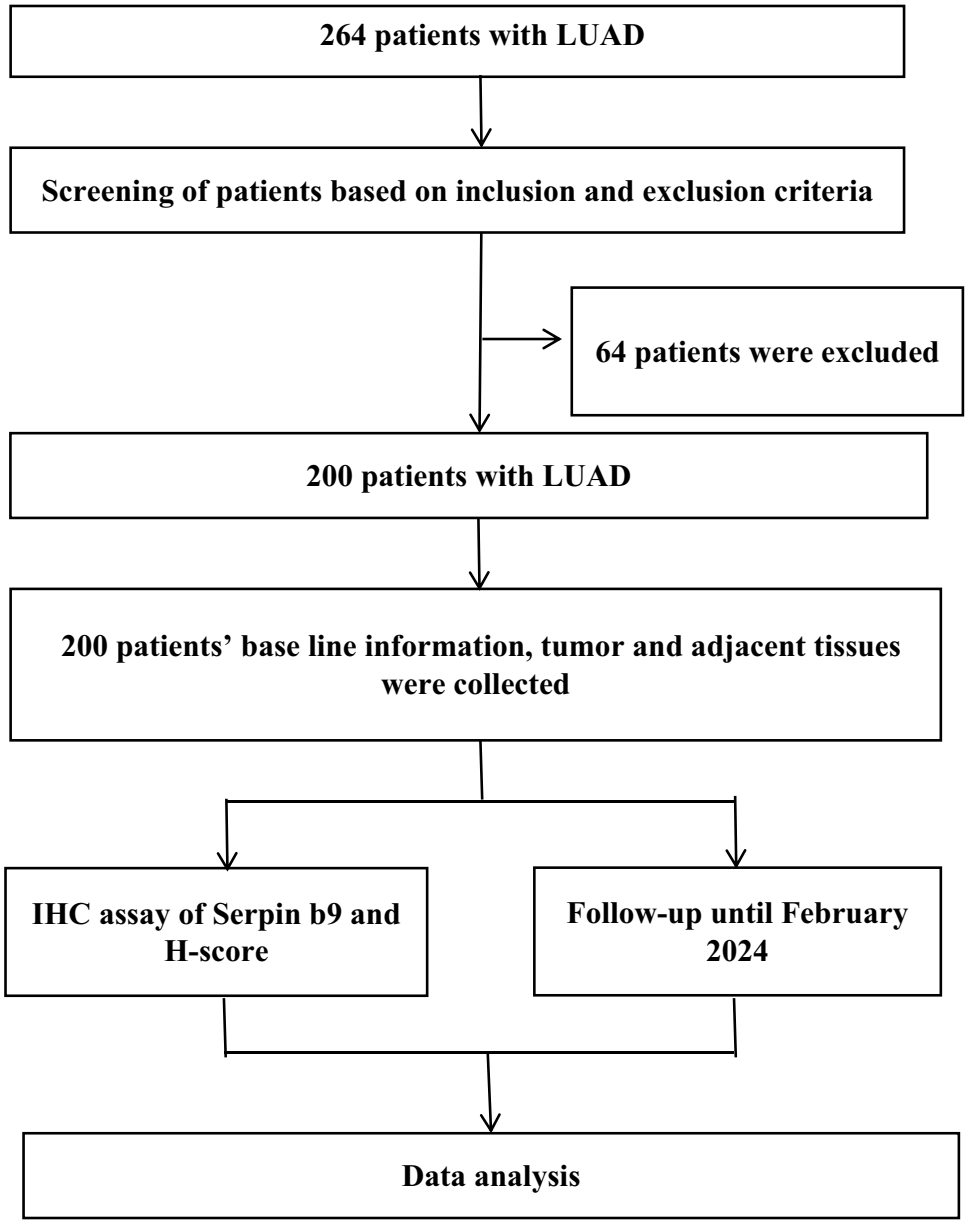

Figure 1 The flow chart for this study.

xylene (20 min, once). Finally, an appropriate amount of resin was added to the tissue for sealing and the sections were observed and photographed under a microscope (OLYPMUS BX43). All IHC in this study was done manually by the researcher and no automated machines were used.

Follow-Up

Follow-up of patient survival time by telephone, outpatient, and inpatient, with a deadline of 1 February 2024. The imaging results of all patients were interpreted by two specialised imaging physicians, computed Tomography (CT) and magnetic resonance imaging (MRI), and subsequently assessed for efficacy by two specialised oncologists using RECIST 1.1 criteria.[14]

Progression-free survival (PFS) was defined as the time from the start of treatment to disease progression; complete response (CR) was defined as complete disease remission; partial response (PR) was defined as partial disease remission (≥30% reduction in the sum of the longest diameters of each lesion (SLD)); stable disease (SD) was defined as stable disease (<30% reduction in the SLD or <30% increase in the SLD); progressive disease (PD) was defined as disease

progression (≥30% increase in the SLD); and objective response rate(ORR) was defined as the objective remission rate, which is usually the proportion of patients who have had a reduction in SLD of up to 30% and maintained it for more than 4 weeks; ORR rate = PR rate + CR rate. The above are defined according to CTCAE version 5.0.

Statistical Analyses

Data were analysed and plotted using spss 22.0 and GraphPad Prism 7.0. All count data were expressed as M ± SD, *t*-test and X^2 or Fisher's test was used to compare differences between groups. The COX regression model was used to investigate the independent risk factors affecting PFS, and the Kaplan-Meier (K-M) curve and Log rank test were used to compare the differences in survival levels between groups. $p < 0.05$ was considered statistically significant and is indicated by * and *** is defined as $p < 0.001$.

Results

The Expression of Serpin B9 in Tumor and Adjacent Tissues in Patients with LUAD

The tumor and adjacent tissues of 200 patients with LUAD were both stained by IHC and photographed under a microscope; IHC scoring was performed by two pathologists and the results showed that the expression level of Serpin B9 protein was higher in the tumor than in adjacent tissues($p < 0.001$, Figure 2A, C and E). As shown in the Figure 2B and D, the main components in lung adenocarcinoma tissues are cancer cells and some immune cells, and Serpin B9 is highly expressed in the cytoplasm of these cells, whereas in adjacent tissues it is mostly alveolar epithelial cells, and the expression of Serpin B9 in these cells is low.

Clinical Features of Patients with LUAD

A total of 200 patients (84 [42%] women and 116 [58%] men) with LUAD were included in this study, with an average age of 54.04±9.55 years, and 154 (77%) patients had a smoking history.

1) Moreover, 92(46%) and 108(54%) patients had stages II, III+IV disease, respectively. Among them, 98 (49%) and 102 (51%) of the patients were in the T1+T2, T3+T4 stages, respectively. Lymph node invasion (N1, N2, and N3) and distant metastasis (M1) were observed in 156 (78%) and 20 (10%) patients, respectively. Analysis of serum tumor marker levels in 200 patients showed that 100% of the patients had carcinoembryonic antigen (CEA) levels greater than 5 ng/mL, 62% had neuron-specific enolase (NSE) levels greater than 15 ng/mL, and 86% had soluble

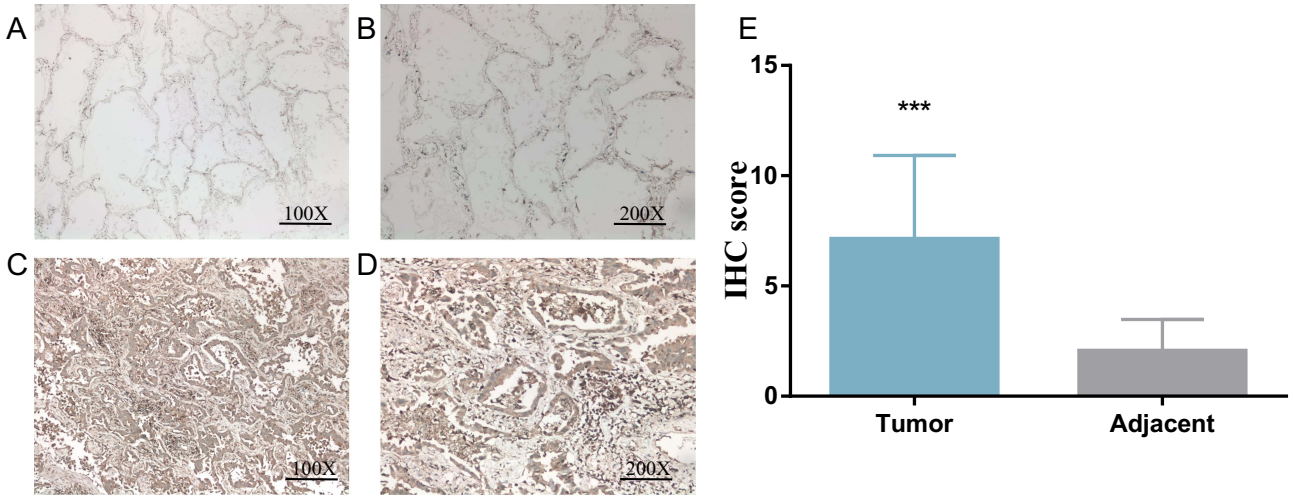

Figure 2 Expression levels, grey values and IHC score of Serpin B9 in different tissues. (**A**) Serpin B9 IHC staining of adjacent tissue (x100), (**B**). Serpin B9 IHC staining of adjacent tissue (x200), (**C**). Serpin B9 IHC staining of tumor tissue (x100), (**D**). Grey values of Serpin B9 protein in different tissues (x200), (**E**). IHC score for Serpin B9 protein expression in different tissues. ***p<0.001.

fragment of cytokeratin-19 (CYFRA21-1) levels greater than 3.3 ng/mL. There were 71, 87, and 22 patients in the PDL1-negative, low-expression, and high-expression groups.

Serpin b9 h-scores were compared for different subgroups of patients, and the *t*-test showed that the expression level of serpin b9 in the tissues of patients in the T1+2 group was lower than that in the T3+4 group ($p < 0.001$); the expression level in the N0 group was lower than that in the N1+2+3 group (p=0.048); the expression level in the tissues of patients in the M0 group was lower than that in the M1 group (p=0.03); and the expression level in the tissues of patients in the stage II group was lower than that in the stage III+IV group (p=0.009, Table 1). And the expression of Serpin B9 was no statistical difference between the age, sex, BMI, smoke, drink, CEA level, NSE level, CYFRA21-1 level and PDL1 expression level ($p>0.05$, Table 1).

Correlation Analysis Between Serpin B9 and PFS

Univariate Cox regression analysis showed that T stage, N stage, M stage, TNM stage, and Serpin b9 level were associated with PFS (Table 2). Multivariate Cox regression analysis confirmed that the N stage, M stage, TNM stage, and Serpin b9 level (HR=0.008, 95% CI, 0.50–0.92) were independent risk factors for PFS (Table 3).

The median PFS period of all patients was 15.4 (range, 4.00–24) months, and 112 patients showed disease progression during the follow-up period. The median PFS of patients in the low-value group was longer by 5.03 months than that of patients in the high-value group (19.24 [range, 12.00–24.00] months vs 14.21 [range, 4.00–24.00] months). In addition, the K–M curve and Log rank test confirmed that the PFS periods of patients in the high-value group were lower than those of patients in the low-value group ($p < 0.05$, Figure 3).

Table 1 Baseline Clinical Characteristics of LUAD Patients(N=200)

Clinicopathological Parameters	Variables	N	IHC score (M±SD)	*t*	*P*-value
Age	≥60	60	7.53±0.62	0.73	0.48
	<60	140	6.93±0.47		
Sex	Male	116	6.86±0.49	0.77	0.45
	Female	82	7.45±0.56		
BMI	≥18.5	140	6.97±0.45	0.56	0.58
	18.5	60	7.43±0.70		
Smoke	Yes	154	7.26±0.42	0.72	0.47
	No	46	6.61±0.85		
Drink	Yes	126	6.89±0.60	0.44	0.66
	No	74	7.20±0.49		
T	1+2	98	5.96±0.53	3.73	<0.001
	3+4	102	8.53±0.44		
N	0	44	5.91±0.83	2.10	0.048
	1+2+3	156	7.65±0.40		
M	0	180	7.01±0.39	2.16	0.030
	1	20	9.60±0.67		
Stage	II	92	6.24±0.57	60.28	0.009
	III+IV	108	8.15±0.44		
CEA	<5ng/mL	0	-	-	-
	≥5ng/mL	200	7.13±4.12	-	-
CYFRA21-1	<3.3ng/mL	28	5.64±1.08	1.80	0.07
	≥3.3ng/mL	172	7.54±0.38		
NSE	<16.3ng/mL	76	7.71±0.56	0.94	0.35
	≥16.3ng/mL	124	7.00±0.48		
PDL1	negative	36	7.07±0.49	0.83	0.44
	low-expression	40	7.19±1.21		
	high-expression	12	6.93±0.56		

Table 2 Univariate Survival Analyses of 200 Patients

Univariate Analysis	Progression-Free Survival	
	HR (95% CI)	p value
Age(≥60 vs<60)	0.79(0.51, 1.07)	0.19
BMI(≥18.5vs<18.5)	0.57(0.42, 0.76)	0.11
Smoke	0.97(1.46, 2.57)	0.24
Drink(Yes VS No)	2.51(1.72, 2.44)	1.78
T	23.70(18.27, 42.71)	0.012
N	4.21(3.44, 6.73)	0.004
M	2.21(1.60, 2.73)	<0.001
Stage	2.17(0.9, 2.58)	<0.001
CEA	1.21(0.75, 1.97)	0.058
SCC	3.47(2.19, 5.49)	0.15
NSE	1.51 (1.02, 2.23)	1.37
IHC score	7.42(4.19, 11.89)	0.016

Table 3 Multivariate Survival Analyses of 200 Patients

Multivariate Analysis	Progression-Free Survival	
	HR (95% CI)	*p* value
T	1.22(0.89, 1.67)	0.215
N	2.33(1.24, 3.89)	0.017
M	1.23(0.57, 1.88)	<0.001
Stage	13.46(6.85, 24.59)	<0.001
IHC score	0.81 (0.50, 0.98)	0.008

Correlation analysis of Serpin b9 Expression and the Patients' Treatment Efficacy after 4-cycles of Treatments

142 patients studied underwent 4 cycles of chemotherapy with platinum-containing regimens, 127 and 15 patients in the high- and low-value groups, respectively. As shown in Table 4, the CR and PD rates were similar between the two groups, but the PR and ORR rates were higher in the low-value group than in the high-value group.

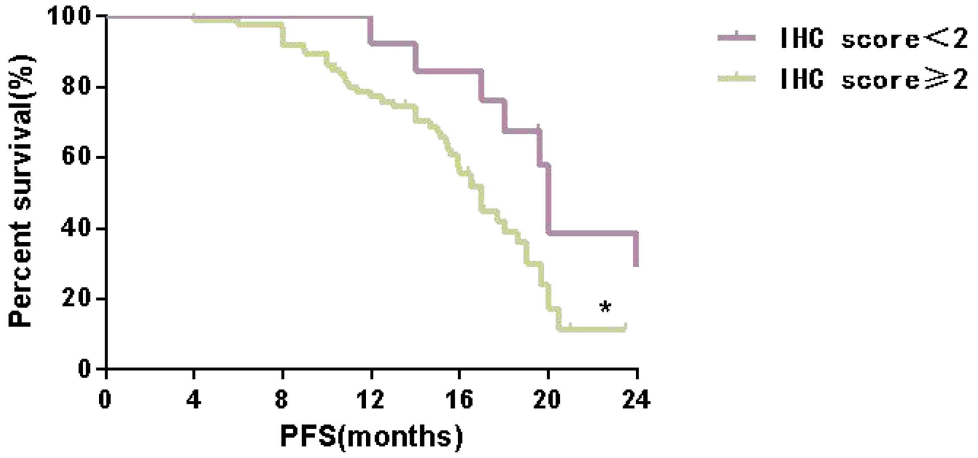

Figure 3 K-M curves for patients in the two groups of 200 LUAD patients. *p<0.05.

Table 4 Treatment Efficacy Evaluation After 4 Cycles of Treatments in 142 Patients

	IHC score<2(n=15)	IHC score≥2(n=127)
CR	1(6.67%)	8(6.30%)
PR	5(33.33%)	33(25.98%)
SD	6(40.00%)	62(48.82%)
ORR	6(40.00%)	41(32.28%)
PD	3(20.0%)	24(18.90%)

Table 5 Treatment Efficacy Evaluation After 4 Cycles of Anti-PD1/PDL1 Treatments in 80 Patients

	IHC score<2(n=11)	IHC score≥2(n=69)
CR	1(9.09%)	2(2.90%)
PR	3(27.27%)	16(23.19%)
SD	6(54.55%)	40(57.97%)
ORR	4(36.36%)	18(26.09%)
PD	1(9.09%)	11(15.94%)

A total of 80 patients who were treated with anti-PD1/PDL1+chemotherapy with platinum-containing regimen treatments participated in the complete 4-cycle efficacy assessment, with 69 and 11 patients in the high- and low-value groups, respectively. As shown in Table 5, the CR, PR, and ORR rates were higher in the low-value group than in the high-value group, whereas the PD and SD rates were the opposite.

Discussion

Serpins represent a superfamily of protease inhibitors that share a common origin and exhibit highly conserved structural sequences that are ubiquitous across the natural world.[15] Initially identified and isolated from human plasma, serpins encompass 13 members within the B subgroup in humans, designated as serpins B1–B13.[16] Serpin B9 is a single-chain protein comprising 376 amino acids that is prominently expressed in various human tissues, including the placenta, testes, ovaries, eyes, and immunocompromised sites.[17] Serpin B 9 is highly expressed in a variety of malignant tumours and promotes tumour progression, but the specific mechanism is not clear. Previous studies have confirmed that granzyme B (GrB) is a protease released by cytotoxic immune cells for the clearance of target cells such as viral infections and tumour cells, and that the reaction centre loop of Serpin B9 is able to form a complex with the active site of GrB to inhibit its activity, suggesting that Serpin B9 may promote tumour progression by inhibiting the cell-killing activity when it binds to GrB.[11,18,19]

Recent evidence has established notable upregulation of Serpin B9 in various malignant tumors, implicating its involvement in cancer progression. Luo et al[20] conducted a comprehensive analysis of gene data from melanoma patients and TCGA database, identifying a high expression of the Serpin B9 gene in malignant melanoma tissues, correlating significantly with patient prognosis. Similarly, in our study involving 200 patients with lung adenocarcinoma, we observed markedly elevated levels of Serpin B9 expression in tumor tissues compared to adjacent tissues, consistent with the findings of Jiang et al[11,15] in lung adenocarcinoma cell lines.

Subsequent statistical analysis of the clinical data from our cohort revealed a correlation between Serpin B9 expression levels and TNM staging in patients with lung adenocarcinoma. Cox regression analysis further confirmed Serpin B9 as an independent risk factor impacting patient PFS, N stage, M stage, and TNM stage. Notably, Zhou et al[21] reported similar findings in hepatocellular carcinoma, where Serpin B9 expression was positively correlated with tumor differentiation, TNM stage, and tumor size and inversely correlated with overall survival (OS). However, our study's limited 2-year follow-up period precluded us from obtaining OS data for all the patients.

Mechanistically, Serpin B9 has been implicated in inhibiting apoptosis by binding to caspases-1, altering its conformation, and impeding substrate binding or catalytic activity, thereby fostering a pro-carcinogenic milieu.[22] Furthermore, mass spectrometry-based proteomic analysis in a mouse model of lung cancer bone metastasis revealed a strong association between elevated Serpin B9 expression and an increased burden of bone metastasis. Consistent with prior research, our findings and those of Luo et al[20] underscored the correlation between Serpin B9 expression and N and M stages, affirming its role in promoting tumor invasiveness.

Previous studies have substantiated the capacity of Serpin B9 to facilitate tumor progression by impeding the natural killer (NK) cell-mediated cytotoxic effect induced by GrB.[11] Serpin B9 has been shown to reduce the immune activity of chimeric antigen receptor-T cells.[23] And Jiang et al[11] observed in mice deficient in Serpin B9 a reduction in the population of immunosuppressive cells and a concurrent increase in the levels of protective T cell immunity associated with tumors. Furthermore, the concurrent inhibition of Serpin B9 expression in both mice and tumors led to a further reduction in tumor growth. These findings underscore the association between Serpin B9 and the quantity and functionality of immunosuppressive cells within the tumor microenvironment, suggesting its potential as a novel immune checkpoint. Combining conventional anti-PD1/PDL1 therapy with interventions targeting Serpin B9 may yield enhanced therapeutic efficacy.

To evaluate the feasibility of combined Serpin B9 and anti-PD1/PDL1 therapy at the patient level, we conducted a subgroup analysis to assess the treatment efficacy after four cycles of therapy in patients receiving anti-PD1/PDL1 treatment in our study cohort. Our findings revealed that patients exhibiting low Serpin B9 expression demonstrated higher rates of CR, PR, and overall ORR than those with high Serpin B9 expression. Conversely, higher rates of progressive PD and SD were observed in the group with high Serpin B9 expression. These results suggest that downregulation of Serpin B9 expression levels during anti-PD1/PDL1 therapy may enhance treatment efficacy.

In conclusion, our study revealed elevated Serpin B expression levels in the tumor tissues of patients with lung adenocarcinoma compared to those in the adjacent tissues. Serpin B9, akin to the N stage, M stage, and overall stage, emerged as an independent prognostic factor for PFS. Notably, patients with diminished Serpin B9 expression exhibited a more favourable ORR to anti-PD1/PDL1 therapy. These findings suggest the potential utility of Serpin B9 as a therapeutic target for managing lung adenocarcinoma. Combining Serpin B9 inhibition with anti-PD1/PDL1 therapy may offer enhanced treatment efficacy.

Nevertheless, our study was limited to a subset of lung adenocarcinoma patients exclusively from Anhui Province, China, potentially introducing ethnic and regional biases. Additionally, we did not evaluate PD1/PDL1 expression levels in all patients, thereby precluding elucidation of the correlation between Serpin B9 and PD1/PDL1 expression levels. These constraints underscore the necessity for future investigations to address these limitations comprehensively and provide a more profound understanding.

Data Sharing Statement

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Ethics Approval Statement

This study was approved by the Ethics Review Committee of the Hefei Cancer Hospital, Chinese Academy of Sciences. [Ethics Approval number: Quick-PJ 2022-05-34].

Patient Consent Statement

Informed consent was obtained from all patients.

Clinical Trial Registration

This study is retrospective and not clinical trial.

Author Contributions

Yue Fang and Yi Yue are co-first authors. All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part

in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work. Yue Fang and Yi Yue made the same contributed equally to this study.

Funding

This study was supported by the Outstanding Young Medical Talent Program of the Hefei Cancer Hospital, Chinese Academy of Sciences, China(No. 2000000005).

Disclosure

All authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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