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RESEARCH

Molecular subtyping dictates therapeutic response to anti-PD-L1 immunotherapy in ES-SCLC

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Abstract

Anti-PD-L1 immunotherapy is recommended as standard of care for patients with extensive stage small cell lung cancer (ES-SCLC); however, there are no reliable biomarkers guiding patient selection and the survival benefit of PD-L1 inhibitors in the overall population is limited. In this study, we retrospectively analyzed a total number of 61 cases of ES-SCLC who underwent anti-PD-L1 immunotherapy. Patient demographic characteristics and laboratory findings were processed for univariate and multivariate analysis. Subgrouping of SCLC was performed on IHC platform using antibodies against ASCL1, NEUROD1 and POU2F3. The tumor microenvironment (TME) of ES-SCLC was evaluated by CD8 + T cell infiltration, granzyme B production and PD-L1 expression. We found limited efficacy of defined variable factors conferring therapeutic outcomes of anti-PD-L1 immunotherapy in patients with ES-SCLC. Intriguingly, there was a profound difference in TME and response to anti-PD-L1 immunotherapy when classifying SCLC into A/N/P/I subgroups. Although accounted for a small proportion of SCLC, the SCLC-P and SCLC-I subtypes manifested as T cell-enriched "hot" tumor and elicited more favorable response to immunotherapy, whereas the SCLC-A and SCLC-N subgroups were T cell-absent "cold" tumor. There was also a significant difference in progression free survival and overall survival across these subsets. Moreover, we found the SCLC-P and SCLC-I tumors revealed features of low neuroendocrine (NE) differentiation and showed clinicopathologic features overlapping with the SCLC non-NE lineage. These findings may aid clinicians to select ES-SCLC patients who were more likely to gain higher response rate and longer survival to anti-PD-L1 immunotherapy. Revisiting SCLC according to A/N/P/I subtyping and NE/non-NE differentiation is a reliable approach to guide therapeutic strategy in patients with ES-SCLC.

Keywords ES-SCLC · Molecular subtyping · NE differentiation · TME · Immunotherapy

Abbreviations

ASCL1	Achaete–scute homolog 1
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
dNLR	Derived neutrophil to lymphocyte ratio
ECOG-PS	Eastern Cooperative Oncology Group Performance Status
EP	Etoposide–platinum
FFPE	Formalin-fixed, paraffin-embedded
GZMB	Granzyme B
irAEs	Immune-related adverse events
LDH	Lactate dehydrogenase
LIPI	Lung immune prognostic index
NE	Neuroendocrine
NEUROD1	Neurogenic differentiation factor 1
NSCLC	Non-small cell lung cancer
NSE	Neuron-specific enolase
PD-1	Programmed cell death protein-1
PD-L1	Programmed death ligand-1
POU2F3	POU class 2 homeobox 3
SCLC	Small cell lung cancer
Syn	Synaptophysin
TME	Tumor microenvironment

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Introduction

In the light of growing insights into the molecular basis for tumorigenesis, emerging therapeutic targets for non-small cell lung cancer (NSCLC) have been identified [1, 2]. Targeted therapy with tyrosine kinase inhibitors in patients harboring defined oncogenic alterations markedly improved patient survival and quality of life. Unfortunately, patients with extensive stage small cell lung cancer (ES-SCLC) still elicited a dismal prognosis and have very limited therapeutic options [3]. Despite an initial response to etoposide–platinum (EP) doublet regimen at first-line setting could be achieved, most patients rapidly progressed on chemotherapy within 6 months of treatment [4, 5]. The comprehensive genomic profiling of SCLC suggested a pivotal of *Rb1* and *TP53* tumor suppressor during SCLC tumorigenesis and progression [6, 7]. By using genetically engineered mouse models, scientists have demonstrated that loss-of-function mutations in *Rb1* and *TP53* is sufficient to drive SCLC [8]. However, targeting these mutant tumor suppressors is technologically challenging and there is no targeted therapy approved for ES-SCLC at the moment.

During the past ten years, immune checkpoint blockade with anti-PD-L1 (programmed death ligand-1) antibodies has been a milestone in clinical practice for patients with ES-SCLC. Two global randomized phase III clinical trials, CASPIAN and IMPOWER 133, demonstrated that the addition of anti-PD-L1 antibodies to EP chemotherapy, followed by immunotherapy consolidation, provided more survival benefits over EP chemotherapy alone in ES-SCLC [9, 10]. In contrast, blockade of the immune inhibitory signaling by antibodies that target PD-1 (programmed cell death protein-1) or CTLA-4 (cytotoxic T-lymphocyte associated protein 4) did not show significant improvement in survival outcomes [11, 12]. These findings led to the approval of chemotherapy plus anti-PD-L1 immunotherapy combinations as standard-of-care in ES-SCLC. Although the CASPIAN and IMPOWER 133 studies have reached their primary endpoints, it is worthy to note that the addition of Durvalumab or Atezolizumab to chemotherapy only yielded a modest extension in patient survival in ITT cohort (ΔmOS = 2.7 months in Durvalumab group; ΔmOS = 2.0 months in Atezolizumab group; versus EP chemotherapy), whereas there was still a small fraction (~ 20%) of patients could survive over 2 years. How to distinguish these long-term responders from transient responders before the initiation of anti-PD-L1 immunotherapy remains confusing due to lack of predictive biomarkers in patients with ES-SCLC.

In order to decipher the discrepancy in therapeutic vulnerabilities, scientists have proposed a novel classification of SCLC based on the expression of three distinct transcriptional factors: achaete–scute homolog 1 (ASCL1), neurogenic differentiation factor 1 (NEUROD1), POU class 2 homeobox 3 (POU2F3), which classified SCLC into SCLC-A (ASCL1-dominant), SCLC-N (NEUROD1-dominant), SCLC-P (POU2F3-dominant), and SCLC-I (triple negative) subtypes, respectively [13]. This molecular classification provides a platform to understand the difference in SCLC biology and unravels a feasible approach toward precision immunotherapy for patients with SCLC. For example, BCL-2 as a transcriptional target of ASCL1 selectively overexpressed in the SCLC-A subtype and impended increased susceptibility to BCL-2 inhibitors [14]. The c-Myc oncogene is frequently amplified in the SCLC-N subtype and a combination strategy involving Aurora kinase and c-Myc inhibitors may improve patient survival in this specific subtype [15, 16]. POU2F3 encodes enzymes implicated in DNA damage repair, and patients with SCLC-P subtype showed a benefit from PARP inhibitors, such as Olaparib and Veliparib [13]. Intriguingly, the SCLC-A/N/P/I classification may also overlap with the canonical neuroendocrine (NE)/non-NE lineages. Evidence supports the overlapping is based on the fact that the SCLC-A and SCLC-N subtypes constitute the majority of SCLC and are usually strongly positive for NE-related proteins (NE subset), whereas the SCLC-P and SCLC-I tumors were generally recognized as the non-NE phenotype [17, 18]. Overall, SCLC is a quite heterogeneous disease characterized by different molecular features and different therapeutic response to anti-tumor treatment. Screening A/N/P/I and NE states to categorize SCLC hold the promise to select long-term responders in the era of immunotherapy.

Herein, we reported the SCLC-A/N/P/I and NE/non-NE classification as a feasible approach to distinguish ES-SCLC patients who are likely to gain long-term survival benefit from anti-PD-L1 immunotherapy. Notably, PD-L1 status was generally negative in most cases, but its expression was enriched in the SCLC-P/I and non-NE cohort. In comparison with the SCLC-A/N and NE subsets, the SCLC-P/I and non-NE cohort are preferentially associated with a “hot” tumor microenvironment (TME) that dictated a favorable response to anti-PD-L1 immunotherapy. Further prospective study to assess the efficacy of SCLC-A/N/P/I and NE/non-NE subtyping as feasible approaches to select SCLC patients would be clinically meaningful.

Methods

Patient selection and study design

We retrospectively collected 61 cases of formalin-fixed, paraffin-embedded (FFPE) tumor tissue blocks from patients with ES-SCLC who received anti-PD-L1 immunotherapy in Jinling Hospital between April 2019 and May 2024. The patient characteristics, including age, gender, smoking history, lines of treatment, anti-tumor regimen, are listed in Table 1.

Table 1 Baseline characteristics of SCLC patients ($n=61$)

Clinical characteristics	*n* (%)
Age, Median (range)	64 (47–84)
< 65 y	31 (50.8)
≥ 65 y	30 (49.2)
Gender	
Male	50 (82.0)
Female	11 (18.0)
Smoking status	
Former/current smoker	43 (70.5)
Non-smoker	18 (29.5)
ECOG-PS	
0	31 (50.8)
1	30 (49.2)
Site of metastasis	
Brain	9 (14.8)
Liver	7 (11.5)
Bone	14 (23.0)
Adrenal gland	3 (4.2)
Lymph node	28 (45.9)
Previous anti-cancer treatments	
Chemotherapy	21 (34.4)
Radiotherapy	9 (14.8)
Laboratory examination, mean ± SD	
CEA, μg/L	17.56 ± 86.24
AFP, μg/L	2.65 ± 0.92
LDH, IU/L	303.44 ± 218.79
NSE, μg/L	68.25 ± 72.39
SCC, ng/mL	1.15 ± 2.61
CA125, IU/mL	66.05 ± 85.70
LIPI	
0	36 (59.0)
1	23 (37.7)
2	2 (3.3)
NLR	3.12 ± 1.34
LMR	3.36 ± 1.87
PLR	180.92 ± 97.11
dNLR	2.00 ± 0.70

Ethics statement

This retrospective study was reviewed and approved by the Institutional Medical Ethics Committee of Jinling Hospital (2024DZKY-050-01). The study complies with the ethical standards for research involving human participants, as outlined in the Declaration of Helsinki. All patients have provided informed consent for data acquisition and analysis.

Clinical data acquisition and analysis

Clinical data, including medical histories, laboratory findings, treatment regimens, therapeutic responses, and survival outcomes, were retrieved from the electronic medical record system, which was regularly preserved and updated by data managers. The objective response was assessed by clinicians according to the Response Evaluation Criteria in Solid Tumors (RECIST) v1.1. PFS was defined as the duration from the date of diagnosis until disease progression. OS was defined as the duration from the date of diagnosis to the date of death from any cause or the last follow-up.

Pathology, immunohistochemistry staining and assessment

SCLC H&E slides were thoroughly evaluated by pathologists to determine the proportion of viable tumor cells and their representativeness. Consecutive 5 μm-thick tissue sections were cut from biopsy blocks and antigen retrieval was performed with a low pH citrate buffer. Endogenous peroxidase activity was blocked using 0.3% H_2O_2. The slides were incubated with primary antibodies against ASCL1 (Ab211327, Abcam), NEUROD1 (Ab213725, Abcam), and POU2F3 (#36,135, Cell Signaling Technology) at 4 °C overnight. After 3 times washes in PBS, the slides were incubated with horseradish peroxidase-conjugated secondary antibodies and DAB substrate (K3468, Dako). Two-steps Anti-Rabbit IgG Peroxidase Polymer Kit (PV-6001, ZSGB-BIO) and Anti-Mouse IgG Peroxidase Polymer Kit (PV-6002, ZSGB-BIO) were used according to the manufacturer's instructions. All interpretations were conducted and reviewed by two independent pathologists with no access to clinical features and therapeutic outcomes.

Evaluation of PD-L1 expression, NE status and TME

The expression of Synaptophysin (Syn, ZA-0506, ZSGB-BIO), Chromogranin A (CgA, ZA-0507, ZSGB-BIO) and CD56 (ZM-0057, ZSGB-BIO) was derived to determine the NE/non-NE status of SCLC. The expression of PD-L1 was assessed by the anti-PD-L1 22C3 antibody because this antibody clone was approved as a companion diagnostic

antibody for its predictive role in the therapeutic response to immunotherapy. To gain an insight into TME implicated in SCLC, the tumor specimen was processed with anti-PD-L1, anti-CD8 (Ab245118, Abcam), and anti-GZMB (granzyme B, Ab255598, Abcam) antibodies, respectively.

Statistical analysis

Categorical variables were analyzed using chi square test (χ^2). Survival curves were delineated with the Kaplan–Meier method and the difference between survival curves were estimated by log-rank test. Variables with *P* values less than 0.05 in the univariate analyses were processed into the multivariate Cox proportional hazards regression model. Statistical analyses were conducted with SPSS 11.0 software and *P* value below 0.05 of the two-sided tests was considered significantly different.

Result

Patient characteristics

The detailed patient baseline characteristics for 61 ES-SCLC cases in this study are summarized in Table 1. The patients included 50 gentle man (82%) and 11 ladies (18%), with an average age of 64 years-old. Most of the patients were smokers (70.5%). The Eastern Cooperative Oncology Group Performance Status (ECOG-PS) was 0 in 31 patients (50.8%), and the remaining 30 cases presented with an ECOG-PS score of 1. All patients were histologically confirmed SCLC at extensive stage, with involvement of lymph nodes (45.9%), brain (14.8%), liver (11.5%), bone (23%) and adrenal gland (4.2%) metastasis. A majority of the patients (40/61, 65.6%) were treated with first-line combinational anti-PD-L1 immunotherapy, including Durvalumab or Atezolizumab, and 21 patients received anti-PD-L1 immunotherapy at second-line setting. Prior treatment included EP chemotherapy ($n=21$) and radiotherapy ($n=9$). The 9 patients who received radiation therapy also received concurrent EP chemotherapy.

Laboratory examination before the initiation of anti-PD-L1 immunotherapy was also assessed. As listed in Table 1, the average concentrations of cancer-associated serological biomarkers were almost within normal limits, except neuron-specific enolase (NSE). Inflammatory biomarkers that associated with poor prognosis in advanced cancer, such as lactate dehydrogenase (LDH), tended to be increased in patients with ES-SCLC. Another potential biomarker, derived neutrophil to lymphocyte ratio (dNLR) designated to reflect the inflammatory status in various cancers, elicited an average value of 2.00. The combination of dNLR and LDH to yield a lung immune prognostic index (LIPI) has been demonstrated to be a predictive biomarker negatively associated with the survival benefit from anti-PD-1/PD-L1 immunotherapy in patients with NSCLC [19]. In our study, 59% of patients was endowed with a low LIPI score. Accordingly, the patients were categorized into LDH^{low}/LDH^{high} (cutoff value: 245 IU/L), $dNLR^{low}$/$dNLR^{high}$ (cutoff value: 2.01) and $LIPI^{low}$ (LIPI score = 0)/$LIPI^{high}$ (LIPI ≥ 1) groups. Unfortunately, patient survival was not significant different across each group, regardless of LDH, dNLR and LIPI status. Although the LDH^{low} cohort ($n=36$) possessed a marginally favorable PFS over the LDH^{high} cohort ($n=25$), a comparable level of OS was found between the two groups (Fig. 1A). Similar findings were noticed between the $dNLR^{low}$ cohort ($n=30$) and $dNLR^{high}$ cohort ($n=31$) (Fig. 1B), and the $LIPI^{low}$ ($n=36$) cohort and $LIPI^{high}$ ($n=25$) cohort (Fig. 1C). Thus, these established biomarkers failed to discriminate long-term responders from transient responders in patient with ES-SCLC.

Analysis of prognostic factors

Univariate Cox regression suggested that patients with baseline liver metastasis (HR = 2.613, 95% CI 1.143–5.969, $P=0.023$), high level of LDH (HR = 1.001, 95% CI 1.000–1.002, $P=0.032$) and NSE (HR = 1.006, 95% CI 1.002–1.010, $P=0.002$) had shorter PFS. Therefore, liver metastasis, LDH and NSE that elicited reverse association with PFS on univariate analyses were selected for multivariate analysis (Table 2). Among these parameters, only baseline liver metastasis showed a higher risk of disease progression compared to that without metastasis (HR = 2.642, 95% CI 1.118–6.244, $P=0.027$). In consistent with this notion, patients with liver metastasis tended to have shorter OS according to both univariate analysis (HR = 3.644, 95% CI 1.587–8.368, $P=0.002$) and multivariate analysis (HR = 4.241, 95% CI 1.716–10.485, $P=0.002$) (Table 3). Patients with male gender (HR = 2.584, 95% CI 1.006–6.637, $P=0.049$), smoking history (HR = 2.157, 95% CI 1.026–4.537, $P=0.043$), lymph node metastasis (HR = 2.060, 95% CI 1.093–3.885, $P=0.025$) and high NSE level (HR = 1.008, 95% CI 1.004–1.013, $P<0.001$) experienced deteriorated OS when analyzed by univariate Cox regression; however, these variables were not prognostic factors under multivariate analysis except NSE level (HR = 1.007, 95% CI 1.002–1.012, $P=0.005$). Collectively, these results suggested that there were limited variables capable of dictating therapeutic outcomes to anti-PD-L1 immunotherapy in ES-SCLC patients; thus, identification of reliable biomarkers for patient selection to improve therapeutic efficacy was urgently needed.

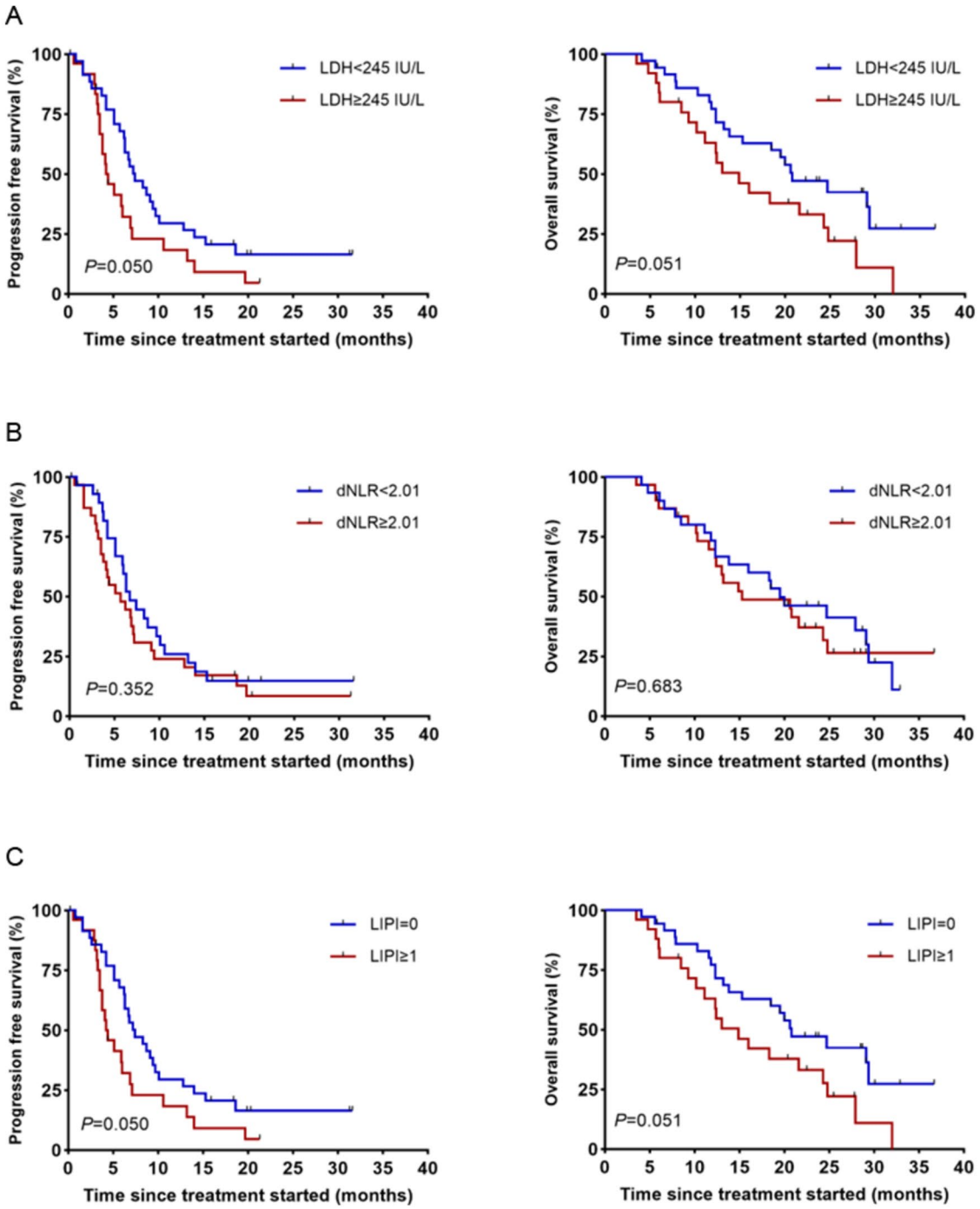

Fig. 1 Progression free survival and overall survival in patients with ES-SCLC receiving anti-PD-L1 immunotherapy stratified by LDH (**A**), dNLR (**B**) and LIPI (**C**)

Classification of SCLC-A/N/P/I subtypes

We performed IHC staining of ASCL1, NEUROD1, POU2F3 and assigned SCLC subtypes based on the expression of defined transcriptional factors, which resulted in a novel category consisting of SCLC-A, SCLC-N, SCLC-P and SCLC-I subgroups. The exact number of ES-SCLC patients ($n = 61$) met corresponding category criteria in the present study was 36, 12, 4 and 9, respectively. These transcriptional factors preferentially localized in the nucleus and seemed to be mutually exclusive from each other (Fig. 2). Notably, 5

Table 2 Univariate and multivariate Cox analysis of PFS in ES-SCLC

Variables	PFS			
	Univariate HR (95% CI)	*P*	Multivariate HR (95% CI)	*P*
Age, < 65 vs. ≥ 65	0.657 (0.376–1.146)	0.139		
Gender, male vs. female	0.972 (0.485–1.947)	0.935		
Smoking, smoker vs. non-smoker	1.242 (0.677–2.278)	0.484		
ECOG-PS, 0 vs.1	0.630 (0,361–1.102)	0.106		
Brain metastasis, Yes vs. No	1.300 (0.582–2.902)	0.523		
Liver metastasis, Yes vs. No	2.613 (1.143–5.969)	0.023	2.642 (1.118–6.244)	0.027
Bone metastasis, Yes vs. No	1.404 (0.732–2.694)	0.307		
Adrenal gland metastasis, Yes vs. No	1.034 (0.321–3.337)	0.955		
Lymph node metastasis, Yes vs. No	1.280 (0.729–2.246)	0.390		
Molecular subtypes, A/N vs. P/I	2.101 (1.010–4.371)	0.047	2.180 (1.038–4.580)	0.040
CEA, μg/L	1.001 (0.998–1.003)	0.697		
AFP, μg/L	1.185 (0.865–1.624)	0.290		
LDH, IU/L	1.001 (1.000–1.002)	0.032	1.001 (0.999–1.002)	0.232
NSE, μg/L	1.006 (1.002–1.010)	0.002	1.004 (0.999–1.008)	0.112
SCC, ng/L	1.029 (0.937–1.129)	0.555		
CA125, IU/mL	1.002 (0.999–1.005)	0.208		
LIPI, 0 vs. 1 and 2	0.575 (0.327–1.012)	0.055		
NLR	1.127 (0.892–1.424)	0.316		
LMR	0.964 (0.789–1.179)	0.723		
PLR	1.002 (0.999–1.005)	0.117		
dNLR	2.070 (1.251–3.426)	0.005	1.970 (1.232–3.148)	0.005

Table 3 Univariate and multivariate Cox analysis of OS in ES-SCLC

Variables	OS			
	Univariate HR (95% CI)	*P*	Multivariate HR (95% CI)	*P*
Age, < 65 vs. ≥ 65	0.696 (0.372–1.302)	0.257		
Gender, male vs. female	2.584 (1.006–6.637)	0.049	1.552 (0.349–6.907)	0.564
Smoking, smoker vs. non-smoker	2.157 (1.026–4.537)	0.043	1.005 (0.315–3.204)	0.993
ECOG-PS, 0 vs.1	0.846 (0.450–1.590)	0.604		
Brain metastasis, Yes vs. No	1.198 (0.463–3.099)	0.710		
Liver metastasis, Yes vs. No	3.644 (1.587–8.368)	0.002	4.241 (1.716–10.485)	0.002
Bone metastasis, Yes vs. No	1.439 (0.700–2.958)	0.323		
Adrenal gland metastasis, Yes vs. No	1.151 (0.276–4.804)	0.847		
Lymph node metastasis, Yes vs. No	2.060 (1.093–3.885)	0.025	1.609 (0.812–3.185)	0.173
Molecular subtypes, A/N vs. P/I	2.937 (1.134–7.609)	0.027	3.437 (1.119–10.562)	0.031
CEA, μg/L	1.001 (0.999–1.004)	0.311		
AFP, μg/L	0.936 (0.647–1.354)	0.726		
LDH, IU/L	1.001 (1.000–1.002)	0.163		
NSE, μg/L	1.008 (1.004–1.013)	< 0.001	1.007 (1.002–1.012)	0.005
SCC, ng/L	1.072 (0.970–1.185)	0.171		
CA125, IU/mL	1.002 (0.999–1.005)	0.221		
LIPI, 0 vs. 1 & 2	0.546 (0.294–1.013)	0.055		
NLR	1.106 (0.853–1.435)	0.446		
LMR	0.946 (0.784–1.142)	0.564		
PLR	1.001 (0.998–1.004)	0.433		
dNLR	1.496 (0.873–2.562)	0.143		

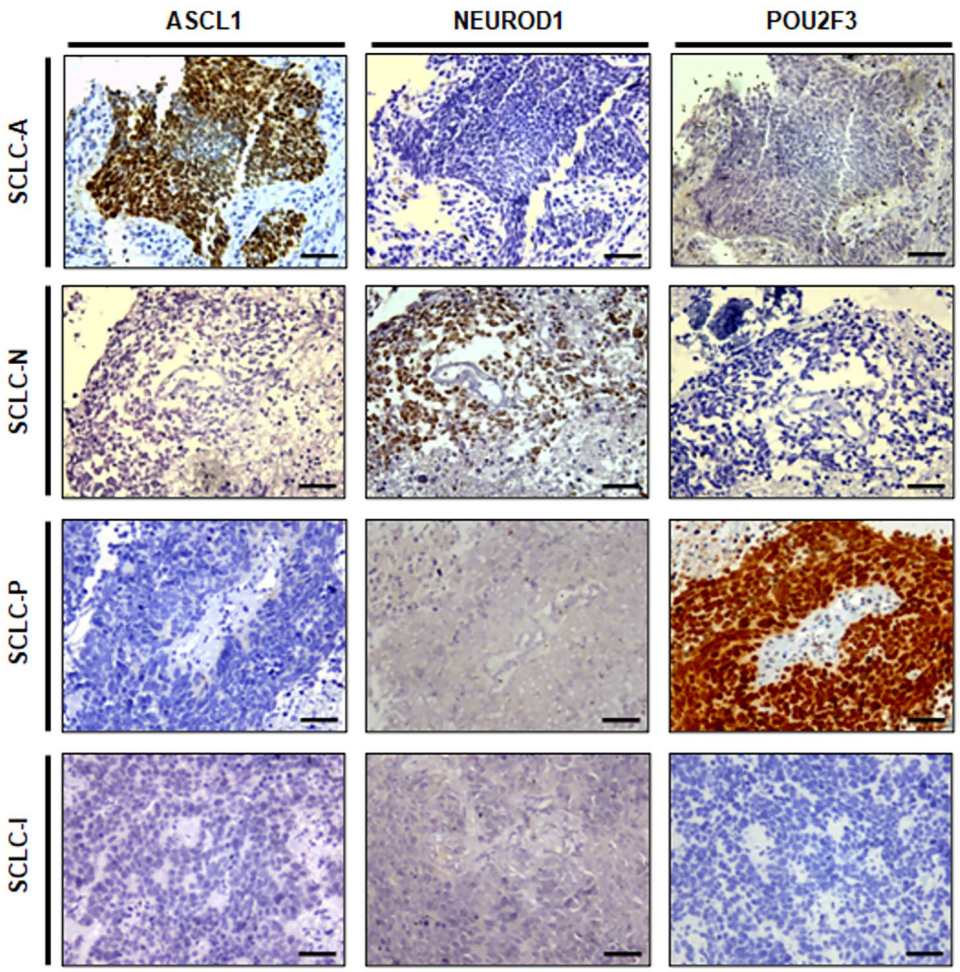

Fig. 2 Representative IHC images of SCLC-A, SCLC-N, SCLC-P and SCLC-I subtypes. Scale bar = 40 μm

patients were found to be positive for both ASCL1 and NEUROD1; however, the magnitude of NEUROD1 expression was marginally positive in the 5 patients, of whom IHC staining of ASCL1 was strongly positive. These 5 patients therefore were assigned to the SCLC-A subgroup (Supplementary Fig. S1A, B). In agreement with the distribution pattern reported in IMPOWER133 trial (SCLC-A 51%, SCLC-N 23%, SCLC-P 7%, SCLC-I 18%) [13], the most prominent subtype in our study was SCLC-A ($n = 36$, 59%), followed by SCLC-N ($n = 12$, 19.7%) and SCLC-I ($n = 9$, 14.8%), with SCLC-P ($n = 4$, 6.5%) being the least common subtype. Given the difference in the expression profile of certain transcriptional factors, each subtype may possess distinct biological characteristics. It was anticipated that the less common SCLC-P and SCLC-I subtypes may elicit different clinical features and therapeutic vulnerabilities to anti-PD-L1 immunotherapy compared with the more common SCLC-A and SCLC-N subtypes.

Distinct SCLC TME under A/N/P/I subtyping

To decipher whether there was a difference in TME based on the SCLC-A/N/P/I classification, we characterized TME of each subtype by analyzing the status of T cell infiltration, GZMB secretion and PD-L1 expression, respectively. IHC staining of CD8, GZMB and PD-L1 in SCLC-A biopsy samples showed the cancer cells were negative for PD-L1 expression, and there was no detectable CD8 + T cell infiltration and GZMB secretion (Fig. 3A). Therefore, the SCLC-A subtype was non-inflamed tumor, which has been characterized by the absence of cytotoxic T cells in the TME. The immune landscape of SCLC-N tumor shared many similarities with SCLC-A tumor and it also lacked active CD8 + T cells infiltration. As such, both SCLC-A and SCLC-N subtypes were defined as "cold" tumor.

Strikingly, we got opposite results of TME profiling in SCLC-P and SCLC-I subtypes. These less common subtypes of SCLC revealed features of inflamed "hot"

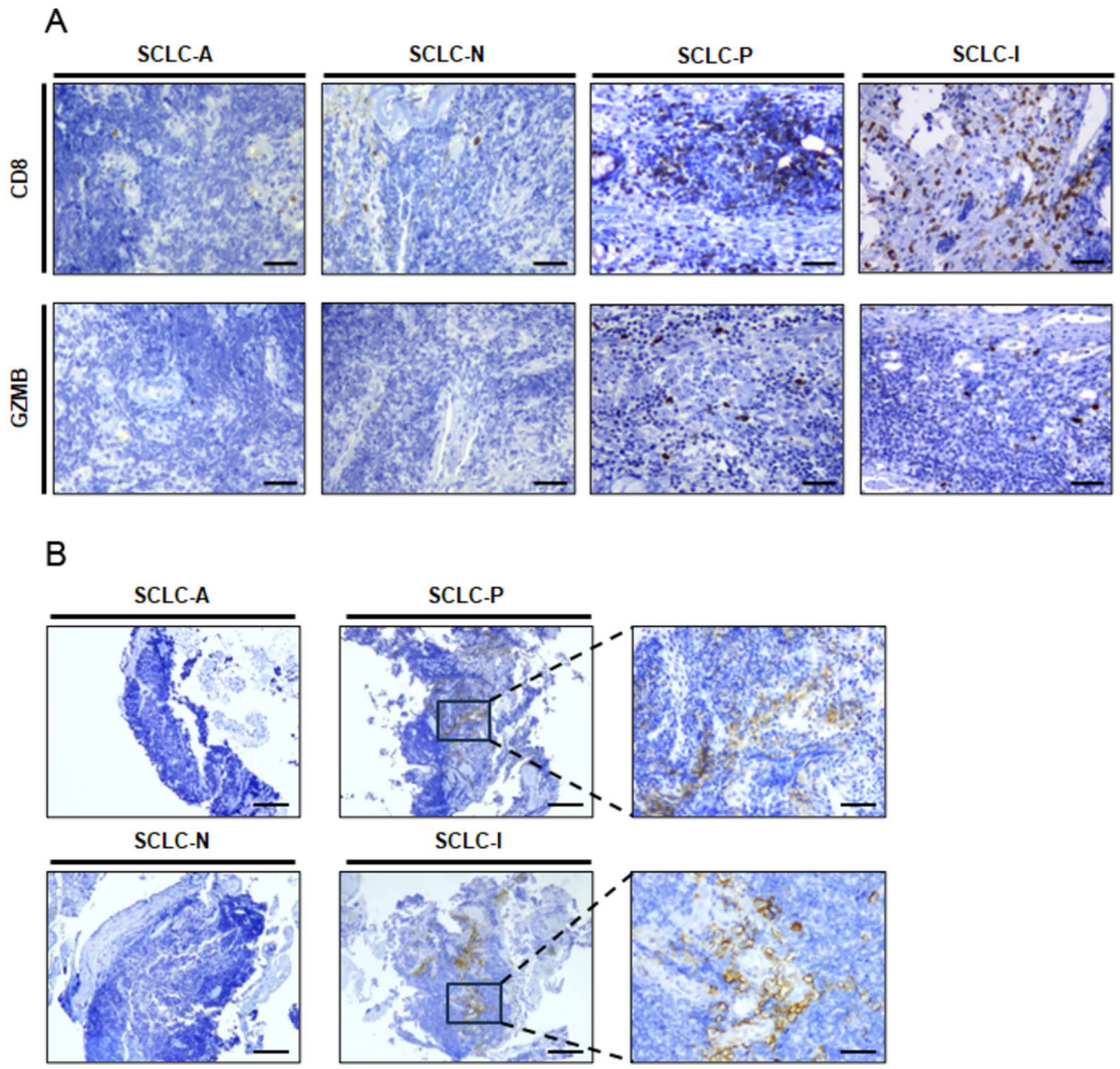

Fig. 3 TME analysis under SCLC-A/N/P/I subtyping. **A** Evaluation of T cell infiltration and GZMB production in subtypes of ES-SCLC. The infiltrated T cells were stained with an anti-CD8 antibody and their activities were assessed by GZMB staining. Scale bar = 40 μm. **B** The expression of membrane-anchored PD-L1 protein in each subtype was determined by the authorized 22C3 antibody. Scale bar = 40 μm under low magnification, and = 200 μm under high magnification, respectively

tumors, as judged by CD8 and GZMB IHC staining. PD-L1 immunolabeling identified a high number of membrane-anchored PD-L1 positive tumor cells within tumor nests in SCLC-P and SCLC-I tumor (Fig. 3B). It was noted that the distribution of CD8 + T cells was inside tumor, along with GZMB production. These findings suggested that the TME of SCLC-P and SCLC-I subtypes discriminated from that of SCLC-A and SCLC-N subtypes. In line with this notion, the SCLC-A and SCLC-N (mentioned as SCLC-A/N thereafter) tumors were annotated as "cold" tumor, whereas the SCLC-P and SCLC-I (SCLC-P/I) tumors were "hot" tumor.

Overlapping of SCLC-NE/non-NE lineages and A/N/P/I classification

As a hallmark of SCLC, most SCLC tumors were positive for NE differentiation. We next explored whether there is a correlation between SCLC-A/N/P/I subtyping and the expression of canonical diagnostic NE differentiation markers of SCLC, including Syn, CgA and CD56. We found that nearly 70% of patients ($n = 41$) were positive for NE markers (NE lineage), and the remaining 20 patients were negative for at least one of the three NE markers (non-NE lineage) (Fig. 4A). We noted the expression magnitude of

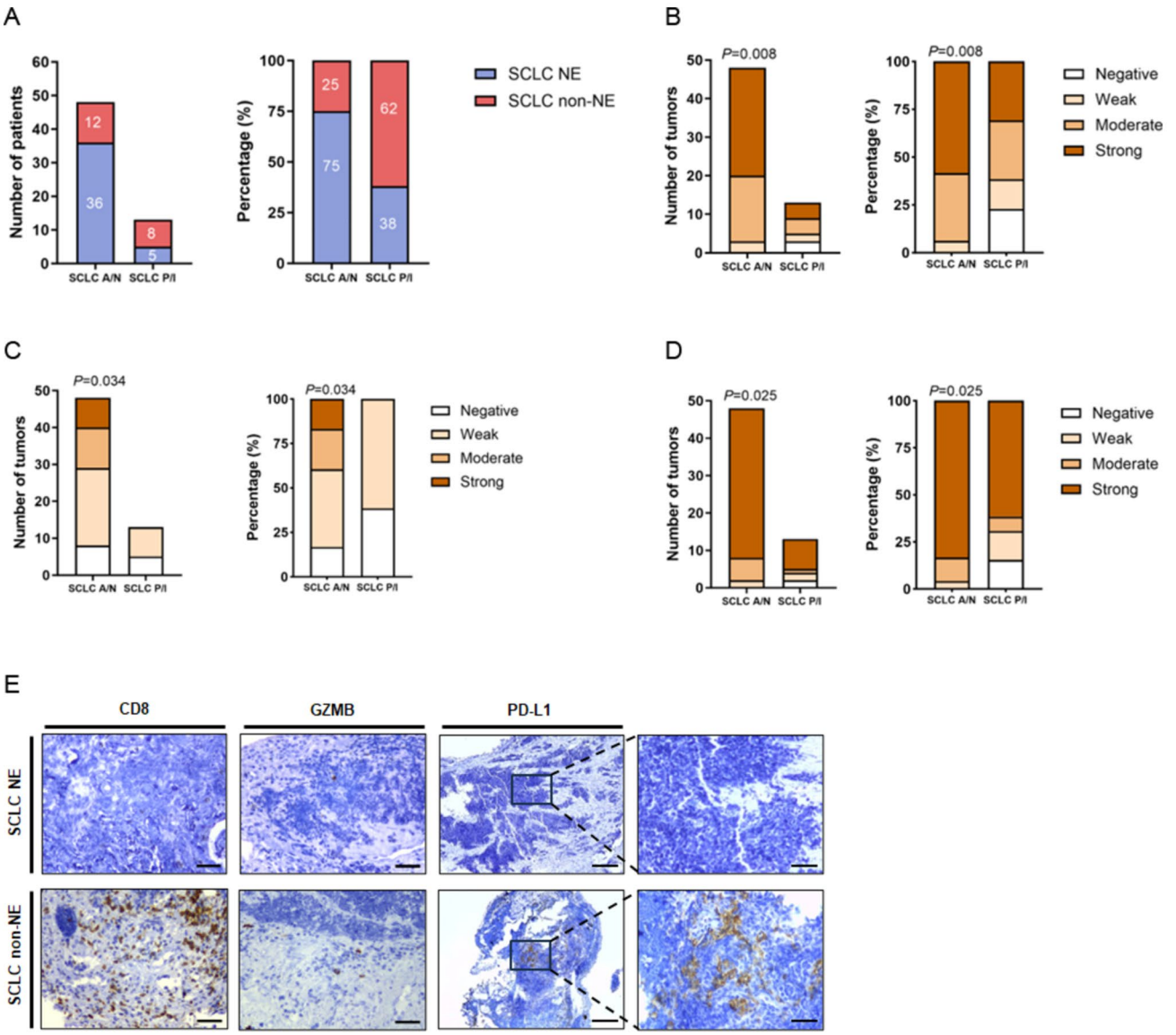

Fig. 4 Overlapping between SCLC-A/N/P/I and NE/non-NE classifications. **A** Comparisons of NE differentiation status (NE subset versus non-NE subset) between SCLC-A/N and SCLC-P/I subgroups. **B**, **D** Comparisons of Syn (**B**), CgA (**C**) and CD56 (**D**) composition in SCLC tumors grouped into SCLC A/N and SCLC-P/I subtypes. **E** Representative IHC images of CD8 + T cells, GZMB and PD-L1 expression in SCLC with different NE status. Scale bar = 40 μm under low magnification, and = 200 μm under high magnification, respectively. Statistical significance was calculated using chi square test (χ^2)

conventional NE markers was significantly lower in the SCLC-P/I subtype. In the SCLC-A/N cohort ($n = 48$), 36 patients (75%) with high level of NE markers were annotated as the NE subset, whereas 12 patients (25%) were negative for NE markers (non-NE subset). By contrast, the number of patients exhibiting NE features was 5 (38%), versus 8 patients (62%) annotated as non-NE subset, in the SCLC-P/I cohort ($n = 13$).

When analyzed individually, the differences in the three conventional NE markers between SCLC-A/N and SCLC-P/I cohorts were more significant. As shown in Fig. 4B, both the absolute number and proportion of Syn-positive SCLC in SCLC-A/N cohort were markedly higher in comparison with the SCLC-P/I cohort ($P = 0.008$). Specifically, a total number of 58 cases was positive for Syn to different degrees (weakly positive, moderate positive and strongly positive). Among these Syn-positive patients, 48 cases were found in the SCLC-A/N cohort and the remaining 10 cases manifested as SCLC-P/I. Moreover, more than 50% SCLC-A/N cases were strongly positive for Syn (28/48, 58.3%), whereas only 30.8% SCLC-P/I cases (4/13) yielded a strongly positive staining result. In agreement with these

notions, the incidence of Syn-negative tumor was higher in the SCLC-P/I subgroup (3 vs. 0 case, 23.1 vs. 0%). Similar findings were recaptured for the expression patterns of CgA ($P=0.034$, Fig. 4C) and CD56 ($P=0.025$, Fig. 4D) based on the classification of SCLC-A/N and SCLC-P/I. Although accounted for a minority of ES-SCLC, the non-NE SCLC subset also manifested as an inflamed "hot" tumor due to large amounts of CD8 + cytotoxic T cells infiltration. The expression of membrane-anchored PD-L1 also elevated in the non-NE subgroup. When evaluating TME of NE SCLC, it revealed features of uninflamed "cold" tumor, judged by absence of CD8 + T cell infiltration, lack of GZMB production, and negative expression of PD-L1 (Fig. 4E). Therefore, the SCLC-NE lineage shared overlapping histological and immunological features with the SCLC-A/N subset, and vice versa. The NE/non-NE feature of SCLC is likely to mirror TME status and aid clinicians to categorize the A/N/P/I classification.

Using novel classification to stratify therapeutic outcomes in ES-SCLC

Having demonstrated the SCLC-A/N/P/I and NE/non-NE classifications dictated distinct status of TME, we proposed the inflamed SCLC-P/I and non-NE subsets preferentially benefited from anti-PD-L1 immunotherapy (Tables 2, 3). In line with this notion, a total number of 23 patients responded to the treatment, with the best objective response of PR. Intriguingly, only 31.3% patients expressing ACSL1 or NEUROD1 achieved PR, whereas 61.5% of patients in the SCLC-P/I subgroup impended PR. There were 16 cases progressed on immunotherapy, in which 15 cases were found in the SCLC-A/N subgroup. The disease control rates (DCR) were 68.8% and 92.3% for SCLC-A/N and SCLC-P/I groups, respectively (Supplementary Table S1, Fig. 5A, Supplementary Fig. S2A, B). Consistently, patients with non-NE differentiation achieved superior PR (55.0 vs. 29.3%) and DCR (80.0 vs. 70.8%) rates over those with NE features.

Therapeutic outcomes were analyzed according to A/N/P/I and NE/non-NE subtyping with stratification at the initial stage. In the overall cohort, the median follow-up time was 28.5 months, the median PFS (mPFS) and median OS (mOS) were 6.3 months and 19.5 months, respectively. However, significant differences in patient survival were observed according to SCLC-A/N/P/I classification, showing that patients with SCLC-A/N had an mPFS of 6.0 months and an mOS of 15.65 months (Fig. 5B, C). In contrast, the SCLC-P/I arm achieved mPFS of 12.8 months (HR = 0.439, 95% CI 0.285–0.974, $P=0.041$) and mOS of 29.4 months (HR = 0.527, 95% CI 0.219–0.880, $P=0.02$). The 1-year PFS rate was significantly higher in the SCLC-P/I arm than in the SCLC-A/N arm (51.28 vs. 17.89%). The 2-year OS rate was 63.64% in the SCLC-P/I subgroup,

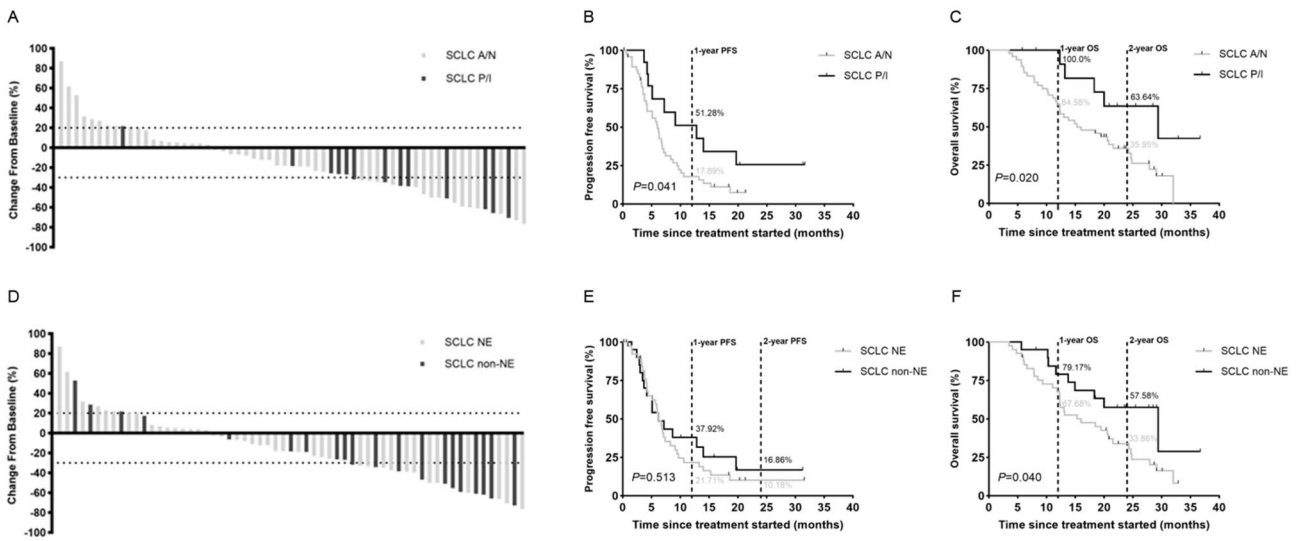

Fig. 5 Therapeutic outcomes of anti-PD-L1 immunotherapy according to A/N/P/I and NE/non-NE subtyping. **A** Maximum percentage of change from baseline in the longest diameter of target lesions, as assessed using RECIST version 1.1 by investigator review. Dotted lines indicate 20% increase (cutoff for determination of progressive disease) and 30% decrease from baseline (cutoff for determination of partial response per RECIST v1.1 criteria). Data are shown for ES-SCLC patients classified into A/N ($n=48$) and P/I ($n=13$) subgroups. **B**, **C** Kaplan–Meier analysis of progression free survival and overall survival for SCLC-A/N and SCLC-P/I patients treated with anti-PD-L1 immunotherapy. **D** Best percentage reduction in the line length of the target lesion compared to baseline. Data are shown for ES-SCLC patients classified into NE ($n=41$) and non-NE ($n=20$) subsets. **E**, **F** Kaplan–Meier analysis of progression free survival and overall survival for SCLC-NE and SCLC non-NE patients treated with anti-PD-L1 immunotherapy

which was more than 1.5-fold higher than the SCLC-A/N subgroup (35.95%).

As we have demonstrated earlier, patients without NE differentiation possessed similarities with SCLC-P/I subgroup. When analyzing patient survival according to NE status, we got similar findings (Supplementary Table S1, Fig. 5D, Supplementary Fig. S2C, D). Although there was no statistic difference in mPFS between NE (6.27 months) and non-NE (6.23 months) subsets (HR = 0.82, 95% CI 0.452–1.486, $P = 0.513$), there was a 1.75-fold increase in 1-year PFS rate in the SCLC non-NE arm (37.92 vs. 21.71%, Fig. 5E). The difference in mOS was significant between NE (16.0 months) and non-NE (29.4 months) arms (HR = 0.515, 95% CI 0.273–0.970, $P = 0.04$), along with a 1.7-fold increase in 2-year OS rate (57.58 vs. 33.86%, Fig. 5F).

Safety profiles based on A/N/P/I and NE/non-NE classification

All enrolled patients were evaluated for safety. Adverse events related to anti-PD-L1 immunotherapy occurred in 25 patients (40.98%). The most common immune-related adverse events (irAEs) were pneumonitis (19.67%), followed by hypothyroidism (9.84%) and diabetes (6.56%). Other

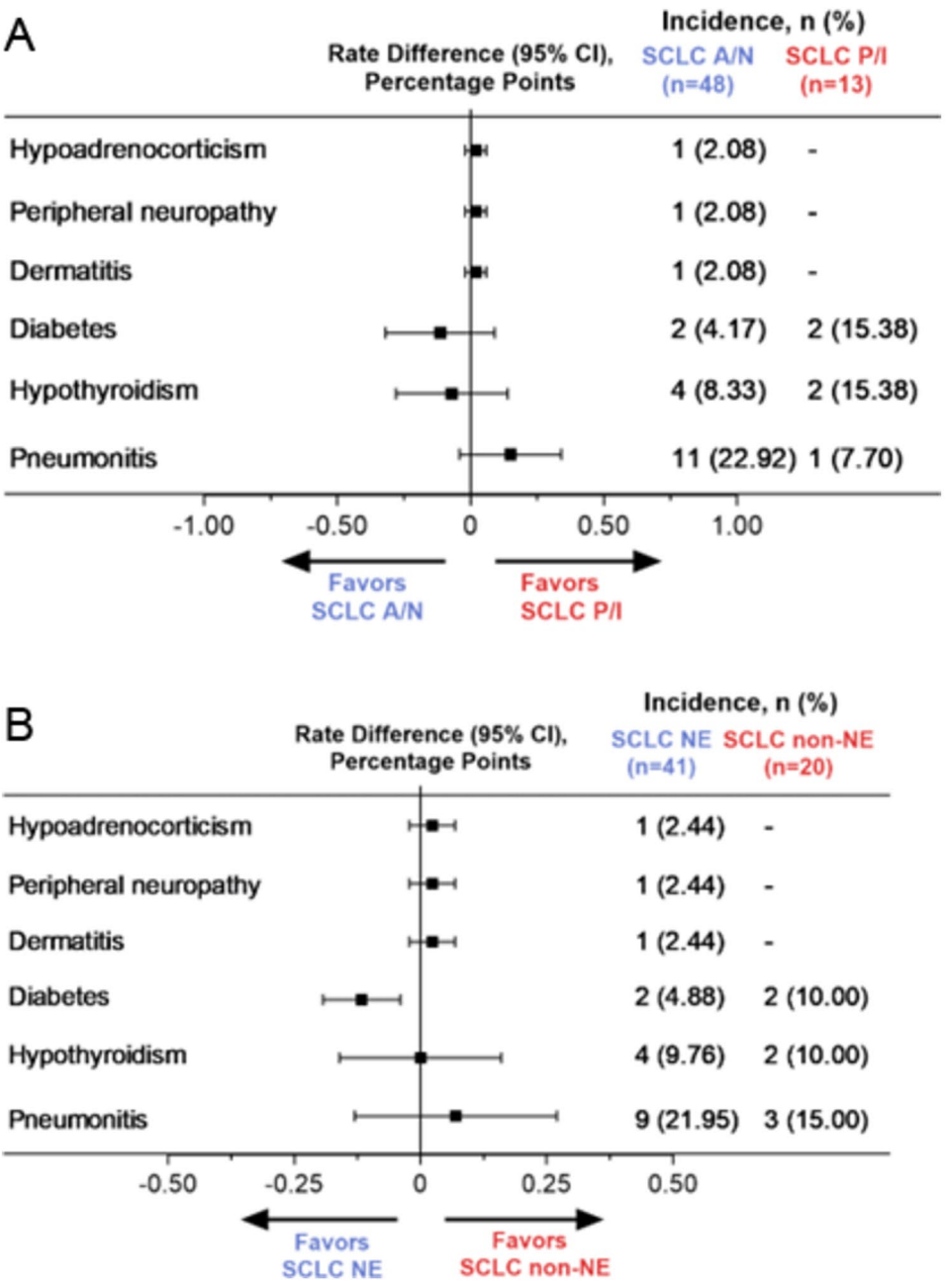

Fig. 6 Incidence of irAEs according to SCLC-A/N/P/I subtyping (**A**) and NE/non-NE classification (**B**)

rare irAEs, including dermatitis, peripheral neuropathy and hypoadrenocorticism, were observed in individual patient. No patient death due to irAEs occurred during the treatment.

Although the overall incidence of irAEs across each subgroup seemed comparable, there was a tendency of increased prevalence of pneumonitis in the SCLC-A/N and NE subgroups. We found that 11 (22.92%) out of 48 patients in the SCLC-A/N arm experienced pneumonitis, while only one patient (7.7%) in the SCLC-P/I arm developed pneumonitis during treatment (Fig. 6A). There were 9 (21.95%) patients and 3 (15%) patients experienced pneumonitis in the NE arm and non-NE arm, respectively (Fig. 6B). The incidence of other irAEs was not significant according to A/N/P/I and NE/non-NE subtyping; thus, this novel classification may also act as predictive factors for predicting immune-related pneumonitis in patients with ES-SCLC.

Discussion

Although ES-SCLC usually possesses a TMB^{high} state, it is generally defined as a "cold" tumor due to lack of immune cells infiltration [20]. The expression of PD-L1 in most SCLC cases is also very weak [21]. To this end, both TMB and PD-L1 expression are not reliable predictive biomarkers for the clinical efficacy of immunotherapy in ES-SCLC. Herein, by using IHC-based subtyping and TME profiling, we demonstrated a direct association between SCLC-A/N/P/I classification and inflamed/non-inflamed TME phenotype. Importantly, we also found the inflamed "hot" SCLC-P/I tumors tended to exhibit pathological features of non-NE differentiation, and vice versa. Although identified as a minority of SCLC, the SCLC-P/I and non-NE entities correlated with more favorable responses to anti-PD-L1 immunotherapy. Our findings may have real-world implications for biomarker-guided patient selection and would be helpful to augment therapeutic outcomes for patients with ES-SCLC (Fig. 7).

SCLC has been recognized as a homogeneous disease and now recommended unexceptionally with EP chemotherapy plus anti-PD-L1 immunotherapy as standard treatment. However, hierarchical response to this combination may underlie the heterogeneity of ES-SCLC; thus, the "all SCLC for immunotherapy" proposal may not be reliable. The emerging SCLC-A/N/P/I subtyping elicited distinct transcriptomic profiles and was expected to segment potential responders to anti-PD-L1 immunotherapy [13]. In our study, eligible patients were constructed with SCLC-A (59%), SCLC-N (19.7%), SCLC-P (6.5%) and SCLC-I (14.8%) subtypes. It was noted the SCLC-A/N/P/Y classification, which is proposed a few years earlier, may also indicate sensitivity to immunotherapy [22, 23]. The SCLC-Y tumor was thought to be positive for YAP, a key

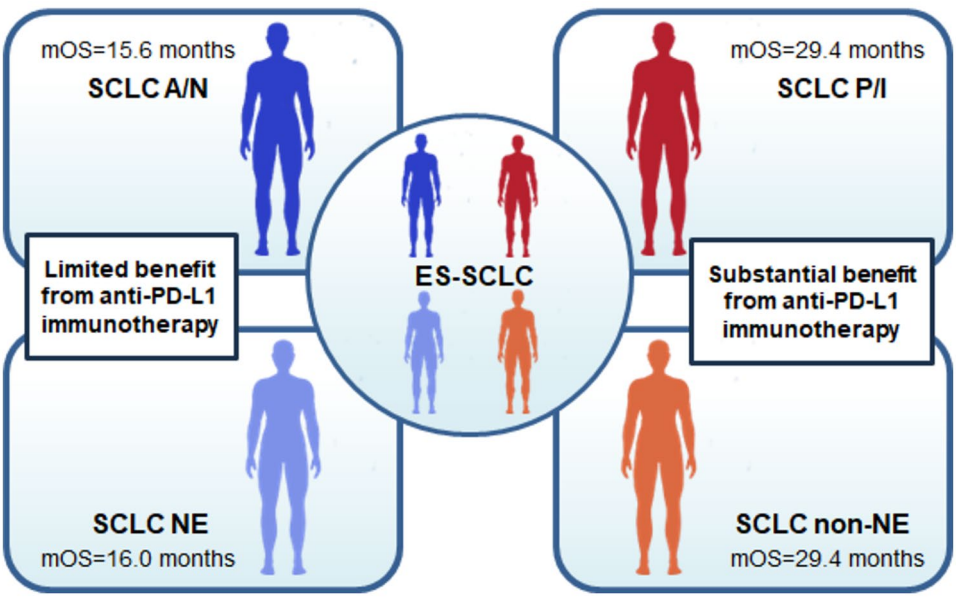

Fig. 7 Diagram illustrating molecular classification and therapeutic outcomes of anti-PD-L1 immunotherapy in patients with ES-SCLC. In the circle, patients were labeled with different colors according to A/N/P/I subtyping and NE/non-NE status. Patients labeled with dark/light red color elicited similarities in TME feature and were recognized as "hot" SCLC, whereas patients in dark/light blue color were "cold" SCLC. These tumors differentially responded to anti-PD-L1 immunotherapy, in which the SCLC-P/I and non-NE subgroups gained long-term survival benefit from immunotherapy. However, the response to anti-PD-L1 immunotherapy in SCLC-A/N and NE subsets was transient and the survival benefit from immunotherapy was limited

component of Hippo signaling. Indeed, expression of YAP was found, to a lesser extent, correlated with increased T cell infiltration in SCLC [24]; however, it failed to define a unique YAP-specific subtype of SCLC because the expression of YAP was not common and often overlapped with ASCL1, NEUROD1 and POU2F3. In our patient cohort, we also found YAP expression in SCLC-A, SCLC-N, SCLC-P and SCLC-I subgroups, with the SCLC-I subtype being the most prevalent. Intriguingly, the distribution of YAP-positive tumor cells seemed to be mutually exclusive from ASCL1/NEUROD1/POU2F3-positive tumor cells, as a consequence, it would be difficult to assign SCLC into YAP or other subtypes when these transcriptional factors were concurrently expressed (Supplementary Fig. S3). In addition, transcriptional classification of the YAP subtype did not necessarily correspond with its classification at protein-level due to the low incidence of YAP protein expression in SCLC. Take together, the SCLC-Y subtype was replaced by the pan-negative SCLC-I subtype since 2021, thus, we did not use the SCLC-A/N/P/Y subtyping in our study.

SCLC showed an uninflamed "cold" TME status in most cases; however, the SCLC-P and SCLC-I subtype tended to possess PD-L1 positive and T cell infiltrated TME. These "hot" SCLC revealed higher response rate and gained longer survival benefit over SCLC-A and SCLC-N. The difference in therapeutic susceptibility to immunotherapy indicates the heterogeneity of SCLC, and thus grouping SCLC into four distinct subtypes according to the expression of ASCL1, NEUROD1 and POU2F3 is a paradigm of biomarkers-guided therapy for patients with SCLC. A key question is how SCLC that looks very similar in histology pertains diverse subtypes and how these differences impact therapeutic vulnerability. Although it is generally believed that SCLC prominently arises from lobar or main bronchi NE cells, type 2 alveolar cells may be a relative rare source of SCLC tumorigenesis [25]. Apart from *Rb1* and *TP53*, accumulating evidence from genetically engineered mouse models reveals that SCLC has different genetic and epigenetic events, and undergoes subclonal evolution during tumorigenesis and disease progression [26]. Therefore, SCLC is not one single disease, but rather a cluster of various subtypes that are not distinguishable at morphological level. The SCLC-A/N/P/I subtyping endows a pristine view toward subset-specific genetic alterations and immunological characteristics. Each subtype takes distinct molecular pathway to shape TME and affects response to specific treatment. To this end, revisiting SCLC at molecular level switches this histologically homogenous tumor into immunity heterogenous malignancy, which gives rise to the development of SCLC subtype-specific therapeutic strategy.

Unfortunately, there are no diagnostic antibodies approved for SCLC-A/N/P/Y subtyping at the moment, and antibodies used in literatures to determine ASCL1, NEUROD1 and POU2F3 state are sometimes arbitrary and are designated for research use only. While we found IHC evaluation of NE differentiation using defined diagnostic antibodies against Syn, CgA and CD56 offered a compensatory approach to partially reflect A/N/P/I status. The TME features and response patterns to immunotherapy based on NE/non-NE category are very similar to what we have observed under A/N/P/I subtyping, thus, the overlapping between the two SCLC classifications may have clinical implications for patient selection before the initiation of immunotherapy [27]. It is therefore proposed to concentrate SCLC-P/I patients from the non-NE cohort to improve therapeutic outcomes and prolong patient survival.

Another impressive finding in our study is a substantial survival advantage of anti-PD-L1 immunotherapy over previous studies. According to CASPIAN and IMPOWER 133 trials, the mOS of ES-SCLC patients receiving first-line EP chemotherapy plus anti-PD-L1 immunotherapy marginally exceeded 12 months [9, 10]. However, despite 34.4% of cases received anti-PD-L1 immunotherapy at second-line setting, the mOS of overall patient cohort in our study was 19.5 months and it was even longer when patients were classified into subgroups. We found the SCLC-P/I arm and non-NE arm reached a mOS of 29.4 months with 2-year survival rate over 50%. The discrepancy in patient survival might be attributed to the difference in patient characteristics. Unlike CASPIAN and IMPOWER 133 trials, all the participants in our study were Asian ethnicity. Race has been considered as a prognostic factor for NSCLC [28], but there is limited information about the significance of ethnicity in the survival outcome of SCLC. In a retrospective study analyzing 4,782 cases of SCLC, Asian ethnicity possesses a superior prognosis over Caucasian (HR = 0.785, $P = 0.0076$) [29]. Consistently, irinotecan in combination with platinum had been shown to confer longer OS from randomized trials performed in Japan but not in United States [30, 31]. Our results strengthen this notion, showing that anti-PD-L1 immunotherapy elicits profound improvement in patient survival in a Chinese cohort of ES-SCLC. It is unclear why there is such a difference between different SCLC populations, but caution should be taken when designing clinical trials for Eastern SCLC patients and Western SCLC patients.

Our study has several limitations. First, it is a retrospective study in a small patient cohort from a single medical center, further randomized clinical trials are warranted to verify our findings. Second, we have not evaluated whether there is a correlation between SCLC subtyping and the efficacy of immunotherapy in limited stage SCLC (LS-SCLC). Anti-PD-L1 immunotherapy may be applicable in LS-SCLC-A/N and NE subsets after careful evaluation. Finally, IHC was critical for SCLC subtyping and TME assessing, authorized

diagnostic antibodies should be developed to enable reproducible and consistent IHC results.

Taken together, our study validated novel classification in primary ES-SCLC tumors by IHC. Our findings may have real-world implications for biomarker-driven therapy in ES-SCLC and ultimately help to improve clinical outcomes for patients with ES-SCLC.

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Author contributions TL and MY conceived and designed the research. QZ, GW, WY, DW, JY, and YS carried out the experiments and analyzed the data. TL, MY, YS, and QZ wrote, reviewed, and edited the manuscript. TL and MY supervised the research and provided funding support. All authors reviewed and approved the final manuscript.

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Data availability No datasets were generated or analyzed during the current study.

Declarations

Competing interest The authors declare no competing interests.

Ethical approval and consent to participate The handling and processing of patient tumor samples were done in accordance with Institutional Ethics Committee Review Board Approved protocols (2024DZKY-050-01).

Consent for publication Written consent was obtained from each participate.

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