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Beyond PD-L1: B7-H6 emerges as a potential immunotherapy target in small cell lung cancer

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Abstract

Introduction: The PD-L1 immune checkpoint inhibitors atezolizumab and durvalumab have received regulatory approval for the first-line treatment of patients with extensive-stage small cell lung cancer. However, when used in combination with platinum-based chemotherapy, these PD-L1 inhibitors only improve overall survival by 2–3 months. This may be due to the observation that <20% of SCLC tumors express PD-L1 at >1%. Evaluating the composition and abundance of checkpoint molecules in SCLC may identify molecules beyond PD-L1 that are amenable to therapeutic targeting.

Methods: We analyzed RNA-Seq data from SCLC cell lines (n=108) and primary tumor specimens (n=81) for expression of 39 functionally validated, inhibitory checkpoint ligands. Further, we generated tissue microarrays containing SCLC cell lines and SCLC patient specimens to confirm expression of these molecules by immunohistochemistry. We annotated patient outcomes data, including treatment response and overall survival.

Results: The checkpoint protein B7-H6 (*NCR3LG1*) exhibited increased protein expression relative to PD-L1 in cell lines and tumors ($P < 0.05$). Higher B7-H6 protein expression correlated with longer progression-free survival ($P = 0.0368$) and increased total immune infiltrates (CD45+) in patients. Furthermore, increased B7-H6 gene expression in SCLC tumors correlated with a decreased activated NK cell gene signature, suggesting a complex interplay between B7-H6 expression and immune signature in SCLC.

Conclusions: We investigated 39 inhibitory checkpoint molecules in SCLC and found that B7-H6 is highly expressed and associated with progression-free survival. In addition, 26/39 immune checkpoint proteins in SCLC tumors were more abundantly expressed than PD-L1, indicating an urgent need to investigate additional checkpoint targets for therapy in addition to PD-L1.

Keywords

small cell lung cancer (SCLC); immunotherapy; checkpoint molecules; immune checkpoint inhibitor; B7-H6

Introduction

Small cell lung cancer (SCLC) is a high-grade neuroendocrine tumor that affects ~15% of lung cancer patients and causes ~200,000 deaths annually worldwide.[1] Approximately 70% of SCLC patients present with distant metastases at initial diagnosis and median overall survival is ~1 year for this disease.[1] SCLC treatment has consisted of a combination of platinum-based chemotherapy plus etoposide for the last 30 years with no significant advances in therapeutic paradigms.[2] However, in 2019, the United States Food and Drug

Administration (FDA) approved the addition of the PD-L1 inhibitor, atezolizumab, in combination with chemotherapy for first-line treatment of patients with extensive-stage SCLC (ES-SCLC).[3] Nearly one year later, another PD-L1 inhibitor, durvalumab, received an analogous approval.[4] Chemotherapy (platinum/etoposide) plus immune checkpoint inhibitors (ICIs) is now the accepted standard of care for ES-SCLC. However, addition of PD-L1 inhibitors in this setting only improves overall survival by 2–3 months.[3, 5] This may be due to the fact that despite high tumor mutation burden,[6] SCLC tumors express minimal PD-L1,[7-10] although additional factors may also explain the poor response. In addition, large clinical trials utilizing alternative checkpoint inhibitors such as anti-PD-1[11-14] and anti-CTLA-4[15] have either been negative or achieved minimal response rates (10–30%).[10, 12, 16] Notably, these trials did not stratify patients based on PD-L1 expression, and as a result, it is unclear who benefits from current FDA-approved ICIs in SCLC. These data reveal a significant knowledge gap in the SCLC clinical realm; immune biomarkers which are standardly accepted in other tumor types, including non-small cell lung cancer (NSCLC), are either 1) inappropriate targets or 2) not predictive of response to immune checkpoint blockade in SCLC.[17]

There is an urgent need to define the immune landscape in SCLC in order to identify the best biomarker(s) of response and resistance to immune checkpoint blockade, maximize the utility of ICIs, and personalize medicine for patients with SCLC. Through these studies, we sought to characterize inhibitory checkpoint molecules in SCLC in an effort to identify targets amenable to ICIs.

Materials and Methods

RNA-Seq Datasets

Details of the RNA-seq datasets and analyses are provided in the Supplementary Methods.

SCLC cell line tissue microarray

A tissue microarray (TMA) was constructed using formalin-fixed and paraffin-embedded (FFPE) cell pellets from 19 unique SCLC cell lines (DMS53, DMS114, COR-L51, H69, H82, H128, H187, H196, H209, H211, H524, H526, H740, H841, H889, H1048, H1607, H2195, H2679) gifted from John Minna, M.D. (University of Texas Southwestern), Pierre Massion, M.D. (Vanderbilt University Medical Center), and Vito Quaranta, M.D. (Vanderbilt University). The TMA consisted of 1 mm diameter tissue cores from 19 SCLC cell lines, each in at least 3 replicates (n=66), human lung adenocarcinoma (n=3), and control tissues from normal human lung (n=3) and brain cortex (n=3). 5μm thick sections were cut from the array and mounted onto charged slides for immunohistochemistry (IHC) analyses. A sequential section was stained with H&E to confirm presence of the histological feature of interest (normal or tumor). The actual number of samples reported for each marker was lower than the total samples analyzed due to unavoidable tissue loss.

SCLC patients and tissue microarray

FFPE tumor samples from 51 SCLC patients treated at Vanderbilt University Medical Center (VUMC) were used to construct a TMA. Patient demographics can be found in

Supplementary Table 1. All tissue was used in accordance with the VUMC institutional review board (IRB) under protocols #030763 and #160769, which approved the patient consent forms or waiver of consent. Archival tissue blocks were stained with H&E and reviewed by a pathologist. The TMA consisted of 0.6 mm diameter tissue cores from 51 unique patients, each in at least 2 replicates (n=164), and 7 control tissues including normal human tonsil (n=9), lung (n=2), brain cortex (n=2), pancreas (n=2), adrenal (n=2), thyroid (n=2), and parathyroid (n=2). Clinicopathological characteristics of the patients were compiled from medical records. 5μm thick sections were cut from the array and mounted onto charged slides for IHC analyses. A sequential section was stained with H&E to confirm presence of the histological feature of interest (normal or tumor). The actual number of samples reported for each marker was lower than the total samples analyzed due to unavoidable tissue loss and absence of or limited tumor cells.

B7-H3, B7-H6, PD-L1, and immune cell quantification in SCLC cell lines and tumors.

Quantification of immunohistochemistry staining is described in detail in Supplementary Methods.

Statistical Analyses

Statistics were performed as indicated using Python, R, or GraphPad Prism. $P < 0.05$ was considered statistically significant for all studies. For paired analyses, a 2-tailed paired *t*-test was utilized. The Chi-square (χ^2) test was used to analyze the correlation of B7-H6 expression and clinical parameters. The Kaplan-Meier method and Cox regression were used to estimate survival and hazard ratios, and survival between groups was analyzed for significance using log-rank (Mantel-Cox) test. Where error bars are presented, all data are mean ± SD.

Results

PD-L2 and B7-H6 emerge as potential candidates of immune checkpoint blockade in SCLC

To evaluate inhibitory checkpoint molecules in SCLC, we first performed an extensive literature review and assembled a list of checkpoint molecules that were 1) expressed by tumors, 2) T cell suppressive, and 3) potentially amenable to ICI or other targeted therapies (Supplementary Tables 2 and 3). We evaluated the presence of these inhibitory checkpoint molecules (n=39) in SCLC cell lines (n=108) using bulk RNA-Seq compiled from three publicly available datasets: the Cancer Cell Line Encyclopedia (CCLE),[18] Genomics of Drug Sensitivity in Cancer (GDSC),[19] and SCLC cBioPortal for Cancer Genomics[20, 21] (Fig. 1A). Previous studies report that most SCLC tumors exhibit low PD-L1 expression with <1% PD-L1 positive tumor and immune cells.[7-10] Quantified expression of each inhibitory checkpoint molecule revealed that 18/38 (47%) had significantly increased gene expression ($P < 0.01$) relative to PD-L1 (Fig. 1B).

Lung adenocarcinoma (LUAD) and melanoma have each been reported to have high response rates to immune checkpoint blockade therapy.[22, 23] We reasoned that genes that show similar or increased expression levels in SCLC compared to these tumor types would be more likely to have clinical relevance. Using the CCLE dataset, we compared gene

expression of inhibitory molecules in SCLC cell lines (n=50) to LUAD (n=188) and melanoma (n=49) cell lines. Many checkpoint ligands (n=16/39, 41%) exhibited reduced gene expression in SCLC cell lines (Fig. 1C). *CD274* expression was higher in SCLC relative to melanoma but not LUAD. However, *PDCD1LG2* (PD-L2) and *NCR3LG1* (B7-H6) exhibited increased expression relative to both LUAD and melanoma cell lines (Fig. 1c). Additional comparison of B7-H6 expression of cell lines in the CCLE revealed an increased expression in SCLC relative to most tumor types (Fig. 2A). PD-L2 showed decreased expression relative to other tumor types (data not shown). Additionally, we examined the potential co-expression of *PDCD1LG2* (PD-L2) and *NCR3LG1* (B7-H6) with *CD274* (PD-L1) by assessing their correlation. *NCR3LG1* (B7-H6) demonstrated minimal correlation with *CD274* (PD-L1) and *PDCD1LG2* (PD-L2) in SCLC cell lines (Fig. 2B).

To validate the findings from SCLC cell lines, a publicly available SCLC tumor RNA-Seq dataset (EGAS00001000925[24]; n=81) was analyzed for expression of inhibitory checkpoint molecules (Supplementary Table 2) in SCLC tumors (Supplementary Data 1A). Quantified expression of inhibitory checkpoint molecules revealed that 26/38 (68%) had significantly increased expression ($P < 0.01$) relative to PD-L1 (Supplementary Data 1B). The TCGA dataset was used to compare gene expression of inhibitory molecules in SCLC tumors (n=81) to LUAD (n=576) and melanoma (n=473) tumors. Analyses did not validate increased *PDCD1LG2* (PD-L2) and *NCR3LG1* (B7-H6) gene expression in SCLC tumors relative to LUAD and melanoma tumors (Supplementary Data 1C) as observed in SCLC cell lines (Fig 1C). In fact, most checkpoint ligands (n=32/33, 97%) exhibited reduced gene expression in SCLC tumors. *PDCD1LG2* (PD-L2) and *CD274* (PD-L1) showed strong correlation (R^2=0.727) in SCLC tumors, indicating a pattern of co-expression (Fig. 2C). A weaker correlation was observed in SCLC cell lines (R^2 = 0.310, Fig. 2B). As observed in SCLC cell lines, *NCR3LG1* (B7-H6) demonstrated little correlation with *CD274* (PD-L1) and *PDCD1LG2* (PD-L2) in SCLC tumors (Fig. 2C).

Immunohistochemistry validates B7-H6 as a potential therapeutic target for immune checkpoint blockade

Although *NCR3LG1* (B7-H6) did not demonstrate increased gene expression relative to *CD274* (PD-L1) in SCLC tumors (Supplementary Data 1A, B) and relative to LUAD and melanoma tumors (Supplementary Data 1C), it displayed increased expression in SCLC cell lines (Fig. 1B) and increased gene expression relative to both LUAD and melanoma cell lines (Fig. 1C). Therefore, we chose to validate B7-H6 protein expression in SCLC. We examined B7-H6 (*NCR3LG1*) via immunohistochemistry (IHC) in SCLC cell lines (n=21) and clinical specimens (n=120). For comparison, we also analyzed SCLC cell lines for the presence of PD-L1 and B7-H3 protein; B7-H3 (*CD276*) is an inhibitory checkpoint ligand previously identified to exhibit high expression in SCLC[7] that showed increased gene expression relative to PD-L1 (Fig. 1b and Supplementary Data 1B). Correlating with the RNA-Seq data (Fig. 1B and Supplementary Data 1B) and previous studies, total PD-L1 (*CD274*) positivity was low (< 20% tumor cells) (Supplementary Data 2A, B). Over half (59%, n=10) of cell lines had >1% total cells positive for PD-L1 immunoreactivity (Supplementary Data 2B). Although B7-H6 average protein expression was similar to PD-L1, >1% total cells positive were detected in all (n=17) cell lines (Supplementary Data 2B).

In addition, median B7-H6 cell positivity was 10-fold higher than PD-L1 (data not shown). Consistent with gene expression data (Fig. 1B and Supplementary Data 1B), B7-H3 was highly expressed at the protein level and showed >1% positive staining in all cell lines (Supplementary Data 2B).

Primary normal human lung tissue was assessed for B7-H6 staining and we observed no expression (Fig. 3A). Cells showing immunoreactivity are hemosiderin-laden macrophages. Negative staining (Fig. 3B), weak staining (Fig. 3C), moderate staining (Fig. 3D), and strong staining (Fig. 3E-F) were observed in SCLC tumors. Similar to SCLC cell lines, most (87%, n=34/39) SCLC tumors showed >1% positive staining. B7-H6 expression was also more abundant in tumors ($P < 0.0001$) when compared to PD-L1 (Fig. 3G).

B7-H6 correlation with clinicopathological features in SCLC

Previous reports in other tumor types have indicated that B7-H6 protein expression correlates with prognoses and other clinicopathological parameters (tumor progression, stage, metastasis, treatment response, and tumor differentiation).[25-30] Therefore, we investigated the association of B7-H6 expression with clinicopathological features of SCLC (Table 1). Since current clinical practices utilize PD-L1 positivity and not H-score to determine ICI administration, we stratified patients based on B7-H6 tumor positivity. In addition, since no clinical guidelines exist for B7-H6 positivity in tumors, we based stratification on the median B7-H6 tumor positive percentage of 24.3% in our cohort. Patients with B7-H6 tumor positive < median (n=19, B7-H6^{low}) or ≥ median (n=20, B7-H6^{high}) were analyzed for SCLC clinical parameters (Fig. 3H). Strong correlation was seen between B7-H6 and gender, with males exhibiting higher B7-H6 expression ($P < 0.0154$). All other clinical parameters analyzed were insignificant as noted in Table 1.

B7-H6 is associated with longer progression-free survival and increased immune infiltrates in SCLC

In order to further investigate the prognostic value of B7-H6 in SCLC, we conducted survival analyses based on B7-H6 tumor expression. B7-H6 expression had no significant correlation ($P = 0.3994$) with overall survival (OS) (Fig. 4A). Interestingly, median OS was nearly 2-fold higher (14.3 versus 7.7 months – CI) in B7-H6^{high} patients (Fig. 4A). When evaluating progression-free survival (PFS) from start of treatment, B7-H6^{high} patients experienced a significantly longer disease control ($P = 0.0368$) and a more than 3-fold improvement in median PFS (10.7 versus 3.8 months) compared to B7-H6^{low} patients (Fig. 4B). Considering that stage is a major factor of OS and PFS in SCLC, we further assessed PFS in stage-matched patients. B7-H6^{high} patients with limited-stage SCLC (LS-SCLC) had a nearly 3-fold higher median PFS than B7-H6^{low} low patients (21.5 versus 8.5 months), although this difference was not statistically significant (Fig. 4C). Median PFS was similar when comparing patients with B7-H6^{high} and B7-H6^{low} ES-SCLC (4.7 versus 3.8 months, respectively; Fig. 4D). These data suggest B7-H6 expression is a contributing factor in length of PFS, although stage cannot be completely excluded.

Based on the observation of increased PFS in B7-H6^{high} patients, we next sought to investigate the influence of B7-H6 expression on immune complexity. Further investigation

of B7-H6$^{high/low}$ cohorts via IHC revealed increased leukocyte infiltration (CD45+) in primary SCLC lung tumors of B7-H6^{high} patients (n=7), a feature which has been reported to be predictive of improved patient outcomes and response to immunotherapy[31-33] (Fig. 5A, B). To establish the relationship between B7-H6 expression and immune infiltration in SCLC tumors, we estimated the inferred relative proportions of immune cell populations using CIBERSORTx[34] and bulk tumor RNA-Seq data (EGAS00001000925[24]; n=81). High B7-H6 expression was associated with increased CD8+ T cells, plasma cells, and follicular helper T cells, which have been reported to be associated with better patient prognosis[31, 35, 36] (Fig. 5C and Supplementary Data 3A, B). We confirmed the increased presence of these immune cell types in B7-H6^{high} SCLC primary lung tumors via IHC (Fig. 5D). Although NK cell relative proportions did not differ between the B7-H6$^{high/low}$ cohorts, we further examined the relationship between B7-H6 and its only known cognate receptor, NKp30,[37] on inferred NK cell populations identified using the CIBESORTx[34] methodology and the LM22 gene signature. We also examined the relationship between B7-H6 expression and abundance of NK cells. The analyses revealed a significant negative correlation between resting NK cells and NKp30 (*NCR3*) expression (R = −0.29, P = 0.0085; Supplementary Data 4A), but a significant positive correlation between activated NK cells and NKp30 expression (R = 0.64, P = 9.9E-11; Supplementary Data 4B). B7-H6 (*NCR3LG1*) expression was also inversely correlated with abundance of activated NK cells (R = −0.23, P = 0.043; Supplementary Data 4C), indicating a potential NK-inhibitory role for B7-H6 in SCLC.

SCLC cell lines and tumors display subtype-specific expression of inhibitory checkpoint molecules

Developing in parallel to these practice-changing clinical trials involving use of ICIs in SCLC are large-scale - omics efforts to bring more precision to the diagnosis and treatment of SCLC at the molecular level. At present, SCLC is approached clinically as a single disease with no standard biomarker assessments (such as tumor DNA sequencing) to personalize care. Attempts to better define specific patient populations and understand SCLC heterogeneity at the molecular level have led to the identification of SCLC subtypes within the previously described, broader neuroendocrine (NE) and non-neuroendocrine (non-NE) classifications.[38-40] These subtypes are defined by differential gene expression of four main transcription regulators—Achaete-Scute Complex Homolog-Like 1 (*ASCL1*), Neurogenic Differentiation Factor 1 (*NEUROD1*), POU Class 2 Homeobox 3 (*POU2F3*), and Yes-Associated Protein-1 (*YAP1*)—and exhibit potentially unique therapeutic susceptibilities.[41, 42] At present, little is known about the relationship between the immune microenvironment and SCLC subtypes.

SCLC cell lines exhibited distinct subtypes—*ASCL1*^{high} (SCLC-A), *NEUROD1*^{high} (SCLC-N), *POU2F3*^{high} (SCLC-P), *YAP1*^{high} (SCLC-Y)—based on relative RNA-Seq expression of four well-defined transcription regulators[42] (Supplementary Data 5A and Supplementary Table 4). Since therapeutic implications amongst individual subtypes have not been fully clarified, the more well-elucidated classifications of neuroendocrine (NE; SCLC-A and SCLC-N) and non-neuroendocrine (non-NE; SCLC-P and SCLC-Y) were used for downstream analyses. Insulinoma-associated 1 (*INSM1*) gene expression, which is a marker of neuroendocrine tumors,[43] was also quantified as a measure of subtyping validation

(Supplementary Data 5A). Subtyping analyses classified most SCLC cell lines as NE (n=92) and the rest as non-NE (n=16). Quantified expression of inhibitory checkpoint molecules in NE and non-NE subtypes revealed differential expression of several genes (Supplementary Data 5B). Notably, PD-L1 and B7-H6 were not differentially expressed amongst SCLC subtypes. Checkpoint genes that were validated in downstream patient analyses are displayed in Supplementary Data 5C. Notably, *CD70* (TNFSF7), *CD81* (CD81), *HLA-B* (MHCI), *HLA-E* (HLA-E), *MICA* (MICA), and *VTCN1* (B7-H4) all showed increased expression ($P < 0.05$) in the non-NE subtype (Supplementary Data 5C). Similar to SCLC cell lines, tumors were first classified into NE (n=67) and non-NE (n=14) subtypes, with NE being the predominant subtype (Supplementary Data 6A). NE and non-NE tumors were then analyzed for expression of checkpoint molecules (Supplementary Data 6b). Differentially expressed markers in SCLC cell lines (Supplementary Data 5C) were validated ($P < 0.05$) in tumor samples (Supplementary Data 6C). The trend of increased gene expression in non-NE subtypes observed in cell lines was also evident in tumor samples.

Discussion

ICIs have significantly altered survival outcomes for some cancer patients with demonstrated success in many tumor types.[44] The FDA has approved PD-L1 inhibitors for use in more than 10 cancer types,[45] including small cell lung cancer, in which it is approved for first-line treatment of advanced disease. However, the number of patients who respond is few, and the benefit gained by responders is minimal. Even blockade of alternative inhibitory checkpoints molecules—CTLA-4 and PD-1—does not substantially prolong overall survival in SCLC patients.[11-15] One explanation could be low or absent expression of these molecules in SCLC,[7-10] precluding their use as viable targets for immune checkpoint blockade. Alternatively, expression of these checkpoint molecules might not serve as predictive biomarkers for ICI administration. Nonetheless, existing ICIs could prove successful if administered within the proper tumor context. To date, there are no guidelines surrounding the administration of ICIs in SCLC and no predictive biomarkers have been established.

In these studies, we aimed to provide context for ICI administration by considering inhibitory checkpoint molecules beyond PD-L1 for immune checkpoint blockade. Additionally, using large datasets, we have highlighted the necessity of considering subtypes in SCLC immune checkpoint blockade by identifying subtype-specific, differential gene expression of inhibitory checkpoint molecules—*CD70* (CD70), *CD81* (CD81), *HLA-B* (MHCI), *HLA-E* (HLA-E), *MICA* (MICA), *VTCN1* (B7-H4)—which are putative targets for immune-based therapies (Supplementary Table 2). Further, we identified two potential candidates for immune checkpoint inhibition in SCLC—*NCR3LG1* (B7-H6) and *PDCD1LG2* (PD-L2)—and validated B7-H6 as a potential target. PD-L2 has been investigated in SCLC but showed no association with clinicopathological factors or prognosis in initial studies.[46] However, the strong correlation of PD-L2 (*PDCD1LG2*) and PD-L1 (*CD274*) gene expression might indicate that a combined PD-L1 / PD-L2 ICI regimen would be more efficacious. B7-H6 is a novel ligand belonging to the B7 family. Several B7 family members, including PD-L1, have been revealed to be overexpressed in tumors and implicated in tumor progression and overall worse prognosis. Distinct from other family members, B7-H6 is abnormally expressed in tumor tissues[29, 47] with little to no

expression in normal human tissues, making it an attractive target for existing immunotherapies such as ICI, chimeric antigen receptor (CAR) T cells,[48-50] or bispecific T cell engagers (BiTEs).[51] Aberrant B7-H6 overexpression has been reported in a breadth of tumor types including glioma, hepatocellular carcinoma, triple-negative breast cancer, and non-small cell lung cancer.[27, 30, 47, 52] However, unlike PD-L1, B7-H6 can also be co-stimulatory, by binding to NKp30 and enhancing antitumor NK cell cytotoxicity and cytokine secretion.[37] Due to its dual immune regulatory nature, the clinical implications of abnormal B7-H6 expression in human cancer remain elusive. While some studies report strong correlation of B7-H6 expression with tumor progression[26, 52-55] and chemoresistance[55, 56] in multiple tumor types, others do not.[30, 57]

Although our study herein revealed a positive correlation of B7-H6 expression with progression-free survival and increased immune infiltration, we observed a negative correlation with B7-H6 and activated NK cells. These findings support previous reports of a context-specific, immune regulatory role for B7-H6 in SCLC. Of note, we did not assess for the presence of soluble B7-H6, which has been demonstrated to be inhibitory to NK cell function.[54, 58] Higher expression of B7-H6 on tumor cells could be indicative of lower serum amounts of soluble B7-H6, which could explain the longer PFS in B7-H6^{high} SCLC patients. We also do not know how the expression of B7-H6 changes dynamically over time and in response to chemotherapy. Furthermore, the comparison of inhibitory checkpoint molecules in SCLC to PD-L1 at the gene expression level might not correlate directly with therapeutic relevance due to various factors that affect protein expression, including protein stability and membrane trafficking. Our studies also assume that higher protein expression correlates with ICI effectiveness, which might not be the case for all checkpoint molecules.

In conclusion, our studies revealed that several evaluated inhibitory checkpoint molecules were more highly expressed than PD-L1, indicating that globally PD-L1 may not be the most optimal target for ICIs in SCLC. We also report that SCLC cell lines could serve as valid models for checkpoint ligand studies in lieu of the relative paucity of SCLC tissues available for research. Future studies will seek to validate B7-H6 as a target amenable to immune checkpoint inhibition and investigate checkpoint molecules beyond PD-L1 as candidates for immune checkpoint blockade in SCLC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ASCL1	Achaete-Scute Complex Homolog-Like 1
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
FPKM	Fragments Per Kilobase of transcript per Million
GDSC	Genomics of Drug Sensitivity in Cancer
ICI	Immune checkpoint inhibitor
IHC	Immunohistochemistry
INSM1	Insulinoma-associated 1
LUAD	Lung adenocarcinoma
NE	Neuroendocrine
NEUROD1	Neurogenic Differentiation Factor 1
NSCLC	Non-small cell lung cancer
OS	Overall survival
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PFS	Progression-free survival
RNA-Seq	RNA sequencing
SCLC	Small cell lung cancer
TMA	Tissue microarray
TPM	Transcripts per million
YAP1	Yes-Associated Protein-1

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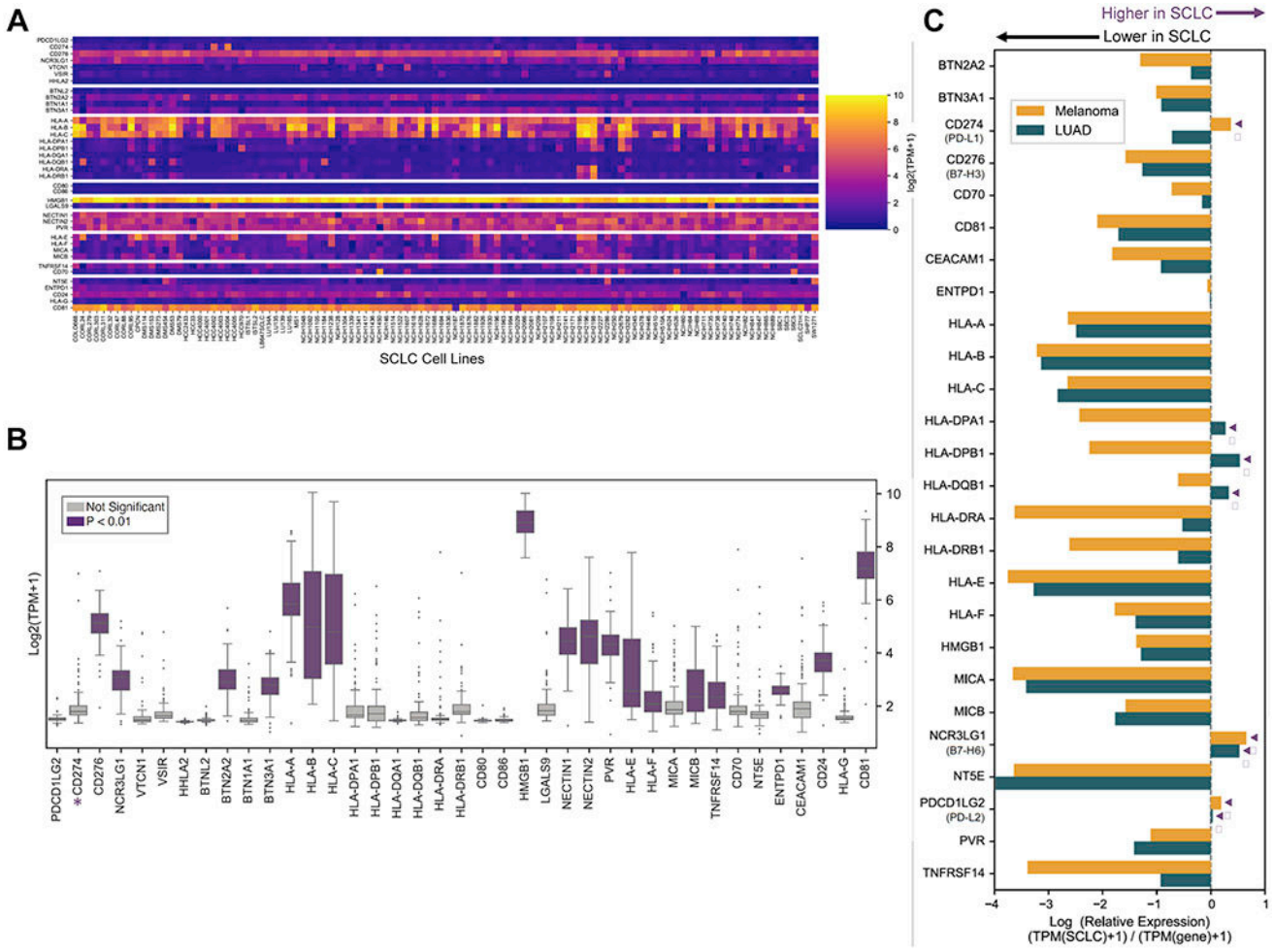

Figure 1: Expression of immune checkpoint molecules in SCLC cell lines.

(**A**) Heatmap depicting log-transformed absolute gene expression of checkpoint molecules (n=39) described in Supplementary Table 2 in SCLC cell lines (n=108). Color scale bar represents absolute expression on a log scale. (**B**) Boxplots of log-transformed absolute expression of checkpoint molecules quantified in all SCLC cell lines. Genes that are significantly increased ($P < 0.01$) compared to PD-L1 (*CD274*, starred) are highlighted in purple. (**C**) Analysis of SCLC checkpoint molecule expression relative to melanoma (n=49) and LUAD (n=188) CCLE cell lines. Relative expression of log-transformed median gene expression of inhibitory checkpoint molecules with SCLC as baseline log(SCLC+1/[melanoma or LUAD]+1). Only genes present in all three datasets are shown. SCLC, small cell lung cancer; LUAD, lung adenocarcinoma. Arrowheads, increased relative expression in SCLC.

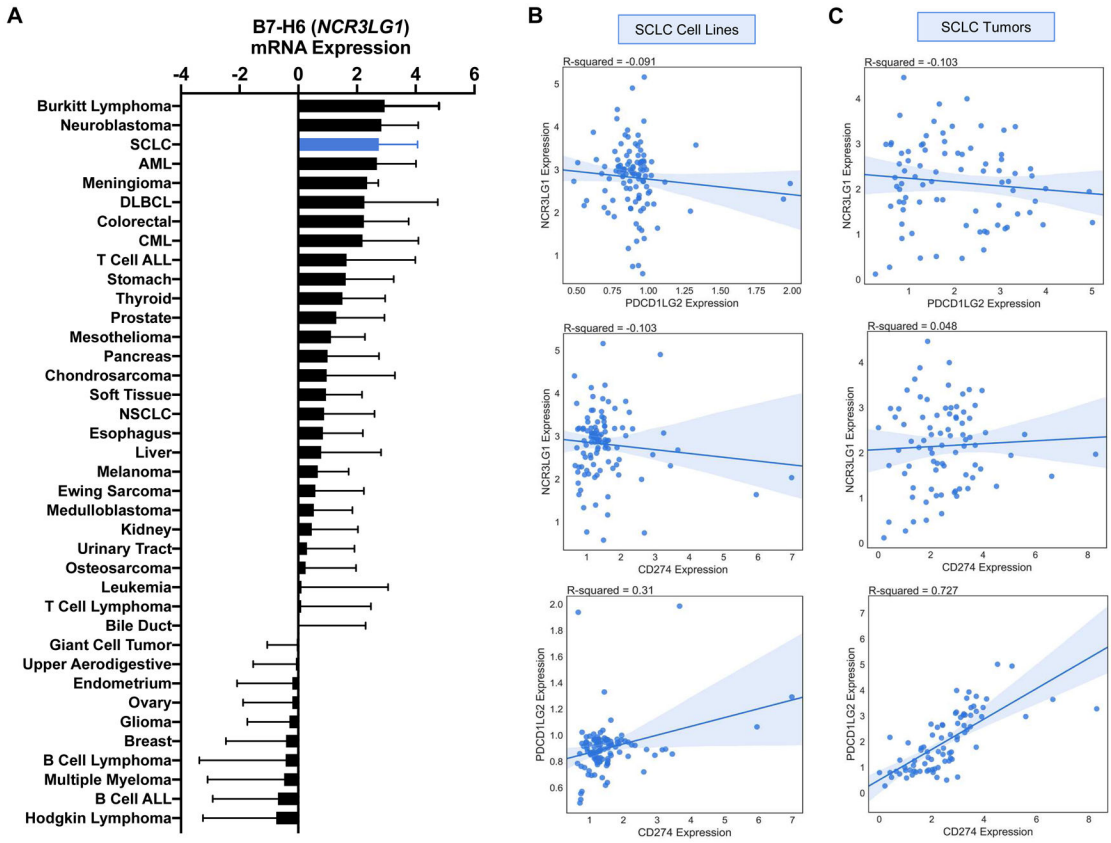

Figure 2: Co-expression of PD-L1, PD-L2, and B7-H6 in SCLC cell lines and tumor.

(**A**) Quantification of mean log-transformed B7-H6 expression in CCLE cell lines. Error bars are standard deviation. Co-expression of *NCR3LG1* (B7-H6), *CD274* (PD-L1), and *PDCD1LG2* (PD-L2) in (**B**) SCLC cell lines and (**C**) SCLC tumors with associated R-squared value (Pearson correlation) and 95% confidence interval for fit using linear regression. SCLC, small cell lung cancer; AML, acute myeloid leukemia; DLBCL, diffuse large B cell lymphoma; CML, chronic myelogenous leukemia; ALL, acute lymphocytic leukemia; NSCLC, non-small cell lung cancer.

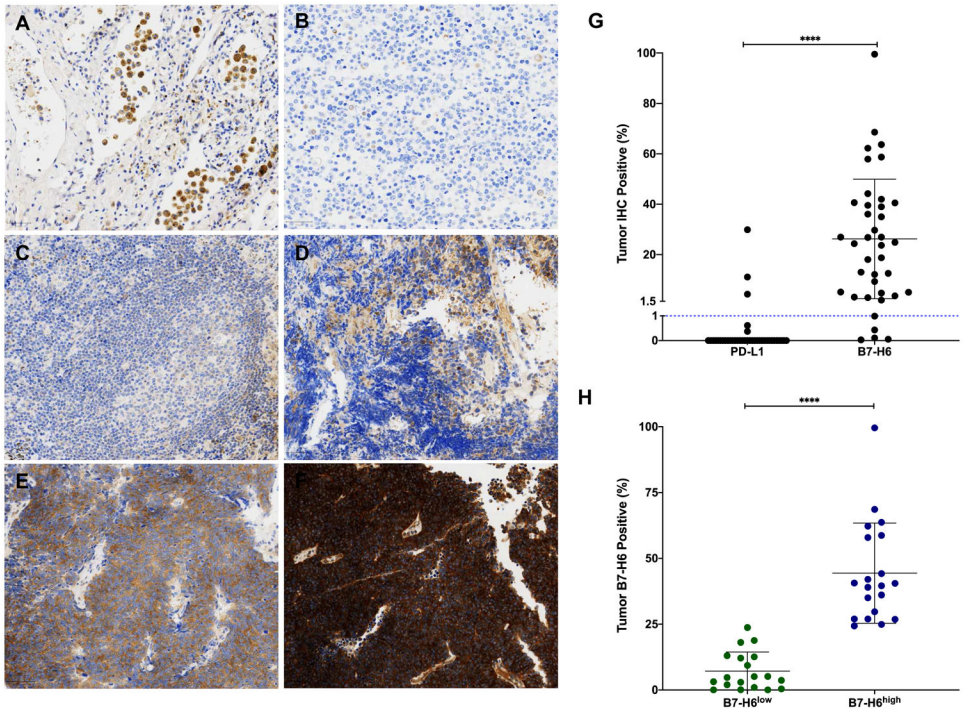

Figure 3: B7-H6 immunohistochemistry staining in normal lung and SCLC tissues.

Representative images, 40X magnification; (**A**) No staining in normal lung (cells showing immunoreactivity are hemosiderin-laden macrophages). (**B**) Negative staining in SCLC. (**C**) Weak staining in SCLC. (**D**) Moderate staining in SCLC. (**E, F**) Strong staining in SCLC. (**G**) Comparison of PD-L1 and B7-H6 positive staining in SCLC tumors. Line represents 1% tumor positive IHC staining. (**H**) Stratification of SCLC tumors into B7-H6^{low} (n=19) and B7-H6^{high} (n=20) based on median expression (24.3%). ****, $P < 0.0001$; SCLC, small cell lung cancer.

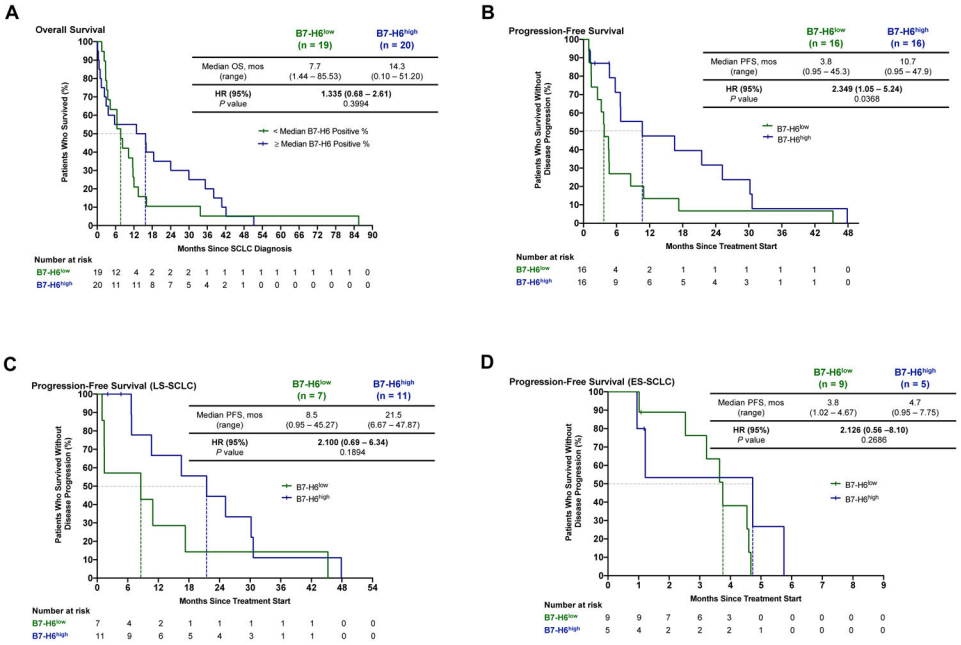

Figure 4: Overall survival (OS) and progression-free survival (PFS) in B7-H6^{high} and B7-H6^{low} patients with SCLC.

Kaplan-Meier estimates of (**A**) OS and (**B**) PFS in patients with SCLC according to low or high B7-H6 expression. Kaplan-Meier estimates of PFS limited to only patients who received systemic therapy with (**C**) limited-stage or (**D**) extensive-stage SCLC. SCLC, small cell lung cancer; OS, overall survival; PFS, progression-free survival; HR, hazard ratio; LS, limited-stage; ES, extensive-stage.

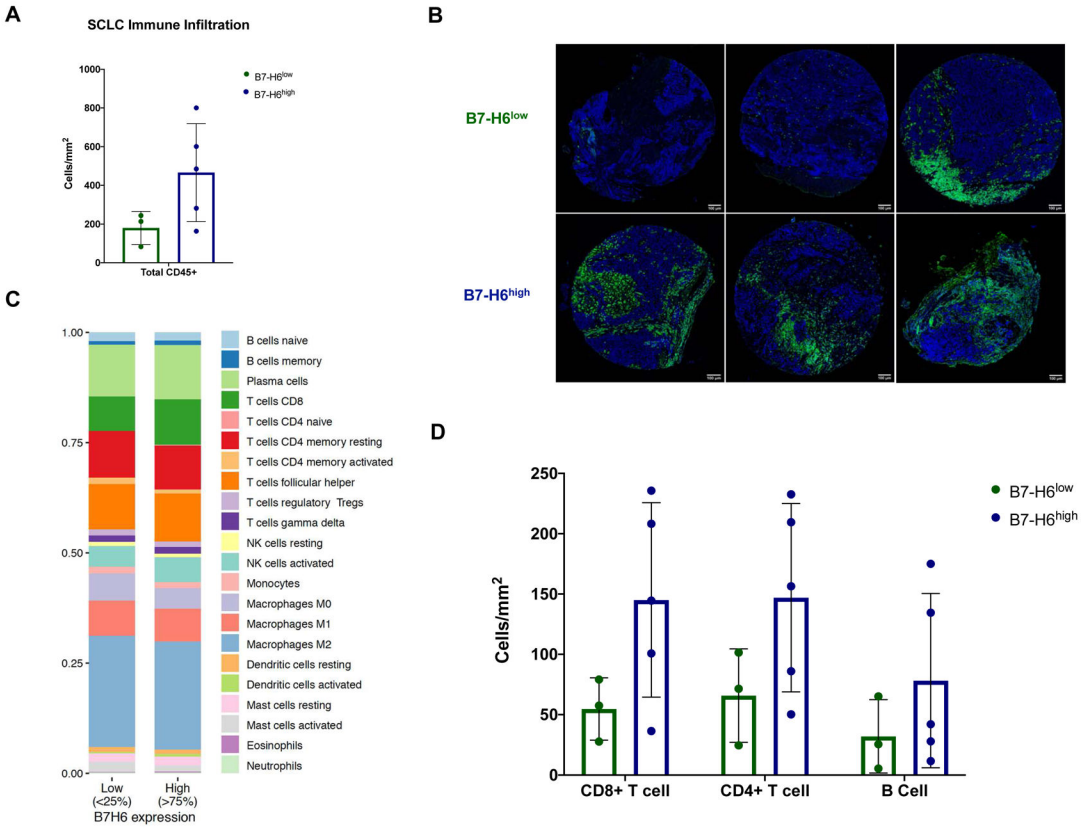

Figure 5: Correlation of B7-H6 and immune signature in SCLC tumors.

(**A**) Quantification of tumor-infiltrating immune cells (CD45+) in B7-H6^{low} (n=3) and B7-H6^{high} (n=5) primary lung SCLC tumors and (**B**) representative images showing nuclei (blue) and CD45+ infiltration (green); scale bar, 100 μm. (**C**) Relative abundance of immune cell populations determined by CIBERSORTx[34] methodology in B7-H6^{low} (n=20) and B7-H6^{high} SCLC tumors (n=20). (**D**) Quantification of CD8+ and CD4+ T cells, and B cells in B7-H6^{low} (n=3) and B7-H6^{high} (n=5) primary lung SCLC tumors. SCLC, small cell lung cancer.

Table 1.

Correlation Between B7-H6 Expression and Clinicopathologic Parameters of Patients With SCLC

		Tumor B7-H6 Positive %			
Clinical Parameters	**Cases**	**< Median (B7-H6^{low})**	**≥ Median (B7-H6^{high})**	**Chi-Square**	***p* Value**
Median survival (range)	39	7.65 mo (1.44-85.53)	15.86 mo (0.10-51.20)	–	0.4576
Gender					
Male	18	5	13	5.867	0.0154[a]
Female	21	14	7		
Age at diagnosis					
< 65 y	25	11	14	0.6205	0.4309
≥ 65 y	14	8	6		
Stage at diagnosis					
Limited-stage SCLC	18	7	11	1.293	0.2556
Extensive-stage SCLC	21	12	9		
Stage at biopsy					
Limited-stage SCLC	14	5	9	1.216	0.2241
Extensive-stage SCLC	25	14	11		
Site of biopsy					
Lung	16	5	11	3.327	0.1895
Lymph node	8	5	3		
Other[b]	15	9	6		
Distant metastases					
M0	18	7	11	1.293	0.2556
M1	21	12	9		
Survival time					
<1 y	24	14	10	1.520	0.1286
≥ 1 y	15	5	10		
Response to first-line therapy					
CR	9	3	6	2.272	0.5180
PR	13	8	5		
Stable disease	3	2	1		
POD	8	5	3		
Did not receive therapy	5	1	4		
Data not available	1	0	1		

[a] Signifies $p < 0.05$.

[b] Liver, bone, brain, neck, bowel, and adrenal.

CR, complete response; PR, partial response; POD, progression of disease.