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Correlation of PD-L1 expression with tumor mutation burden and gene signatures for prognosis in early stage squamous cell lung carcinoma

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Conflicts of Interest

Dr. Ballman reports grants from NCI, during the conduct of the study.

Dr. Govindan reports personal fees from Inivata, personal fees from Pfizer, honorarium and consulting fees from Genetech, personal fees from NeoHealth, personal fees from BMS, personal fees from Nektar, outside the submitted work.

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Dr. Shepherd reports honoraria from Merck, AstraZeneca, Roche and BMS

Dr. Hirsch is co-inventor of a University of Colorado owned patent: "EGFR IHC and- FISH as predictive biomarkers for EGFR Therapy". Dr. Hirsch has participated in advisory boards for: BMS, Genentech/Roche, HTG, Lilly, Merck, Pfizer, Ventana. Dr. Hirsch's laboratory has received research grants (through University of Colorado) from: Genentech, BMS, Lilly, Bayer and Clovis.

All of the other authors have nothing to disclose.

Abstract

Purpose : Anti-PD1/PD-L1 immunotherapy has demonstrated success in the treatment of advanced non-small cell lung cancer (NSCLC). Recently, PD1/PD-L1 blockade also has demonstrated interesting results in small trials of neo-adjuvant treatment in Stage IB-IIIA NSCLC. In addition, several clinical trials using anti-PD1/PD-L1 as an adjuvant or neo-adjuvant treatment in resectable stage NSCLC patients are ongoing. However, few analyses of anti-PD1/PD-L1 immunotherapy related biomarkers in early stage squamous cell lung carcinoma (SqCLC) have been reported. In this study, we evaluated PD-L1 protein expression, tumor mutation burden, and expression of an immune gene signature in early stage SqCLC, providing data for identifying the potential role for anti-PD1/PD-L1 treatment in early stage SqCLC patients.

Experimental Design and Results : A total of 255 early stage SqCLC patient specimens were identified within the Strategic Partnering to Evaluate Cancer Signatures (SPECS) program participating centers. PD-L1 protein expression by IHC was evaluated using the Dako PD-L1 22C3 pharmDx kit on the Dako Link 48 auto-stainer. Tumor Mutation Burden (TMB) was calculated based on data from targeted genome sequencing. The T-effector and IFN-γ gene signature was determined from Affymetrix gene chip data from frozen specimens. The prevalence of PD-L1 expression was 9.8% at a tumor proportion score (TPS) cutoff of ≥ 50%. PD-L1 mRNA and PD-L2 mRNA positively correlated with PD-L1 protein expression on tumor cells (TCs) and tumor-infiltrating immune cells (TIICs). PD-L1 protein expression on TIICs was correlated with the T-effector and IFN-γ gene signature ($P<0.001$), but not with TMB. For tumor cells, all of these biomarkers were independent of each other. And neither PD-L1 protein expression, TMB, or T-effector and IFN-γ gene signatures were independently prognostic for patient outcomes.

Conclusions : Evaluation of PD-L1 expression, TMB, and T-effector and IFN-γ gene signatures in the early-stage SqCLC cohort were found to be independent of each other and none were associated with overall survival. Results also support the hypothesis that PD-L1 expression is

regulated by an intrinsic mechanism on tumor cells and an adaptive mechanism on immune cells.

Introduction

Non-small cell lung cancer (NSCLC) represents 87% of all lung cancers, and of these, approximately one third are squamous cell carcinoma (SqCLC)[1]. Several therapy options have become available for patients with adenocarcinoma, but there has been little progress for patients with SqCLC. Patients with early stage SqCLC (I-IIIA) are treated by surgery with curative intent after which adjuvant chemotherapy following surgery is offered to those patients with stage IB (>4 cm), II and IIIA. Post-operative adjuvant chemotherapy improves survival to a modest degree in stage II-III NSCLC [2,3], but it is not recommended for stage I, even though patients with stage IA and IB NSCLC experience only 82% and 66% five-year survival respectively after complete surgical resection[4].

Based on the recent success obtained with immunotherapy for several types of tumors including advanced NSCLC, there is renewed hope for treatment of SqCLC. Several clinical trials with immune checkpoint inhibitors have been shown to be superior to chemotherapy in the frontline and second line setting (**Figure 1**)[5-9].

Increasingly, clinical data have shown that the expression of PD-L1 in patient tumors predicts the likelihood of a positive response to anti-PD-1/PD-L1 immunotherapy [10-13]. PD-L1 protein expression by immunohistochemistry remains the most frequently used biomarker for predicting clinical benefit from anti-PD-1/PD-L1 therapies, as demonstrated in many clinical trials[5,10,13-15]. Additionally, tumor mutational burden (TMB) may further help predict response. Rizvi *et. al.* recently demonstrated that treatment efficacy was associated with higher mutation burden in advanced NSCLC tumors using a whole-exome sequence assay [16]. In addition, Gandara *et. al*. found that not only was TMB in tissue correlated with the efficacy in first-line immunotherapy treated NSCLC, but TMB in blood measured from cell-free DNA in patients' plasma may predict the clinical efficacy in NSCLC treated with immunotherapy as second-line

care[17]. Tumor mutation burden has also been reported to enhance the predictive power of PD-L1 IHC for selecting patients who benefit from first-line therapy with nivolumab[8].
Several clinical trials in advanced melanoma provide evidence that gene expression profiling can indeed be a useful predictive biomarker[18-20]. Immune gene signatures, especially those induced by interferon-γ (IFN- γ), may serve as robust biomarkers for predicting the clinical benefit to anti-PD-1/PD-L1 therapies, a theory of which has been supported by PD-L1 expression data. In the POPLAR study, a T-effector and IFN- γ gene signature was identified by eight genes, which were previously associated with activated T cells, immune cytolytic activity, and IFN- γ expression[11,21-24]. Patients with pre-existing immunity, defined by high T-effector-IFN- γ-associated gene expression, had improved overall survival with atezolizumab [11].
However, research on the expression of PD-L1 and other immunotherapy related biomarkers in early-stage SqCLC is limited. In this study, we evaluated PD-L1 protein expression level in 255 early-stage SqCLC by IHC. We quantitatively assessed the tumor mutation burden with correlation to PD-L1 protein expression and clinical characteristics. Immune gene expression was analyzed by mRNA profiling using an Affymetrix based gene-expression platform. This study analyzed the anti-PD1/PD-L1 immunotherapy related biomarkers in a large cohort of early-stage SqCLC patients with association to clinical characteristics.

Methods

Patient population: A total of 255 patient specimens were collected from 6 centers [University of Colorado (UC), University of Michigan (UM), Washington University (WU), Duke University (DU), Brigham and Women's Hospital (BWH) and Princess Margaret Cancer Center (PM)] participating in the SPECS program. Each center identified approximately 50 samples from resected patients with stage I-II SqCLC who had either: a) died from lung cancer, or b) had a minimum of 3 years of follow-up without documented disease recurrence. None of the patients had received adjuvant, neoadjuvant or radiotherapy before surgery, with the exception of two patients who had received ajuvant therapy. All primary diagnoses were reviewed by experienced pathologists at each site according to the WHO nomenclature for squamous carcinoma [25]. Specimens chosen for analysis had a confirmed diagnosis of SqCLC and utilized

regions with ≥ 60% tumor cellularity. Data collected from each case included: age, sex, smoking history, ECOG performance status (PS), tumor size, node status, tumor stage, node stage and overall survival. Specimens were collected from patients (in IRB-approved protocols) who underwent resection of stage I and II lung SqCLC. Resected research tumor specimens were immediately frozen and stored at -80°C for gene expression studies. Corresponding formalin fixed, paraffin embedded clinical tissue blocks were used for IHC and targeted genome sequencing.

Immunohistochemistry: Four-micrometer FFPE sections were prepared on charged glass slides and sent to the Hirsch Biomarker Analysis Lab at the University of Colorado. IHC was performed using the Dako PD-L1 22C3 pharmDx kit (Dako, Carpinteria, CA) on the Dako Link 48 platform. For the cohort, one pathologist (H.Y.) scored all the specimens and other pathologists (M.K. and W.F.) scored 20% of specimens as quality control. For discrepant results, a final score was determined by a consensus conference of the pathologists. Scoring was determined according to the tumor proportion score (TPS) criteria on the basis of percentage of tumor cells with partial or complete cell membrane staining at any intensity. The expression of PD-L1 on tumor-infiltrating immune cells (TIICs) was scored as a percentage of tumor area, as described in other studies [10,11].

Tumor mutation burden (TMB): As part of an independent study (manuscript in preparation), 199 of the 255 patients tumors were subject to targeted exome sequencing using a capture panel representing 1,537 cancer-related genes. Tumor DNA was isolated from paraffin section slides and, where necessary, macro-dissected for areas of >60-90% enriched tumor cellularity. Targeted capture sequencing was performed with sequence alignment and variant calling using the Genome Modelling System (GMS) pipeline[26]. To identify true somatic variant calls in the absence of paired, non-malignant specimens, results were further filtered using the Variant Reporting Subsystem (VRS), which implements several additional user-defined filtering steps and annotates the resulting variants with information about their allele frequencies in dbSNP, the 1000 Genomes Project, and the NHLBI database. Variants were required to have a minimum VAF of 10%, minimum coverage of 10X, and to be present strictly in the on-target regions. Any variants exceeding 0.01% in NHLBI European, African-American, or All data sets, or

in the 1000 Genomes Project, were removed. An additional panel of 905 normal breast tissue samples was used as a germline filter. Variants that appeared in five or more samples from that dataset were also removed. Finally, a subset of the ExAC dataset was incorporated into the pipeline[27]to exclude other potential germline variants.

RNA profiling: Frozen specimens were macro-dissected to enrich for neoplastic tissue. RNA isolation, cRNA synthesis and Affymetrix gene expression profiling were performed as described by Raponi et al [28]. Following RNA isolation with Trizol (Invitrogen) and RNeasy column purification (Qiagen), RNA yield and purity were assessed using Nanodrop, RNA integrity was determined with an Agilent 2100 Bioanalyzer and the concentration calculated by OD260 measurement. GeneChip quality was assessed using the Affymetrix Microarray Suite and Mining Tools and recorded for each sample. CHP files were generated from CEL files and probe IDs for all genes retrieved. Spline quantile normalization was applied and analysis of variance (ANOVA) used to remove batch effects or outlier samples from various sites. A T-effector and IFN-γ gene signature was defined by *CD8A, GZMA, GZMB, IFNγ, EMOES, CXCL9, CXCL10* and *TBX21* [11]. The high biomarker group was defined as gene expression at or above the median level, and the low biomarker group as gene expression below the median level as described in the POPLAR study. *PD-L1* and *PD-L2* gene expression were also evaluated.

Data and Statistical analysis:

To assess the association between clinical features and biomarkers, we used the two-sample t-test for continuous variables and Fisher's exact test for categorical variables. The Pearson correlation coefficient was used to evaluate the correlation between two numerical biomarkers. To further assess the linear trend between ordinal variables, we used CMH trend test. A log-rank test was used to test the group differences of survival time. All analyses were two-sided with an alpha of 0.05 as the significance level. The p values were not adjusted for potential multiple comparisons due to the exploratory nature of the study. All analyses were performed in the statistical software SAS9.4 (SAS Institute, Cary, NC) or R (R Foundation for Statistical Computing, Vienna, Austria).

Results

PD-L1 protein expression in early-stage SqCLC and the association with clinical characteristics

Samples were collected from 255 early-stage SqCLC patients. The distribution of PD-L1 expression on tumor cells (TC) and TIICs at different cutoffs (on the basis of the published association of cutoff with clinical response to anti-PD-1 therapy) are shown in **Table 1**. The overlap of populations for PD-L1 protein expression level on TC and TIICs are shown in **Figure 2**. Patients with co-expression of PD-L1 on both tumor cells and TIICs at high level were few (4%). A linear trend association was found between PD-L1 protein expression on TC and the expression on TIICs at a TPS cutoff of ≥ 5% only (P=0.036). PD-L1 positive staining on TC at the cutoff had a higher PD-L1 staining on TIICs.

Among the 208 patients with clinical data, 6 cases were missing TPS value. For 202 cases with both clinical and PD-L1 protein expression data, no significant association was observed between the PD-L1 protein expression in tumor cells and the clinical characteristics (**Table 2**) or prognosis (**Figure 3A**).

Analysis also revealed a positive correlation between PD-L1 protein expression on TC and PD-L1 mRNA level (P<0.0001, r=0.62) and PD-L2 mRNA expression (P<0.0001, r=0.45). PD-L1 mRNA level correlated with PD-L2 mRNA (P<0.0001, r=0.64) as well (**Figure 4**). Both PD-L1 mRNA level and PD-L2 mRNA level were linear associated with PD-L1 protein expression on TIICs as well (both P=0.033).

TMB and clinical characteristics

The TMB was evaluated in 199 SqCLC patient specimens. Preliminary quality filtering on somatic variation calls was performed to choose only on-target regions and those that met other quality thresholds (e.g. min coverage > 10X). Extensive additional mutational filtering on the output of the somatic variation pipeline using various data sets, such as 1000 genomes, ExAC and the existing panel of normal samples, brought the total number of mutations from 36053 to 19094. The bases covered at a min. depth of 20X were used to calculate the mutations/megabase (Mb). The results include all mutation types (missense, nonsense, intronic, intergenic etc.). The median number of somatic mutations /Mb was 31 (range 17 to 195). The mean value of somatic mutations was 35/Mb. We defined the high TMB group as a TMB value at or above the

median level, and the low TMB group as TMB value below the median level. For 155 cases with both TMB data and clinical characteristics, no significant association was observed between TMB and age, gender, smoking history, stage (**Supplement Table 1**) and prognosis (**Figure 3B**).

T-effector and IFN-Y gene signature expression

The T-effector and IFN-γ gene signature was used to evaluate the status of the tumor microenvironment from 208 patients. Eight genes which are associated with T-cell activation and IFN-γ level were included in the panel. The z-scores were calculated for each gene in the 8-gene signature. The median of the averages of the 8 gene z-scores were then used to dichotomize the patients at the median into high and low groups. Data revealed no correlation between the T-effector and IFN-γ gene signature and clinical characteristics or prognosis (**Supplement Table 2, Figure 3C**).

Correlation among PD-L1 protein expression, TMB and T-effector and IFN-γ gene signature

In 153 patients who had all three biomarker data, 4.6% (7/153) of patients showed high levels of all three biomarkers at a PD-L1 TPS cutoff of ≥ 50%. In addition, 15.7% (24/153) of patients demonstrated negative expression for all three biomarkers (**Figure 5**). Neither PD-L1 expression on TC or on TIICs was associated with TMB. Data for TMB also did not correlate with the T-effector and IFN-γ gene signature. Interestingly, a significant association ($P < 0.001$, CMH trend test) was observed between T-effector and IFN-γ gene signature and PD-L1 expression on TIICs (**Figure 6**). Higher T-effector and IFN-γ gene signature scores were associated with higher PD-L1 expression on TIICs. We further explored the prognosis of patients with PD-L1 expression ≥ 50% TPS on TC and a high TMB. These patients did not significantly correlate with a worse prognosis than others (**Figure 7**)

Discussion

Recently, anti-PD1/PD-L1 immunotherapy has produced objective and often durable responses in approximately 20% of previously treated NSCLC patients, not selected on the basis of PDL-1 expression. Despite this low overall response rate, overall survival was superior to docetaxel chemotherapy for most patient subgroups including those with squamous cancers.

Furthermore a modest proportion of patients achieved long-term survival even up to two years or more[5,11,13,14].

Despite complete resection, 25% of stage IA and 45% of stage IB patients develop recurrent disease within five years. Adjuvant chemotherapy can improve survival moderately in stage II-III NSCLC[29]. The objective of our study was to perform a comprehensive analysis of potential immune biomarkers in early-stage SqCLC, to examine the overlap and relationship of these biomarkers to each other, and to evaluate their prognostic role in resected SqCLC.

At the present time, expression of PD-L1 by IHC remains the only validated biomarker that has demonstrated a strong correlation with response in most trials. However, there is variability in marker staining among the approved antibody tests[30]. In this study, we detected PD-L1 expression by IHC in the early-stage SqCLC cohort using the Dako 22C3 pharmDx kit, which has been approved by the FDA as the companion diagnostic for pembrolizumab. Both the IASLC Blueprint study and NCCN-BMS comparison study aimed to evaluate the variability of current assays including FDA approved or laboratory derived tests (LDTs) [31,32]. Data from these studies showed that the Dako 22C3, Dako 28-8, E1L3N (CellSignaling) and SP263 (Ventana) assays were closely aligned on tumor cell staining.

Our data demonstrate the prevalence of PD-L1 expression on tumor cells in the early-stage SqCLC cohort was 9.8% at a TPS cutoff of ≥ 50%. In comparison, data from pembrolizumab clinical trials demonstrated a PD-L1 prevalence ranging from 20% to 30% in advanced NSCLC using the 22C3 antibody at the same cutoff[7,13,15,33,34]. However, in a study which enrolled 678 stage I-III NSCLC patients and detected the prevalence of PD-L1 expression using the 22C3 antibody found high PD-L1 expression in 7.4% of NSCLC cases and 8.1% of the squamous cell carcinomas at a TPS cutoff of ≥ 50%[35], which was close to our result. Another study that enrolled 170 stage I-III NSCLC Chinese patients reported a prevalence in PD-L1 expression of 15.8% in the SqCLC specimens using the same antibody at the same cutoff[36]. These data indicate that increased PD-L1 expression may be associated with tumor progression and advanced tumor stage. One theory is that of heterogeneity selection. This concept involves tumor cells without PD-L1 expression at early stage disease are eliminated by T cells, while tumor cells expressing PD-L1 escaped from the immune response. As the tumor stage advances,

more tumor cells express PD-L1 and have the ability of escaping immune elimination. However, results from studies on the association of PD-L1 expression with the tumor stage in NSCLC or in lung adenocarcinoma alone are often not in agreement with regards to correlations of PD-L1 expression and tumor stage[35,37-39]. Although there was no significant correlation between PD-L1 expression and tumor stage in our study of early-stage SqCLC patient specimens, this may be due to the fact that most of the patients in our cohort were stages I-II.

Tumors with higher TMB have been hypothesized to have more neoantigens that can be recognized by the immune system in response to checkpoint inhibition. Rizvi et al in 2015[16] identified TMB as a promising predictive biomarker for NSCLC immunotherapy. In that single arm study, assessment of somatic, non-synonymous TMB in NSCLC patients who received pembrolizumab was determined using whole-exome sequencing (WES), and correlated with improved overall response rates (ORRs), progression-free survival (PFS), and durable clinical benefit. Recently, several additional studies evaluated TMB by NGS and correlated results with immunotherapy response. Rizvi et al used the MSK-IMPACT (Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets) platform to evaluate TMB in 240 patients with advanced NSCLC and showed a median TMB of 7.4 single-nucleotide variants/Mb[40]. Kowanetz et al reported in a study of 454 patients treated with atezolizumab and assessed for TMB from a 315-gene NGS panel, a median mutational load of 9.9/Mb, and > 75th percentile was classified as high TMB[41]. In our study, the median TMB was determined as 35/Mb which, unlike other studies, includes both synonymous and non-synonymous variants and insertion/deletions. Given differences in NGS gene panel content, variant filtering methods, and definitions of “high” TMB thresholds, it is difficult to compare TMB results across studies.

The immunogenicity of specific mutations may play a more important role in neoantigen recognition by the immune system. In the case of a non-immunogenic mutation, the altered peptide may not present the altered amino acid sequence for recognition or may bind poorly to the MHC class I molecule. The altered amino acids might also not be accessible to the T-cell antigen receptor (TCR). In an immunogenic mutation, the altered amino acid(s) may enable binding of the peptide to an MHC class I molecule, or it may project towards the TCR[42].

Although many clinical trials have shown that patients with high PD-L1 protein expression or high TMB have a greater response to immunotherapy, some patients with low PD-L1 protein expression or low TMB may also respond. This may in part be due to tumor heterogeneity, and biopsy of an area of low expression in a tumor that may have high expression in areas not accessed by a small needle biopsy[43-45]. Another possible reason as we mentioned above, some tumor-specific mutations have higher immunogenicity than others, which means that these mutations can produce the neoantigens that are more easily recognized and would activate the functional T cells. Dr. Tran and his group recently described in a case report that adopting T-cell transfer therapy targeting mutant KRAS G12D mediated effective antitumor immunotherapy in a patient with metastatic colorectal cancer[46], in which KRAS G12D is a promising immunogenic mutation that can be designed as a target. The tumor microenvironment is the constitutive element in cancer immunity. A variety of factors contribute to profiling of the microenvironment, such as PD-L1 expression on the TIICs, the levels of many proinflammatory and effector cytokines, the ratio of effector T cells to exhausted T cells, etc. PD-L1 expression on TIICs has been demonstrated to associate with response to atezolizumab using the SP142 antibody[10], and therefore was adopted in a series of atezolizumab clinical trials as the predictive marker[6,11,47]. Several studies reported tumor immune-related gene profiling predicted the response to PD1/PD-L1 blockade[48-50] We evaluated the mRNA level of the T-effector and IFN-γ gene signature adopted by the POPLAR study employing Affymetrix gene chip and did not find any association with clinical characteristics in early-stage SqCLC.
Data from this study demonstrated that the biomarkers evaluated were not found to be prognostic in the early-stage patients with SqCLC who underwent complete resection. However, less than 10% of patients in the cohort demonstrated high expression of PD-L1 at a TPS cutoff of ≥ 50%. We therefore acknowledge that this low number of patients may have limited the ability to assess the association of PD-L1 expression with survival. No correlation was found between PD-L1 expression on TCs, and TMB, and T-effector and IFN-γ gene signatures. Patients with a higher TMB have a greater chance of generating neoantigens, which can be recognized by T cells. These patients therefore may have high response rates to immunotherapy, when they receive anti-PD1/PD-L1 therapy and activate effector T cells.

Currently, there is no evidence of a direct correlation between TMB and PD-L1 expression and PD-L1 and the T-effector and IFN- γ gene signatures. The regulation processes are dynamic and fluctuate over time, as they occur in response to the host antitumor immunity. Few patients in our study (4.6%) had high levels of all biomarkers, indicating that these biomarkers identified distinct subpopulations in SqCLC. These results suggest that composite biomarker detection is needed as a strategy to select patients who may be more likely to benefit from anti-PD1/PD-L1 immunotherapy. A linear trend was associated with T-effector and IFN-γ gene signature expression and PD-L1 expression on TIICs. This is similar to the results found in the POPLAR study. Higher T-effector and IFN-γ gene signature expression levels could promote the expression of PD-L1 on TIICs. However, we did not find a significant correlation between PD-L1 expression on TC and on TIICs. These results support the hypothesis that the expression of PD-L1 is, in general, regulated by different mechanisms in TC and TIICs. Several intrinsic mechanisms have been identified in tumor cells as responsible for up-regulating PD-L1 expression, including aberrations of signaling pathways induced by oncogenic event, such as activation of EGFR, MAPK, or PI3K-Akt pathways[51], elevated expression of STAT3 and HIF-1 transcription factors[52], and amplification of PD-L1 and PD-L2 together with JAK2[53]. Although inflammatory cytokines are also involved in up-regulation of PD-L1 expression on TC, our data showed that the impact of proinflammatory and effector cytokines on tumor cells may not be as strong as the effect they have on TIICs in the tumor microenvironment, which is described as an adaptive immune reaction[10].

In summary, this study evaluated the level of three biomarkers related to anti-PD1/PD-L1 immunotherapy including, PD-L1 expression, TMB and T-effector and IFN-γ gene signature in an early-stage SqCLC cohort. We found that all of these biomarkers were independent of each other and independently, none was associated with OS of this newly diagnosed, early stage SqCLC cohort. Moreover, these results also support the hypothesis that PD-L1 expression is regulated by an intrinsic mechanism on tumor cells and an adaptive mechanism on immune cells. These findings may be important in the future when evaluating the role of anti-PD1/PD-L1 immunotherapy in the settings of (neo-) adjuvant trials.

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Figure Legends

Table 1. Prevalence of PD-L1 protein expression in early-stage SqCLC tumor cells.

Table 2. Clinico-pathological characteristics of the early-stage SqCLC cohort according to PD-L1 protein status in tumor cells

Figure 1. Forest plot of hazard ratios (HRs) for 5 clinical trials assessing the efficacy of anti-PD1/PD-L1 immunotherapy versus chemotherapy with overall survival in advanced squamous NSCLC. OS, overall survival; CI, confidence interval; mo, month; NSCLC, non-small cell lung cancer.

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Figure 4. Correlation of TC for PD-L1 protein by TPS score with PD-L1 mRNA levels, PD-L2 mRNA levels and PD-L1 mRNA to PD-L2 mRNA. Data indicates a moderate to strong association for all comparisons ($r \geq 0.45$ and $P<0.0001$).

Figure 5. Comparison of PD-L1 protein expression on tumor cells, TMB level and T-effector and IFN-γ gene signature score (Number in Venn diagrams represent the number of patients in each subgroups.)

Figure 6. MOSAIC plots showing the linear Trend association. A linear trend association was determined between T-effector gene signature scores and PD-L1 expression on TIICs. Higher T-effector scores are associated with higher TIICs staining.

Figure 7. Correlation of TMB/PD-L1 TPS score with patient overall survival. Data indicates no correlation for TMB and PD-L1 protein expression with prognosis.

Supplement Table 1. Correlation of SqCLC tissue TMB and patient clinical characteristics.

Supplement Table 2. Correlation of T-effector and IFN- γ gene signature and patient clinical characteristics

Table 1. Prevalence of PD-L1 protein expression in early-stage SqCLC tumor cells

	PD-L1 protein expression on tumor cells (IHC, TPS) **(Dako 22C3 PharmDx)**		
Cutoffs	< 1% (N, %)	1~49% (N,%)	≥50% (N,%)
Prevalence (N=255)	136 (53.3%)	94 (36.9%)	25 (9.8%)

	PD-L1 protein expression on tumor infiltrating immune cells (IHC, percentage of tumor area) **(Dako 22C3 PharmDx)**			
Cutoffs	< 1% (N, %)	1~4% (N, %)	5~9% (N, %)	≥ 10% (N, %)
Prevalence (N=255)	98 (38.5%)	60 (23.5%)	37 (14.5%)	60 (23.5%)

Table 2. Clinico-pathological characteristics of the early-stage SqCLC cohort according to PD-L1 protein status in tumor cells

Variable	Total	TPS 0	TPS 1-49%	TPS >=50%	*P* [1]
Age					
N	202	109	76	17	0.215 [A]
MEAN	70.02	71.02	68.95	68.36	
SD	8.89	8.24	9.83	8.05	
MEDIAN	70.75	70.78	69.52	71.86	
MIN	43.00	44.81	43.00	53.00	
MAX	92.22	92.22	86.18	81.72	
Q1	63.85	66.13	62.23	59.52	
Gender2, [n (%)]					
Female	74 (36.6)	45 (41.3)	21 (27.6)	8 (47.1)	0.099 [F]
Male	128 (63.4)	64 (58.7)	55 (72.4)	9 (52.9)	
SmokingHx, [n (%)]					
never smoked	4 (2.0)	2 (1.8)	2 (2.6)	0 (0.0)	1.000 [F]*
former smoker	135 (66.8)	73 (67.0)	51 (67.1)	11 (64.7)	
current smoker	58 (28.7)	32 (29.4)	22 (28.9)	4 (23.5)	
unknown	5 (2.5)	2 (1.8)	1 (1.3)	2 (11.8)	
Node_status, [n (%)]					
N0	177 (87.6)	96 (88.1)	66 (86.8)	15 (88.2)	1.000 [F]*
N1	23 (11.4)	12 (11.0)	9 (11.8)	2 (11.8)	
missing	2 (1.0)	1 (0.9)	1 (1.3)	0 (0.0)	
Tumor_Size					

N	202	109	76	17	0.645 [A]
MEAN	3.55	3.43	3.69	3.71	
SD	1.97	2.01	2.04	1.43	
MEDIAN	3.00	3.00	3.00	4.00	
MIN	0.50	0.50	1.10	1.30	
MAX	14.00	14.00	12.00	6.00	
T_stage, [n (%)]					
T1a	44 (21.8)	27 (24.8)	15 (19.7)	2 (11.8)	0.132 [F]*
T1b	60 (29.7)	32 (29.4)	26 (34.2)	2 (11.8)	
T2a	65 (32.2)	35 (32.1)	22 (28.9)	8 (47.1)	
T2b	20 (9.9)	11 (10.1)	5 (6.6)	4 (23.5)	
T3	13 (6.4)	4 (3.7)	8 (10.5)	1 (5.9)	
N_stage, [n (%)]					
Nx: unknown	5 (2.5)	2 (1.8)	3 (3.9)	0 (0.0)	0.890 [F]*
N0	175 (86.6)	95 (87.2)	64 (84.2)	16 (94.1)	
N1	22 (10.9)	12 (11.0)	9 (11.8)	1 (5.9)	

[1] P values obtained from the statistical tests: [A] - ANOVA; [F] - Fishers exact test; [F]* - Fishers exact test (excluding level: 'unknown', 'missing', 'other specify' 'not specified', 'none', 'not done', 'Nx: unknown'); [NA] - Not Available;

Note:

1. Out of 208 cases, 6 cases were removed because NAs in TPS group;

Supplement Table 1. Correlation of SqCLC tissue TMB and patient clinical characteristics.

Variable	Total	TMB Level High	TMB Level Low	*P* [1]
Age				
N	155	77	78	0.276 [T]
MEAN	70.10	69.28	70.90	
SD	9.24	8.93	9.53	
MEDIAN	71.33	70.77	71.73	
MIN	43.00	44.81	43.00	
MAX	92.22	92.22	86.18	
Q1	63.50	63.17	64.39	
Sex, [n (%)]				
Female	53 (34.2)	28 (36.4)	25 (32.1)	0.614 [F]
Male	102 (65.8)	49 (63.6)	53 (67.9)	
SmokingHx, [n (%)]				
never smoked	3 (1.9)	2 (2.6)	1 (1.3)	0.889 [F]*
former smoker	106 (68.4)	52 (67.5)	54 (69.2)	
current smoker	40 (25.8)	19 (24.7)	21 (26.9)	
unknown	6 (3.9)	4 (5.2)	2 (2.6)	

Variable	Total	TMB Level High	TMB Level Low	*P* [1]
Node_status, [n (%)]				
N0	138 (89.0)	69 (89.6)	69 (88.5)	1.000 [F]*
N1	17 (11.0)	8 (10.4)	9 (11.5)	
Tumor_Size				
N	155	77	78	0.861 [T]
MEAN	3.75	3.78	3.72	
SD	1.98	2.04	1.93	
MEDIAN	3.10	3.10	3.10	
MIN	1.00	1.10	1.00	
MAX	14.00	14.00	12.00	
T_stage, [n (%)]				
T1a	29 (18.7)	17 (22.1)	12 (15.4)	0.715 [F]*
T1b	45 (29.0)	20 (26.0)	25 (32.1)	
T2a	52 (33.5)	24 (31.2)	28 (35.9)	
T2b	19 (12.3)	10 (13.0)	9 (11.5)	
T3	10 (6.5)	6 (7.8)	4 (5.1)	
N_stage, [n (%)]				
Nx: unknown	3 (1.9)	1 (1.3)	2 (2.6)	1.000 [F]*
N0	134 (86.5)	67 (87.0)	67 (85.9)	

Variable	Total	TMB Level		P [1]
		High	Low	
N1	18 (11.6)	9 (11.7)	9 (11.5)	

[1] P values obtained from the statistical tests: [F] - Fishers exact test; [F]* - Fishers exact test (excluding level: , 'unknown', 'missing', 'other specify', 'not specified', 'none', 'not done', 'Nx: unknown'); [NA] - Not Available; [T] - T-test;

Note:

1. Out of 199 cases, 155 cases could be analyzed with clinical data;

Supplement Table 2. Correlation of T-effector and IFN- γ gene signature and patient clinical characteristics

Variable	Total	T-IFNY High	T-IFNY Low	*P* [1]
Age				
N	208	104	104	0.172 [T]
MEAN	70.05	70.89	69.21	
SD	8.83	8.43	9.18	
MEDIAN	70.75	71.46	69.62	
MIN	43.00	44.81	43.00	
MAX	92.22	85.58	92.22	
Q1	63.96	65.74	63.00	
Gender, [n (%)]				
Female	78 (37.5)	38 (36.5)	40 (38.5)	0.886 [F]
Male	130 (62.5)	66 (63.5)	64 (61.5)	
SmokingHx, [n (%)]				
never smoked	4 (1.9)	1 (1.0)	3 (2.9)	0.470 [F]*
former smoker	139 (66.8)	68 (65.4)	71 (68.3)	
current smoker	59 (28.4)	32 (30.8)	27 (26.0)	
unknown	6 (2.9)	3 (2.9)	3 (2.9)	
Node_status, [n (%)]				
N0	181 (87.0)	93 (89.4)	88 (84.6)	0.143 [F]*
N1	25 (12.0)	9 (8.7)	16 (15.4)	

Variable	Total	T-IFNγ High	T-IFNγ Low	*P* [1]
missing	2 (1.0)	2 (1.9)	0 (0.0)	
Tumor_Size				
N	208	104	104	0.230 [T]
MEAN	3.56	3.39	3.72	
SD	1.95	2.00	1.89	
MEDIAN	3.00	2.70	3.15	
MIN	0.50	0.50	1.20	
MAX	14.00	14.00	12.00	
T_stage, [n (%)]				
T1a	44 (21.2)	27 (26.0)	17 (16.3)	0.320 [F]*
T1b	62 (29.8)	33 (31.7)	29 (27.9)	
T2a	68 (32.7)	29 (27.9)	39 (37.5)	
T2b	21 (10.1)	10 (9.6)	11 (10.6)	
T3	13 (6.3)	5 (4.8)	8 (7.7)	
N_stage, [n (%)]				
Nx: unknown	5 (2.4)	2 (1.9)	3 (2.9)	0.393 [F]*
N0	179 (86.1)	92 (88.5)	87 (83.7)	
N1	24 (11.5)	10 (9.6)	14 (13.5)	

[1] P values obtained from the statistical tests: [F] - Fishers exact test; [F]* - Fishers exact test (excluding level: 'unknown', 'missing', 'not done', 'Nx: unknown'); [T] - T-test;

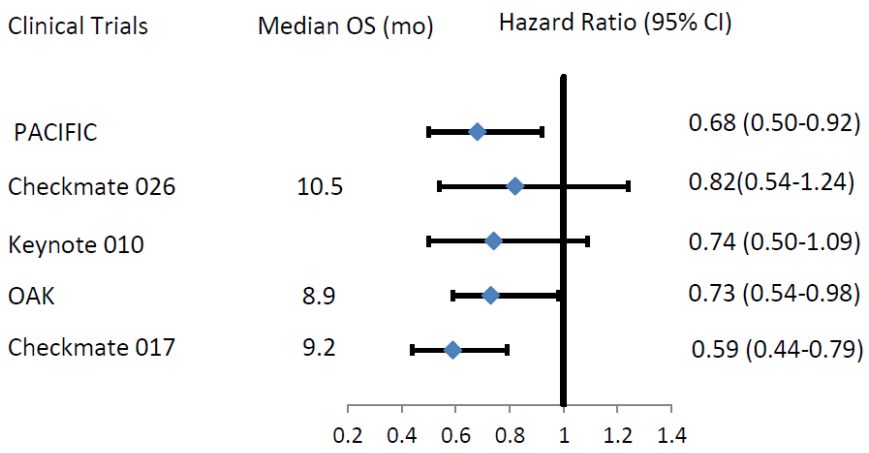

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Figure 2. Overlap between PD-L1 expression on tumor cells (TC) and tumor-infiltrating immune cells (TIIC).

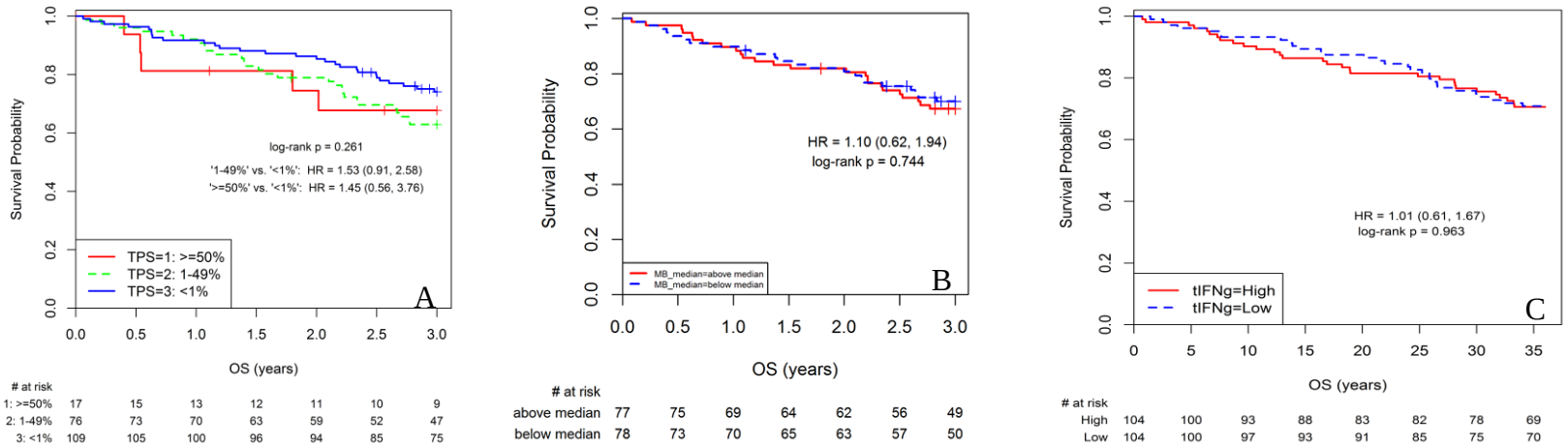
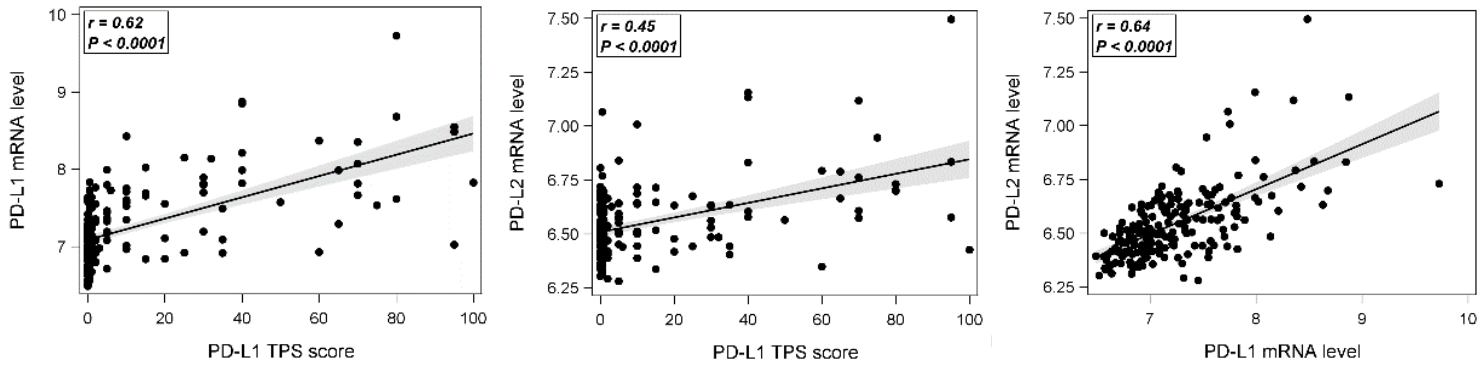


Figure 3. **A.** Correlation of PD-L1 protein expression with patient overall survival in the early stage SqCLC cohort. **B.** Correlation of TMB with patient overall survival in the early stage SqCLC cohort. Data evaluated for TMB high identified as ≥ median value as compared to TMB low which is identified as below the median value. **C.** Correlation of T-effector and IFN-γ gene signature and clinical prognosis for 208 early stage SqCLC patients. Signature high represented as ≥ median and low as < median. Results demonstrated no difference in prognosis by overall survival in years.



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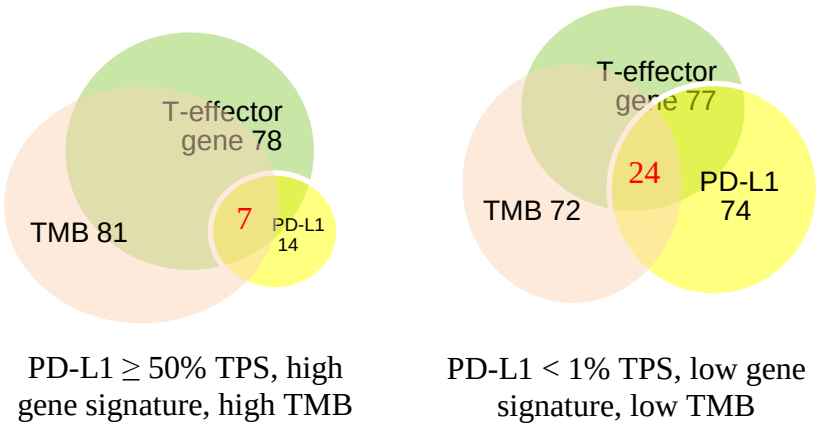
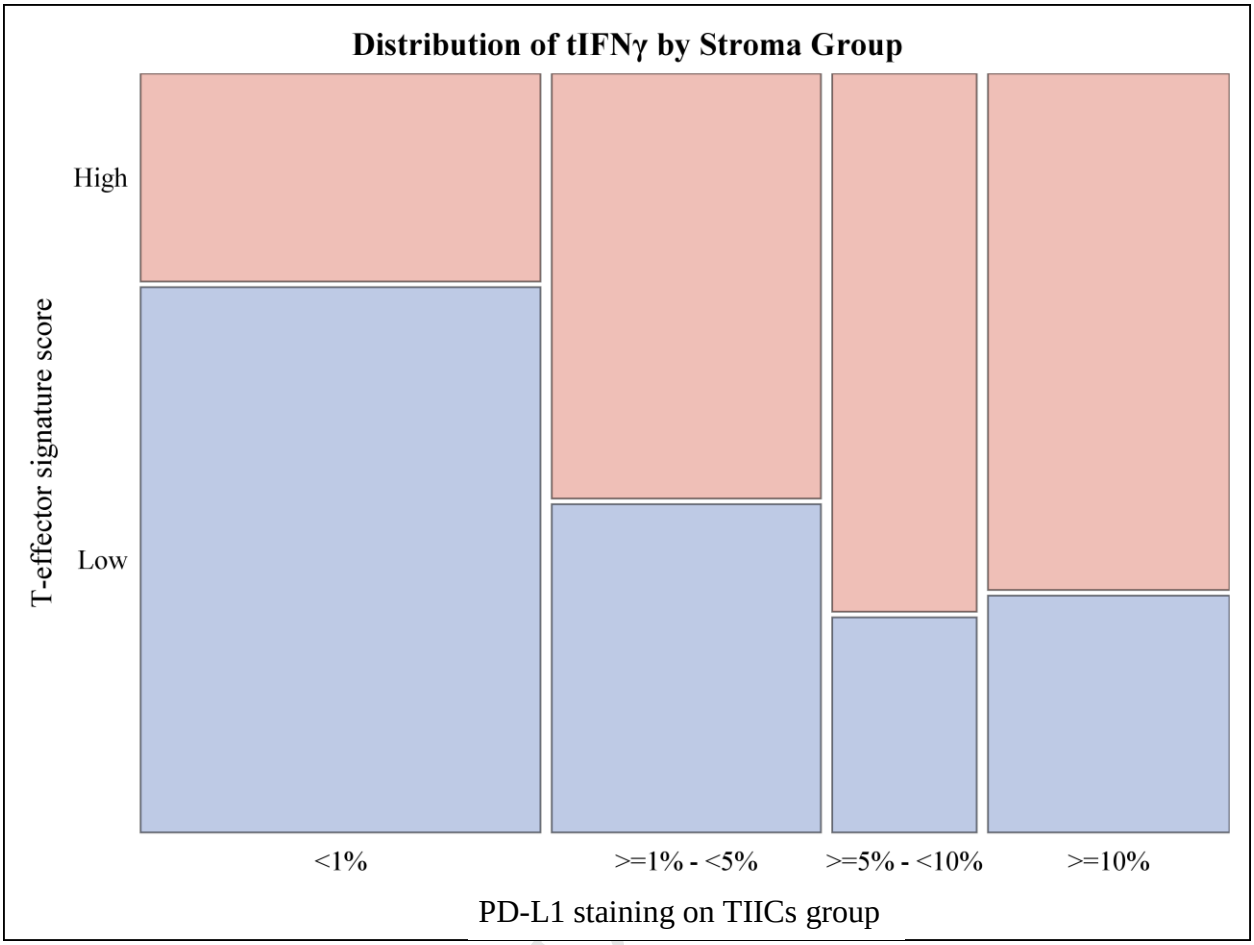


PD-L1 ≥ 50% TPS, high gene signature, high TMB

PD-L1 < 1% TPS, low gene signature, low TMB

Figure 5. Comparison of PD-L1 protein expression on tumor cells, TMB level and T-effector and IFN-γ gene signature score (Number in Venn diagrams represent the number of patients in each subgroups.)



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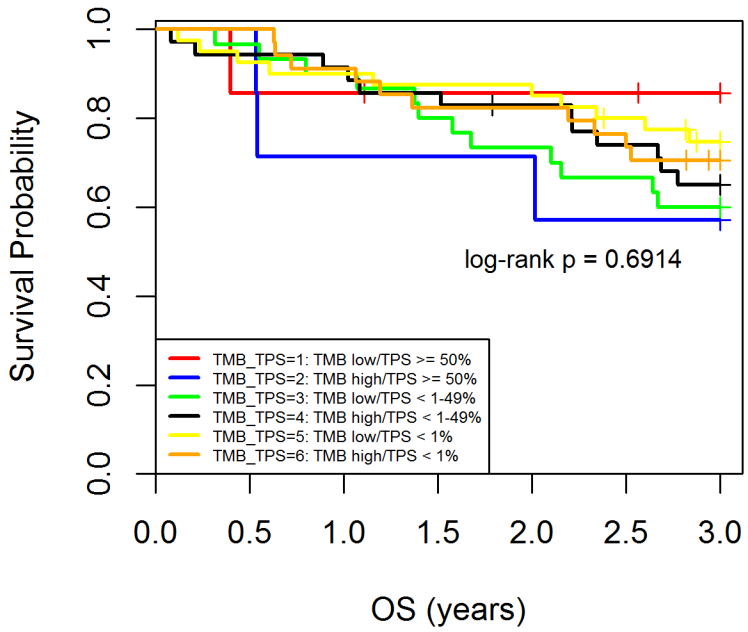

# at risk							
1: TMB low/TPS >= 50%	7	6	6	5	5	5	4
2: TMB high/TPS >= 50%	7	7	5	5	5	4	4
3: TMB low/TPS < 1-49%	30	29	27	24	22	20	18
4: TMB high/TPS < 1-49%	35	33	32	30	28	25	22
5: TMB low/TPS < 1%	40	37	36	35	35	31	27
6: TMB high/TPS < 1%	34	34	31	28	28	26	22

Figure 7. Correlation of TMB/PD-L1 TPS score with patient overall survival. Data indicates no correlation for TMB and PD-L1 protein expression with prognosis.

Table 1. Prevalence of PD-L1 protein expression in early-stage SqCLC tumor cells

	PD-L1 protein expression on tumor cells (IHC, TPS) **(Dako 22C3 PharmDx)**		
Cutoffs	< 1% (N)	1~49% (N)	≥50% (N)
Prevalence (N=255)	136(53.3%)	94 (36.9%)	25 (9.8%)

	PD-L1 protein expression on tumor infiltrating immune cells (IHC, percentage of tumor area) **(Dako 22C3 PharmDx)**			
Cutoffs	< 1% (N)	1~4% (N)	5~9% (N)	≥ 10% (N)
Prevalence (N=255)	98 (38.5%)	60 (23.5%)	37 (14.5%)	60 (23.5%)