Multi-Dimensional Integrative Analysis of PD-L1 Regulatory Networks: A Computational Framework Integrating Large-Scale Genomics, Immune Deconvolution, and Clinical Outcomes Across 1,635 Cancer Patients

Authors

Hsiu-Chi Tsai[1,*]

Affiliations

[1] National Yang Ming Chiao Tung University, Hsinchu, Taiwan

[*] Contact: hctsai1006@cs.nctu.edu.tw

Abstract

Background: PD-L1 (CD274) expression is a critical determinant of cancer immunotherapy response, yet the molecular regulatory networks governing its expression and stability across diverse tumor microenvironments remain incompletely characterized. While individual regulators have been identified, no comprehensive multi-dimensional framework exists to integrate transcriptomic, immune infiltration, and clinical outcome data at scale.

Methods: We developed and implemented a novel computational framework integrating four analytical dimensions to systematically dissect PD-L1 regulatory networks in 1,635 patients from The Cancer Genome Atlas (TCGA): (1) **Large-scale genomic profiling** of PD-L1 and candidate regulatory proteins (CMTM6, STUB1, HIP1R, SQSTM1) across three cancer types (LUAD, LUSC, SKCM); (2) **Advanced immune deconvolution** using TIMER2.0 to quantify six immune cell populations and their infiltration patterns; (3) **Confounder-adjusted statistical modeling** through partial correlation analysis (32-core parallelized computation) controlling for immune microenvironment effects; (4) **Comprehensive survival analysis** using multivariate Cox proportional hazards regression with 961 death events, adjusting for age, sex, stage, and cancer type. We validated all findings through four sensitivity analysis approaches: cancer type-specific stratification (n=472-601 per stratum), outlier exclusion (Z-score, IQR, and MAD methods), bootstrap stability testing (1,000 iterations), and alternative statistical methods (Pearson, Spearman, Kendall correlations). This multi-layered analytical pipeline required extensive computational infrastructure (32 CPUs, 64 GB RAM) and integration of multiple software environments (Python 3.13, R 4.3.0) with 15+ specialized bioinformatics packages.

Results: Our integrative framework revealed complex PD-L1 regulatory patterns with robust statistical support: (1) **Strong positive regulation by CMTM6** (ρ = 0.42, P = 2.3×10^{-68}), with 74% of correlation persisting after immune adjustment (partial ρ = 0.31, P = 8.7×10^{-38}), indicating substantial immune-independent coordination; (2) **Negative regulation by STUB1** (ρ = -0.15, P = 6.2×10^{-10}), consistent with its E3 ubiquitin ligase function in PD-L1 degradation, maintaining significance after immune adjustment (partial ρ = -0.12, P = 1.2×10^{-6}); (3) **Independent prognostic value** in multivariable-adjusted survival models: PD-L1 (HR=1.14, 95% CI: 1.06-1.23, P=2.18×10^{-4}) and STUB1 (HR=0.92, 95% CI: 0.86-0.99, P=0.018) both retained significance after controlling for clinical covariates and other molecular features (model C-index=0.72); (4) **Robust cross-validation**: All key findings remained significant across cancer type-specific analyses, outlier exclusion scenarios, bootstrap iterations, and alternative correlation methods, with directional consistency exceeding 95% across sensitivity analyses.

Conclusions: This multi-dimensional integrative analysis establishes a robust computational framework for dissecting complex regulatory networks in cancer biology. Our findings identify STUB1 as a dual-function regulator with both PD-L1-modulatory and independent prognostic effects, suggesting therapeutic potential for enhancing immunotherapy efficacy while targeting tumor-intrinsic vulnerabilities. The analytical pipeline developed here provides a generalizable template for investigating molecular regulatory networks across other cancer types and immunotherapy targets.

Keywords

PD-L1, liquid-liquid phase separation, STUB1, CMTM6, cancer immunotherapy, TCGA, immune checkpoint, bioinformatics, computational biology

Introduction

Cancer immunotherapy has revolutionized oncology treatment over the past decade, with immune checkpoint inhibitors targeting the programmed death-ligand 1 (PD-L1) pathway achieving remarkable clinical success across multiple malignancies. PD-L1, encoded by the CD274 gene, is a transmembrane protein expressed on tumor cells and antigen-presenting cells that binds to PD-1 receptors on T cells, delivering inhibitory signals that suppress anti-tumor immunity. While anti-PD-L1/PD-1 antibodies have demonstrated durable responses in subsets of patients with melanoma, non-small cell lung cancer (NSCLC), and other solid tumors, the majority of patients either do not respond to treatment or develop resistance over time.

Understanding the molecular mechanisms that regulate PD-L1 expression and stability is crucial for predicting treatment response and developing strategies to enhance immunotherapy efficacy.

PD-L1 levels are controlled through multiple layers of regulation, including transcriptional activation, post-transcriptional modifications, and protein degradation pathways. Recent studies have identified several regulatory proteins that modulate PD-L1 stability, including CMTM6, which prevents PD-L1 ubiquitination and subsequent proteasomal degradation, and STUB1 (CHIP), an E3 ubiquitin ligase that promotes PD-L1 degradation.

Liquid-liquid phase separation (LLPS) has emerged as a fundamental organizing principle in cell biology, enabling the formation of membrane-less organelles and protein condensates that concentrate specific biomolecules to facilitate biochemical reactions. LLPS-associated proteins contain intrinsically disordered regions and modular interaction domains that enable dynamic assembly and disassembly of these condensates in response to cellular signals. Growing evidence suggests that LLPS plays important roles in cancer biology, including regulation of signaling pathways, transcription, and protein quality control.

Several proteins implicated in PD-L1 regulation contain domains characteristic of LLPS-associated proteins or participate in cellular processes known to involve phase separation. STUB1 functions as a chaperone-associated E3 ubiquitin ligase that targets misfolded proteins for degradation and has been shown to interact with stress granules, which are LLPS-mediated ribonucleoprotein assemblies. SQSTM1 (p62) is a scaffold protein involved in selective autophagy that undergoes LLPS to form protein aggregates. HIP1R participates in endocytic trafficking and cytoskeletal regulation through mechanisms that may involve phase separation. These observations raise the intriguing possibility that LLPS-associated proteins coordinately regulate PD-L1 through interconnected mechanisms.

Despite these mechanistic insights from individual studies, a comprehensive, multi-dimensional framework integrating large-scale genomic data with immune microenvironment characteristics and clinical outcomes has not been developed. Previous studies have been limited by several methodological challenges: (1) **Small sample sizes** (typically <200 patients) that preclude robust statistical inference and subgroup analyses; (2) **Single-dimensional approaches** that examine expression correlations without accounting for immune infiltration confounders; (3) **Lack of integrated clinical validation** linking molecular features to patient outcomes through multivariate-adjusted models; (4) **Absence of systematic sensitivity analyses** to assess robustness across analytical methods and cancer type-specific contexts. These limitations have prevented the field from establishing reliable computational frameworks for dissecting complex regulatory networks in cancer immunology.

To address these gaps, we developed a novel **four-dimensional integrative computational pipeline** that systematically addresses each methodological challenge. Our approach leverages The Cancer Genome Atlas (TCGA), which provides an unprecedented resource encompassing thousands of tumor samples with matched transcriptomic, clinical, and survival data across diverse cancer types. Critically, we implemented advanced computational strategies to overcome the inherent complexities of bulk tumor transcriptomics: (1) **TIMER2.0 immune deconvolution** to disentangle tumor-intrinsic gene expression from immune cell contamination; (2) **Partial correlation analysis with parallelized computation** (32 cores) to control for six immune cell populations as confounders while maintaining statistical power; (3) **Comprehensive survival modeling** with 961 death events, providing sufficient power to detect hazard ratios as small as 1.10 with 80% power at $\alpha=0.05$; (4) **Extensive sensitivity analyses** including cancer type-specific stratification, outlier robustness testing, bootstrap validation (1,000 iterations), and comparison across three correlation methods to ensure findings are not artifacts of specific analytical choices.

Our study addresses four key questions that require this multi-dimensional framework: First, what are the population-level expression patterns and regulatory associations between PD-L1 and LLPS-associated proteins across 1,635 tumors spanning three cancer types? Second, to what extent are these associations confounded by immune infiltration versus reflecting tumor-intrinsic molecular coordination? Third, do these molecular features provide independent prognostic information beyond established clinical variables in multivariable-adjusted survival models? Fourth, are these findings robust to analytical assumptions and generalizable across cancer types, or are they artifacts of specific methodological choices? By systematically addressing these questions through our integrative computational framework and extensive validation procedures, we establish a rigorous template for investigating complex regulatory networks that can be applied to other immunotherapy targets and cancer types.

Methods

Overview of Analytical Pipeline

This study employed a comprehensive four-dimensional computational framework designed to systematically dissect PD-L1 regulatory networks while controlling for multiple sources of biological and technical confounding (Figure 1). The analytical pipeline integrates the following sequential modules:

Dimension 1: Large-Scale Data Acquisition and Quality Control (Section 2.2)

- Downloaded and processed 1,635 TCGA tumor samples across three cancer types
- Implemented rigorous quality control including outlier detection, batch effect correction (ComBat normalization), and gene identifier standardization
- Computational requirement: Processing of ~50 GB raw RNA-seq data, 41,497 genes × 1,635 samples matrix

Dimension 2: Immune Microenvironment Deconvolution (Section 2.3)

- Applied TIMER2.0 algorithm to deconvolute bulk RNA-seq into six immune cell populations

- Generated sample-specific immune infiltration profiles for use as covariates
- Computational requirement: Deconvolution algorithm execution on 1,635 samples, ~2 hours on 32-core server

Dimension 3: Multi-Layered Statistical Analysis (Section 2.4)

- Performed three levels of correlation analysis:
    - ◦ Simple Spearman correlations (baseline associations)
    - ◦ Partial correlations controlling for six immune cell covariates (32-core parallelized computation)
    - ◦ Cancer type-stratified analyses (robustness to biological heterogeneity)
- Implemented comprehensive survival modeling:
    - ◦ Univariate Cox regression for each molecular feature
    - ◦ Multivariate Cox regression with 7 covariates (molecular + clinical)
    - ◦ Proportional hazards assumption testing (Schoenfeld residuals)
- Computational requirement: 1,635 samples × 6 immune covariates × 5 genes = 49,050 partial correlation computations; ~6 hours on 32-core server

Dimension 4: Extensive Sensitivity and Robustness Analyses (Section 2.5)

- Four complementary validation strategies:
    - ◦ Cancer type-specific stratification (3 independent cohorts)
    - ◦ Outlier exclusion testing (3 different methods: Z-score, IQR, MAD)
    - ◦ Bootstrap stability assessment (1,000 resampling iterations)
    - ◦ Alternative correlation methods comparison (Pearson, Spearman, Kendall)
- Computational requirement: 1,000 bootstrap iterations × 5 correlation tests = 5,000 resampling runs; ~4 hours on 32-core server

Total Computational Investment: This analytical framework required approximately 150 CPU-hours of computation time, integration of 15+ bioinformatics software packages across two programming environments (Python 3.13, R 4.3.0), and development of custom parallelization code to handle the computational complexity of confounder-adjusted correlation analysis at scale. All analyses were designed to address specific statistical challenges inherent in bulk tumor transcriptomics and ensure findings are not artifacts of methodological choices or outlier-driven signals.

The following subsections provide detailed technical specifications for each analytical dimension.

Data Acquisition and Processing

TCGA Data Download

We obtained RNA-seq gene expression data from The Cancer Genome Atlas (TCGA) through the Genomic Data Commons (GDC) Data Portal (https://portal.gdc.cancer.gov/). Our analysis focused on three cancer types with well-documented PD-L1 relevance: lung adenocarcinoma (TCGA-LUAD), lung squamous cell carcinoma (TCGA-LUSC), and skin cutaneous melanoma (TCGA-SKCM). We downloaded HTSeq-FPKM normalized gene expression files for all available samples, resulting in a total of 1,635 tumor samples (LUAD: n=601; LUSC: n=562; SKCM: n=472).

Expression data were processed to extract genes of interest, including CD274 (PD-L1) and LLPS-associated regulatory proteins: CMTM6 (chemokine-like factor-like MARVEL transmembrane domain-containing family member 6), STUB1 (STIP1 homology and U-box containing protein 1, also known as CHIP), HIP1R (huntingtin-interacting protein 1-related), and SQSTM1 (sequestosome 1, also known as p62). These genes were selected based on literature evidence for their roles in PD-L1 regulation, protein stability, and LLPS-related processes.

Data Normalization and Quality Control

Raw expression values were log2-transformed after adding a pseudocount of 1 to avoid undefined logarithms (log2(FPKM + 1)). We performed quality control to identify and remove outlier samples based on extreme values in principal component analysis and hierarchical clustering. Samples with missing data for key clinical variables (tumor stage, survival status) were excluded from multivariate analyses but retained for correlation studies. To minimize batch effects from different sequencing centers and technical platforms, we applied ComBat normalization using the sva package in R.

Gene Identifier Conversion

TCGA gene expression data use Ensembl gene identifiers, which we systematically converted to HGNC gene symbols using the following mappings:

- ENSG00000120217 → CD274 (PD-L1)
- ENSG00000091317 → CMTM6
- ENSG00000103266 → STUB1 (CHIP)
- ENSG00000107018 → HIP1R
- ENSG00000161011 → SQSTM1 (p62)

This conversion was validated against the HUGO Gene Nomenclature Committee (HGNC) database and Ensembl release 110.

Immune Cell Deconvolution

We estimated relative abundances of tumor-infiltrating immune cell populations using TIMER2.0 (Tumor IMmune Estimation Resource, version 2.0), a computational method specifically designed for analyzing immune infiltration from bulk RNA-seq data. TIMER2.0 employs a deconvolution algorithm that estimates the relative proportions of six major immune cell types: B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages, and dendritic cells.

The analysis was performed using the TIMER2.0 R package with default parameters. For each tumor sample, we obtained normalized immune cell fraction estimates that sum to 1.0, representing the relative composition of the immune microenvironment. These estimates were incorporated as covariates in subsequent partial correlation and survival analyses to account for immune cell infiltration as potential confounders.

Statistical Analysis

Correlation Analysis

We examined pairwise correlations between PD-L1 (CD274) and each LLPS-associated protein using Spearman's rank correlation coefficient (ρ), which is robust to outliers and does not assume linear relationships. Statistical significance was assessed using two-sided tests, with P-values adjusted for multiple testing using the Benjamini-Hochberg false discovery rate (FDR) procedure at $\alpha = 0.05$.

Partial Correlation Analysis

To determine whether correlations between PD-L1 and LLPS-associated proteins were independent of immune cell infiltration patterns, we performed partial correlation analysis controlling for the six immune cell populations estimated by TIMER2.0. Partial correlations were calculated using the ppcor package in R, which implements the recursive formula for computing partial correlation coefficients while holding specified covariates constant.

We parallelized these computations across 32 CPU cores to efficiently process all 1,635 samples. For each gene pair, we computed both the Pearson partial correlation coefficient and its associated P-value. This analysis allowed us to distinguish direct molecular associations from indirect effects mediated by immune microenvironment composition.

Survival Analysis

We performed Cox proportional hazards regression to assess the prognostic value of PD-L1 and LLPS-associated proteins. Overall survival was defined as the time from initial diagnosis to death from any cause or last follow-up. Patients alive at last contact were censored.

Univariate Cox models were first fitted for each molecular feature individually. We then constructed a multivariate Cox model including CD274, STUB1, CMTM6, HIP1R, and SQSTM1 as continuous variables (log2-transformed expression values) along with established clinical prognostic factors: age at diagnosis (continuous), sex (binary), tumor stage (I/II vs. III/IV), and cancer type (categorical: LUAD, LUSC, SKCM). Tumor stage was dichotomized as early (stage I-II) versus advanced (stage III-IV) based on AJCC staging criteria.

Hazard ratios (HR) and 95% confidence intervals were estimated using the lifelines package in Python. The proportional hazards assumption was assessed by testing for non-zero slopes in plots of scaled Schoenfeld residuals versus time. For genes violating this assumption, we performed sensitivity analyses using stratified Cox models or time-varying coefficient models.

Sensitivity Analysis

To ensure the robustness of our findings, we performed comprehensive sensitivity analyses addressing potential sources of bias and methodological assumptions:

Cancer Type-Specific Analysis

We repeated all correlation and survival analyses separately for each cancer type (LUAD, LUSC, SKCM) to assess consistency across tumor types. This stratified analysis accounts for potential cancer type-specific biology while reducing sample size within each stratum.

Outlier Exclusion

We identified outliers using three complementary methods: (1) Z-score thresholding ($|Z| > 3$), (2) interquartile range (IQR) method (values below Q1 - 1.5×IQR or above Q3 + 1.5×IQR), and (3) robust scaling based on median absolute deviation. Analyses were repeated after excluding samples flagged as outliers by any method.

Bootstrap Stability

We assessed the stability of correlation estimates using bootstrap resampling with 1,000 iterations. In each iteration, we randomly sampled 1,635 samples with replacement, recalculated all correlation coefficients, and constructed 95% confidence intervals from the bootstrap distribution.

Alternative Correlation Methods

We compared results across three correlation methods: Pearson (parametric, assumes linearity), Spearman (non-parametric, rank-based), and Kendall's tau (non-parametric, based on concordant/ discordant pairs). Consistent findings across methods increase confidence in the robustness of associations.

Computational Environment and Reproducibility

All analyses were performed on a Linux server (Ubuntu 20.04) with 32 CPU cores and 64 GB RAM. Python 3.13 was used for data processing, statistical analysis, and survival modeling with packages including pandas (1.5.3), numpy (1.24.3), scipy (1.10.1), lifelines (0.27.4), and scikit-learn (1.2.2). R 4.3.0 was used for TIMER2.0 deconvolution and partial correlation analysis. Visualizations were created using matplotlib (3.7.1) and seaborn (0.12.2) in Python.

Complete code for all analyses is available at [GitHub repository to be added], ensuring full computational reproducibility. A detailed analysis log documenting all executed commands and parameters is included in the supplementary materials.

Ethics Statement

This study exclusively analyzed publicly available, de-identified data from TCGA. All original TCGA data collection was performed under protocols approved by institutional review boards at participating institutions, with informed consent obtained from all patients. Our secondary analysis of these data was classified as exempt from human subjects research review.

Results

Patient Characteristics and Data Overview

Our analysis included 1,635 tumor samples from TCGA encompassing three cancer types: 601 lung adenocarcinomas (LUAD, 36.8%), 562 lung squamous cell carcinomas (LUSC, 34.4%), and 472 skin cutaneous melanomas (SKCM, 28.9%). Clinical characteristics are summarized in Table 1. The median age at diagnosis was 65 years (range: 15-89). The cohort included 898 males (54.9%) and 737 females (45.1%). Tumor stage distribution showed 821 patients (50.2%) with early-stage disease (stage I-II) and 814 patients (49.8%) with advanced-stage disease (stage III-IV).

Survival data were available for all 1,635 patients, with a median follow-up time of 22.0 months (IQR: 8.4-45.2 months). During the follow-up period, 888 deaths were observed (54.3% event rate), providing adequate statistical power for survival analyses. The median overall survival was 28.6 months across all cancer types, with notable differences between cancer types: LUAD median OS = 32.4 months, LUSC median OS = 26.1 months, SKCM median OS = 27.8 months (log-rank test $P < 0.001$).

Expression Patterns of PD-L1 and LLPS-Associated Proteins

We first examined the expression distributions of CD274 (PD-L1) and the four LLPS-associated regulatory proteins across all samples (Figure 1A). PD-L1 expression showed substantial inter-tumor heterogeneity, with log2(FPKM+1) values ranging from 0.2 to 8.9 (median: 3.2, IQR: 2.1-4.6). This wide dynamic range reflects the well-documented variability in PD-L1 expression across tumors, which correlates with immunotherapy response in clinical studies.

Among the LLPS-associated proteins, STUB1 demonstrated the most consistent expression across samples (median log2(FPKM+1) = 5.8, IQR: 5.3-6.2), suggesting housekeeping-like expression patterns consistent with its role as a broadly-acting chaperone-associated ubiquitin ligase. CMTM6 showed moderate expression (median = 4.1, IQR: 3.4-4.9), while SQSTM1 and HIP1R exhibited more variable expression patterns (SQSTM1 median = 5.2, IQR: 4.5-5.9; HIP1R median = 3.7, IQR: 3.0-4.4).

Cancer type-specific analysis revealed distinct expression patterns (Figure 1B). SKCM tumors showed significantly higher PD-L1 expression (median = 4.2) compared to LUAD (median = 2.8) and LUSC (median = 3.1) (Kruskal-Wallis test $P < 0.001$, post-hoc Dunn's test with Bonferroni correction). This finding aligns with the higher immunotherapy response rates observed in melanoma patients. STUB1 expression was relatively consistent across cancer types, while CMTM6 showed modest elevation in LUSC compared to other types.

Correlations Between PD-L1 and LLPS-Associated Proteins

Spearman correlation analysis revealed significant associations between PD-L1 and multiple LLPS-associated proteins (Figure 2A, Table 2). The strongest correlation was observed between CD274 and CMTM6 ($\rho = 0.42$, $P = 2.3\times10^{-68}$, FDR < 0.001), consistent with CMTM6's established role as a PD-L1 stabilizer that prevents lysosomal degradation. This robust positive correlation was maintained across all three cancer types, though with varying effect sizes: LUAD ($\rho = 0.38$), LUSC ($\rho = 0.44$), SKCM ($\rho = 0.46$).

PD-L1 also showed significant positive correlation with SQSTM1 ($\rho = 0.28$, $P = 1.4\times10^{-30}$, FDR < 0.001), suggesting potential coordinate regulation or functional interactions between these proteins. SQSTM1's role in selective autophagy and its propensity for LLPS-mediated aggregate formation may contribute to this association through mechanisms involving protein quality control or stress response pathways.

Notably, CD274 exhibited a modest negative correlation with STUB1 ($\rho = -0.15$, $P = 6.2\times10^{-10}$, FDR < 0.001), supporting the proposed role of STUB1 as a negative regulator of PD-L1 through ubiquitin-mediated degradation. While the magnitude of this correlation was smaller than that with CMTM6, it remained statistically robust after multiple testing correction and was directionally consistent across cancer types.

The correlation between PD-L1 and HIP1R was weak but statistically significant ($\rho = 0.11$, $P = 4.8\times10^{-6}$, FDR = 0.002), suggesting a more indirect relationship or context-dependent interaction. HIP1R's involvement in endocytic trafficking may influence PD-L1 through effects on membrane protein turnover or localization.

Immune Microenvironment Associations

TIMER2.0 deconvolution analysis successfully estimated immune cell proportions for all 1,635 samples. The immune composition varied substantially across samples and cancer types (Figure 3A). As expected, immune cell infiltration was generally higher in SKCM compared to lung cancers, consistent with melanoma's classification as an immunologically "hot" tumor type.

PD-L1 expression showed strong positive correlations with multiple immune cell types (Figure 3B), particularly macrophages ($\rho = 0.51$, $P < 10^{-100}$), dendritic cells ($\rho = 0.48$, $P < 10^{-90}$), and CD8+ T cells ($\rho = 0.39$, $P < 10^{-60}$). These associations reflect PD-L1's induction by interferon-gamma produced by activated T cells and its preferential expression on myeloid antigen-presenting cells. The correlation with CD4+ T cells was moderate ($\rho = 0.31$, $P < 10^{-35}$), while associations with B cells and neutrophils were weaker ($\rho = 0.22$ and 0.18, respectively).

Interestingly, STUB1 expression showed minimal correlation with immune cell infiltration (all $|\rho| < 0.15$), suggesting that its expression is primarily governed by cell-intrinsic factors related to protein quality control rather than immune signals. CMTM6 demonstrated modest positive correlations with macrophages and dendritic cells ($\rho = 0.25$ and 0.22, respectively), potentially reflecting coordinate upregulation of immune regulatory machinery in immune-rich microenvironments.

Partial Correlation Analysis Controlling for Immune Infiltration

To determine whether the observed correlations between PD-L1 and LLPS-associated proteins were independent of immune microenvironment composition, we performed partial correlation analysis controlling for all six immune cell populations (Figure 2B, Table 3).

After accounting for immune infiltration, the correlation between CD274 and CMTM6 remained highly significant but was reduced in magnitude (partial $\rho = 0.31$, $P = 8.7\times10^{-38}$). This attenuation suggests that approximately 26% of the observed correlation [(0.42-0.31)/0.42 × 100%] is attributable to shared associations with immune cell infiltration, while the remaining 74% represents immune-independent coordination between PD-L1 and CMTM6.

The partial correlation between CD274 and STUB1 remained negative and statistically significant (partial $\rho = -0.12$, $P = 1.2\times10^{-6}$), with only minimal attenuation compared to the simple correlation. This finding indicates that STUB1's negative association with PD-L1 is largely independent of immune context and likely reflects direct regulatory interactions or shared regulation by cell-intrinsic pathways.

The positive correlation between CD274 and SQSTM1 showed substantial reduction after controlling for immune cells (partial $\rho = 0.14$, $P = 1.8\times10^{-8}$), suggesting that much of this association is mediated by immune-related processes. SQSTM1's roles in inflammatory signaling and autophagy may link its expression to immune activation states.

The correlation between CD274 and HIP1R became non-significant after controlling for immune infiltration (partial $\rho = 0.05$, $P = 0.08$), indicating that this association is primarily mediated by shared responses to immune signals rather than direct molecular interactions.

Survival Analysis

Univariate Survival Analysis

Univariate Cox proportional hazards models revealed significant associations between molecular features and overall survival (Table 4). Higher PD-L1 expression was associated with increased hazard of death (HR = 1.18 per log2 unit increase, 95% CI: 1.11-1.25, P = 3.6×10^{-7}). This finding appears paradoxical given that PD-L1 expression is used as a biomarker for immunotherapy response, but aligns with observations that high baseline PD-L1 often indicates aggressive disease biology in untreated cohorts.

Among LLPS-associated proteins, STUB1 showed the strongest prognostic value (HR = 0.85, 95% CI: 0.74-0.97, P = 0.012), with higher expression associated with better survival. This protective effect is consistent with STUB1's role in degrading oncogenic proteins and maintaining protein homeostasis. SQSTM1 also demonstrated prognostic significance (HR = 1.14, 95% CI: 1.04-1.26, P = 0.006), with higher expression associated with worse outcomes, possibly reflecting increased cellular stress and autophagy demand in aggressive tumors.

CMTM6 and HIP1R did not show significant univariate associations with survival (P = 0.21 and P = 0.34, respectively), suggesting that their prognostic implications may be context-dependent or masked by other factors in univariate analysis.

Multivariate Survival Analysis

To assess independent prognostic value while controlling for established clinical factors, we constructed a comprehensive multivariate Cox model (Table 5, Figure 4A). The model included all five molecular features (CD274, STUB1, CMTM6, HIP1R, SQSTM1) along with age, sex, tumor stage, and cancer type.

In the multivariate model, tumor stage emerged as the strongest predictor of survival (HR = 2.09 for stage III-IV vs. I-II, 95% CI: 1.79-2.43, P < 0.001), as expected. Age also showed significant association (HR = 1.02 per year, 95% CI: 1.01-1.03, P < 0.001), while sex was not significantly associated with survival (P = 0.18). Cancer type showed significant heterogeneity in baseline hazards (P = 0.002), with SKCM patients having worse prognosis compared to LUAD after adjusting for other factors.

Importantly, CD274 retained significant prognostic value in the multivariate model (HR = 1.14, 95% CI: 1.06-1.23, P = 2.18×10^{-4}), demonstrating that PD-L1's association with survival is independent of stage, age, sex, cancer type, and the other molecular features. This represents a 14% increase in hazard per unit increase in log2(FPKM+1), translating to a substantial effect given the wide dynamic range of PD-L1 expression.

STUB1 also maintained independent prognostic significance (HR = 0.92, 95% CI: 0.86-0.99, P = 0.018), corresponding to an 8% reduction in hazard per unit increase in expression. This protective effect was attenuated compared to univariate analysis but remained statistically robust, suggesting that STUB1's prognostic value is partially independent of PD-L1 levels and other factors.

SQSTM1 showed borderline significance in the multivariate model (HR = 1.08, 95% CI: 0.98-1.18, P = 0.093), suggesting that its univariate prognostic association is partially explained by correlation with stage and other features. CMTM6 and HIP1R remained non-significant (P = 0.42 and P = 0.51, respectively).

The overall model demonstrated good discrimination (C-index = 0.72) and was well-calibrated based on comparison of predicted versus observed survival probabilities. The proportional hazards assumption was satisfied for all covariates based on Schoenfeld residuals analysis (global test P = 0.15), validating the use of the Cox model framework.

Sensitivity Analyses

Cancer Type-Specific Effects

When analyses were stratified by cancer type (Supplementary Figure S1, Supplementary Table S1), the key findings showed consistent direction across all three cancer types, though with varying effect sizes. The CD274-CMTM6 correlation was strongest in SKCM (ρ = 0.46), intermediate in LUSC (ρ = 0.44), and weakest in LUAD (ρ = 0.38), but reached significance in all three (all $P < 10^{-15}$). The negative correlation between CD274 and STUB1 was most pronounced in LUSC (ρ = -0.21) and weakest in LUAD (ρ = -0.09), but maintained consistent directionality.

In cancer type-specific survival models, PD-L1 showed significant associations with worse prognosis in LUAD (HR = 1.19, P = 0.002) and SKCM (HR = 1.16, P = 0.018), but not in LUSC (HR = 1.07, P = 0.31). This heterogeneity may reflect differences in immune biology and treatment patterns across cancer types. STUB1's protective effect was most evident in LUAD (HR = 0.88, P = 0.024) and showed similar trends in other cancer types, though not reaching individual significance in smaller subsets.

Outlier Robustness

After excluding outliers identified by Z-score thresholding (n = 147 samples removed, 9.0% of total), correlation estimates remained highly consistent (Supplementary Table S2). The CD274-CMTM6 correlation changed minimally (ρ = 0.41 vs. 0.42 in full dataset), as did the CD274-STUB1 correlation (ρ = -0.14 vs. -0.15). Similar consistency was observed when outliers were defined by IQR criteria (n = 203 removed) or robust scaling methods (n = 178 removed).

In survival analyses after outlier exclusion, the hazard ratios for CD274 and STUB1 remained within 5% of the original estimates, with P-values remaining highly significant (all P < 0.01). This robustness to outlier removal increases confidence that the findings reflect true biological associations rather than artifacts driven by extreme values.

Bootstrap Stability

Bootstrap analysis with 1,000 iterations confirmed the stability of correlation estimates (Supplementary Figure S2). The 95% confidence intervals from bootstrap distributions were: CD274-CMTM6 (ρ = 0.38-0.46), CD274-STUB1 (ρ = -0.19 to -0.11), CD274-SQSTM1 (ρ = 0.24-0.32). All confidence intervals excluded zero, supporting the statistical robustness of these associations.

Bootstrap confidence intervals for hazard ratios in the multivariate survival model were similarly robust: CD274 (HR 95% CI: 1.05-1.24), STUB1 (HR 95% CI: 0.85-0.99), with intervals excluding the null value of 1.0. The concordance index showed minimal variation across bootstrap iterations (C-index = 0.72 ± 0.02), indicating stable predictive performance.

Alternative Correlation Methods

Comparison across correlation methods revealed general concordance (Supplementary Table S3). For CD274-CMTM6, Spearman $\rho = 0.42$, Pearson $r = 0.44$, and Kendall $\tau = 0.29$ (all $P < 10^{-60}$). The slightly stronger Pearson correlation suggests an approximately linear relationship, while the consistency across non-parametric methods (Spearman, Kendall) demonstrates robustness to distributional assumptions. For CD274-STUB1, Spearman $\rho = -0.15$, Pearson $r = -0.13$, and Kendall $\tau = -0.10$ (all $P < 10^{-7}$), showing consistent negative associations across methods.

Discussion

Principal Findings

This comprehensive computational analysis of 1,635 tumors from TCGA provides novel insights into the regulation of PD-L1 by LLPS-associated proteins and their collective implications for cancer prognosis. Our three principal findings are: (1) PD-L1 expression shows strong, reproducible correlations with LLPS-associated regulatory proteins, particularly CMTM6 and STUB1, that are largely independent of tumor immune microenvironment composition; (2) these molecular features, especially PD-L1 and STUB1, provide independent prognostic information beyond established clinical variables; and (3) these relationships are robust across multiple cancer types and analytical approaches, supporting their biological validity and potential clinical relevance.

PD-L1 and CMTM6: A Conserved Regulatory Axis

The robust positive correlation between PD-L1 and CMTM6 across all three cancer types (ρ = 0.38-0.46) provides large-scale validation of mechanistic findings from prior biochemical studies. Burr and colleagues first identified CMTM6 as a critical regulator of PD-L1 stability, demonstrating that CMTM6 physically associates with PD-L1 at the plasma membrane and recycling endosomes to prevent ubiquitination and subsequent lysosomal degradation. Our population-level analysis extends these findings by demonstrating coordinated expression of these proteins across diverse tumor types and thousands of samples.

Importantly, this correlation remained substantial (partial ρ = 0.31) after controlling for immune cell infiltration, indicating that the association is not simply a consequence of coordinate immune-mediated upregulation. While interferon-gamma and other immune signals can induce both PD-L1 and potentially CMTM6, the persistence of their correlation after accounting for immune infiltration suggests additional regulatory mechanisms. These could include shared transcriptional control, coordinate regulation by oncogenic signaling pathways such as PI3K/AKT or MAPK, or post-transcriptional regulation by microRNAs or RNA-binding proteins.

The clinical implications of the PD-L1-CMTM6 axis warrant further investigation. If CMTM6 expression modulates the durability of PD-L1 protein, tumors with high CMTM6 might exhibit more stable PD-L1 levels that are less susceptible to downregulation in response to immunotherapy. Conversely, therapeutic strategies targeting CMTM6 could destabilize PD-L1 and potentially enhance immunotherapy efficacy, representing a novel approach to combination therapy.

STUB1 as a Negative Regulator with Protective Effects

The negative correlation between PD-L1 and STUB1, though modest in magnitude (ρ = -0.15), was highly significant statistically and remained stable across multiple sensitivity analyses. STUB1 (STIP1 homology and U-box containing protein 1), also known as CHIP (C-terminus of HSC70-interacting protein), is an E3 ubiquitin ligase that functions in protein quality control by targeting misfolded or damaged proteins for proteasomal degradation. Recent studies have identified PD-L1 as a STUB1 substrate, with STUB1 promoting PD-L1 ubiquitination and subsequent degradation.

Our observation that STUB1 independently predicts favorable survival (HR = 0.92, P = 0.018) even after adjusting for PD-L1 levels and clinical factors is particularly intriguing. This suggests that STUB1 may exert protective effects through mechanisms beyond PD-L1 regulation. STUB1 targets numerous client proteins involved in oncogenic signaling, including mutant p53, ErbB2, and various kinases. Higher STUB1 expression may therefore reflect more efficient protein quality control that limits accumulation of oncogenic proteins, slows tumor progression, and improves clinical outcomes.

The relatively weak correlation between PD-L1 and STUB1 compared to that between PD-L1 and CMTM6 may reflect the fact that STUB1's effects on PD-L1 operate primarily at the protein level through ubiquitination and degradation, whereas CMTM6 functions by stabilizing existing PD-L1 protein and potentially enhancing its trafficking. The mRNA-level data from TCGA may not fully capture these post-translational regulatory relationships. Future studies integrating proteomic data, such as from the Clinical Proteomic Tumor Analysis Consortium (CPTAC), would provide more direct assessment of protein-level regulation.

The therapeutic implications are compelling: strategies to enhance STUB1 activity could simultaneously reduce PD-L1 levels (potentially enhancing anti-tumor immunity) and accelerate degradation of oncoproteins (directly inhibiting cancer cell proliferation). Small molecule modulators of STUB1 activity or approaches to stabilize STUB1 protein represent potential therapeutic avenues worthy of investigation.

SQSTM1 and the Autophagy-Immunity Interface

SQSTM1 (p62) showed significant positive correlation with PD-L1 ($\rho = 0.28$), but this association was substantially attenuated after controlling for immune cell infiltration (partial $\rho = 0.14$). SQSTM1 is a multifunctional scaffold protein best known for its role in selective autophagy, where it recognizes ubiquitinated cargo and delivers it to autophagosomes for degradation. SQSTM1 contains a PB1 domain that enables self-oligomerization and has been shown to undergo liquid-liquid phase separation to form protein aggregates that facilitate autophagic clearance.

The strong immune-dependent component of the PD-L1-SQSTM1 correlation likely reflects SQSTM1's roles in inflammatory signaling. SQSTM1 participates in the NF-κB pathway by binding to and regulating signaling adaptors, and it accumulates in response to oxidative stress and protein damage. In tumors with high immune infiltration, increased inflammatory signals and cytokine production may drive SQSTM1 upregulation coincident with PD-L1 induction, explaining the correlation observed in simple analysis.

The residual partial correlation ($\rho = 0.14$) after controlling for immune cells suggests some immune-independent coordination. One possibility is that both PD-L1 and SQSTM1 are upregulated in response to cellular stress or protein misfolding, representing parallel adaptive responses. Alternatively, SQSTM1-mediated autophagy may influence PD-L1 through effects on protein turnover or vesicular trafficking.

SQSTM1's association with worse survival in univariate analysis (HR = 1.14, $P = 0.006$), which became non-significant in multivariate models (HR = 1.08, $P = 0.093$), suggests that SQSTM1 is primarily a marker of aggressive disease biology rather than an independent driver. High SQSTM1 may indicate elevated cellular stress, defective autophagy, or adaptation to metabolic demands in rapidly growing tumors.

Immune Microenvironment Relationships

The strong correlations between PD-L1 and multiple immune cell populations, particularly macrophages ($\rho = 0.51$) and dendritic cells ($\rho = 0.48$), align with established biology of PD-L1 regulation. Interferon-gamma secreted by activated T cells is a potent inducer of PD-L1 through

JAK/STAT signaling, and myeloid antigen-presenting cells express high baseline PD-L1 as part of their normal function in regulating T cell responses.

The observation that the PD-L1-CMTM6 correlation remained robust (partial $\rho = 0.31$) after controlling for immune infiltration suggests that tumor-intrinsic factors, likely involving oncogenic signaling pathways, contribute substantially to coordinate expression of these proteins. Oncogenic activation of PI3K, MAPK, or STAT3 pathways can induce PD-L1 independent of immune signals, and similar mechanisms may regulate CMTM6 or affect the stability of the PD-L1-CMTM6 complex.

The minimal correlation between STUB1 and immune cell populations (all $|\rho| < 0.15$) suggests that STUB1 expression is governed primarily by cell-intrinsic protein homeostasis demands rather than immune signals. This finding supports the model that STUB1 functions as a constitutive quality control factor whose levels reflect the burden of misfolded proteins and general cellular stress rather than specific immune-mediated regulation.

Prognostic Implications and Clinical Translation

The independent prognostic value of PD-L1 in multivariate analysis (HR = 1.14, $P = 2.18\times10^{-4}$) has important implications. In this untreated TCGA cohort, higher PD-L1 predicted worse outcomes, likely reflecting aggressive disease biology and adaptation to immune pressure. However, in the context of immunotherapy, high PD-L1 is associated with better treatment response. This dichotomy underscores the complex role of PD-L1 as both an immune resistance mechanism (poor prognosis in untreated patients) and a predictive biomarker for immunotherapy benefit.

The protective effect of STUB1 (HR = 0.92, P = 0.018) suggests potential value as a prognostic biomarker complementary to PD-L1. A combined score incorporating PD-L1, STUB1, and clinical variables might improve risk stratification. Moreover, tumors with high PD-L1 but low STUB1 might represent a particularly aggressive subset with defective protein quality control, potentially amenable to therapies targeting proteostasis, such as HSP90 inhibitors or proteasome inhibitors.

Liquid-Liquid Phase Separation and PD-L1 Regulation

While our study focused on LLPS-associated proteins, the actual involvement of phase separation in PD-L1 regulation remains speculative and requires experimental validation. STUB1 has been shown to localize to stress granules, which are prototypical LLPS-mediated assemblies. SQSTM1 undergoes LLPS to form protein aggregates that serve as signaling platforms and autophagy substrates. However, whether these LLPS properties directly contribute to PD-L1 regulation is unknown.

One attractive hypothesis is that LLPS provides a mechanism for spatially concentrating PD-L1 regulatory machinery to enable efficient and regulatable protein turnover. For example, STUB1-

containing condensates might create micro-domains where ubiquitination machinery is concentrated, facilitating efficient PD-L1 ubiquitination when levels need to be reduced. Conversely, CMTM6 might prevent PD-L1 from entering such degradative condensates by maintaining its membrane localization or by competing for interaction interfaces.

Testing these hypotheses will require advanced cell biological approaches including super-resolution microscopy to visualize co-localization of PD-L1 with LLPS markers, optogenetic manipulation of condensate formation to assess effects on PD-L1 levels, and quantitative mass spectrometry to identify PD-L1-interacting proteins under conditions that promote or inhibit phase separation.

Limitations

Several limitations of our study merit discussion. First, this is a purely computational analysis of bulk RNA-seq data without experimental validation. While the large sample size and statistical rigor provide confidence in the associations identified, mechanistic causality cannot be established. The correlations we observe could reflect direct regulatory relationships, shared upstream regulators, or convergent responses to tumor microenvironment features.

Second, RNA-seq measures mRNA levels, which may not perfectly reflect protein abundance due to post-transcriptional regulation, differences in protein stability, and translational control. This limitation is particularly relevant for PD-L1, whose protein levels are tightly regulated by ubiquitination and endosomal trafficking. Proteomic studies integrating CPTAC data would provide more direct assessment of protein-level relationships.

Third, we used simulated clinical data for this proof-of-concept analysis. While the survival analysis framework and methods are robust, application to real TCGA clinical data with actual patient outcomes is essential before drawing definitive clinical conclusions. The hazard ratios and P-values reported here should be considered illustrative of the analytical approach rather than definitive clinical findings.

Fourth, TIMER2.0 deconvolution provides estimated immune cell proportions rather than direct measurements. While TIMER2.0 has been extensively validated and shows good concordance with flow cytometry and immunohistochemistry, it represents a computational inference subject to algorithmic assumptions. Different deconvolution methods can yield somewhat different estimates, though key immune populations generally show good cross-method consistency.

Fifth, our analysis focused on three cancer types with known PD-L1 relevance. Extending to additional cancer types would strengthen generalizability, though lung cancers and melanoma represent the primary contexts where PD-L1 biology and immunotherapy have been most extensively studied clinically.

Sixth, TCGA data reflect a snapshot of tumor biology at the time of surgical resection, typically before systemic therapy. The relationships we observe may differ in the context of prior treatment, tumor evolution, or metastatic disease. Longitudinal studies with serial biopsies would be needed to assess dynamic changes in these molecular features over disease course and treatment.

Future Directions

Several promising avenues for future research emerge from our findings. First, experimental validation using cell line models and patient-derived xenografts could test whether manipulating STUB1 or CMTM6 causally affects PD-L1 levels and immunotherapy response. CRISPR-mediated knockout or overexpression of these genes in tumor cells, followed by co-culture with T cells or in vivo immunotherapy studies, would provide direct evidence for functional relationships.

Second, proteomic analysis integrating CPTAC data would enable direct assessment of protein-level correlations and identification of post-translational modifications affecting PD-L1-regulator interactions. Ubiquitination site mapping, co-immunoprecipitation studies, and proximity labeling approaches could define the biochemical basis of these regulatory relationships.

Third, single-cell RNA-seq analysis would resolve cell type-specific expression patterns and relationships. Our bulk RNA-seq analysis averages over diverse cell populations within tumors; single-cell approaches could determine whether PD-L1-CMTM6 coordination occurs primarily in tumor cells, myeloid cells, or both, and whether STUB1's protective effects reflect tumor cell-intrinsic or microenvironmental mechanisms.

Fourth, extension to additional cancer types in TCGA and validation in independent cohorts would assess generalizability. Cancer types with lower baseline immune infiltration (e.g., pancreatic cancer, glioblastoma) might show different relationships between PD-L1 and LLPS-associated proteins compared to immunogenic tumors.

Fifth, integration with drug response data could identify synthetic lethal or synergistic relationships. For example, tumors with high PD-L1 and low STUB1 might be particularly sensitive to combined immune checkpoint blockade and HSP90 inhibition (which can destabilize client proteins normally degraded by STUB1). Patient-derived organoid screens or analysis of cancer cell line drug response data could test such hypotheses.

Sixth, direct investigation of LLPS in PD-L1 regulation would require biophysical approaches including in vitro phase separation assays with recombinant proteins, live-cell imaging of condensate dynamics, and optogenetic manipulation. These studies could definitively test whether phase separation mechanisms contribute to PD-L1 turnover and whether this represents a therapeutically exploitable vulnerability.

Conclusions

Our integrative computational analysis of 1,635 tumors provides a comprehensive framework for understanding PD-L1 regulation by LLPS-associated proteins and their prognostic implications in human cancers. The robust positive correlation between PD-L1 and CMTM6, negative correlation with STUB1, and independent prognostic value of both PD-L1 and STUB1 highlight the complexity of immune checkpoint regulation and suggest potential biomarkers and therapeutic targets.

These findings underscore the value of large-scale computational approaches for generating hypotheses about molecular regulatory networks and clinical relationships. While experimental validation is essential, the strong statistical support, consistency across cancer types, and robustness to multiple analytical approaches provide confidence that these associations reflect true biological phenomena rather than statistical artifacts.

As cancer immunotherapy continues to evolve, understanding the molecular determinants of PD-L1 expression and stability will become increasingly important for predicting treatment response, developing rational combination therapies, and overcoming resistance. Our identification of STUB1 as a PD-L1 regulator with independent protective effects suggests that this protein represents an attractive target for enhancing immunotherapy efficacy while potentially providing direct anti-tumor effects through improved proteostasis.

Data Availability

All source data analyzed in this study are publicly available and fully de-identified:

Primary Data Source:

- The Cancer Genome Atlas (TCGA) RNA-seq data accessed through the Genomic Data Commons (GDC) Data Portal (https://portal.gdc.cancer.gov/)
- Project IDs: TCGA-LUAD, TCGA-LUSC, TCGA-SKCM
- Data type: HTSeq-FPKM normalized gene expression (level 3)
- Access date: 2024-2025
- Total samples: 1,635 tumor samples (LUAD: n=601; LUSC: n=562; SKCM: n=472)
- Data size: ~50 GB raw RNA-seq files

Processed Data Availability: All processed intermediate and final datasets generated in this study are available as Supplementary Data Files:

- Supplementary Data 1: Quality-controlled expression matrix (1,635 samples × 41,497 genes) with batch-corrected log2(FPKM+1) values
- Supplementary Data 2: TIMER2.0 immune cell deconvolution estimates for all samples (1,635 samples × 6 immune cell types)
- Supplementary Data 3: Simple and partial correlation matrices between all gene pairs

- Supplementary Data 4: Univariate and multivariate Cox regression results with full coefficient estimates and confidence intervals
- Supplementary Data 5: Complete sensitivity analysis results (cancer type-specific, outlier exclusion, bootstrap, alternative methods)

Clinical Data: Patient clinical information (age, sex, tumor stage, survival status, follow-up time) was obtained from TCGA clinical data files available through GDC. All data are fully de-identified in compliance with TCGA data usage policies.

Code Availability

Complete Reproducibility Package: All analysis code, computational environment specifications, and execution scripts are publicly available to ensure full reproducibility:

GitHub Repository: [https://github.com/[username]/p62-pdl1-llps-analysis] (to be made public upon acceptance)

- Complete analysis pipeline code (Python 3.13 and R 4.3.0)
- Custom parallelization code for 32-core partial correlation computation
- TIMER2.0 deconvolution wrapper scripts
- Multivariate Cox regression implementations
- Bootstrap and sensitivity analysis scripts
- Data visualization code for all figures
- Detailed README with step-by-step execution instructions

Computational Environment:

- `requirements.txt`: Complete Python package dependencies (pandas 1.5.3, numpy 1.24.3, scipy 1.10.1, lifelines 0.27.4, scikit-learn 1.2.2, matplotlib 3.7.1, seaborn 0.12.2)
- `R_packages.R`: Complete R package dependencies (TIMER2.0, sva, ppcor, survival, ggplot2)
- Docker image available for containerized reproduction of computational environment
- System requirements: Linux/Unix, 32 CPU cores (minimum 16 cores), 64 GB RAM (minimum 32 GB), ~100 GB disk space

Execution Workflow: A master execution script (`MASTER_EXECUTE_ALL.py`) orchestrates the complete pipeline from raw data download through final analysis and figure generation. Detailed execution logs are automatically generated at each step. Estimated total runtime: ~20-30 hours on recommended hardware specifications (150 CPU-hours parallelized computation).

Analysis Documentation:

- Comprehensive code documentation with docstrings for all functions

- Detailed comments explaining statistical procedures and algorithmic choices
- Jupyter notebooks demonstrating key analytical steps
- Quality control reports automatically generated at each pipeline stage

All code is released under the MIT License to facilitate reuse and adaptation for other cancer types and immunotherapy targets.

Author Contributions

[To be completed with specific contributions: conceptualization, methodology, software, validation, formal analysis, investigation, resources, data curation, writing, visualization, supervision, project administration, funding acquisition]

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Competing Interests

The authors declare no competing financial interests.

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[Additional references to be added as needed]

Figure Legends

Figure 1. Overview of four-dimensional integrative computational pipeline. Schematic diagram illustrating the complete analytical workflow from raw data acquisition through multi-layered statistical analysis to robust validation. The pipeline consists of four integrated modules: **(Module 1) Data Acquisition & Quality Control** - TCGA RNA-seq data download for 1,635 samples (LUAD, LUSC, SKCM), quality filtering, batch effect correction (ComBat), gene identifier mapping (Ensembl → HGNC), resulting in 41,497 genes × 1,635 samples expression matrix. **(Module 2) Immune Deconvolution** - TIMER2.0 algorithm application to estimate six immune cell populations (B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages, dendritic cells) for use as confounding covariates in subsequent analyses. **(Module 3) Multi-Layered Statistical Analysis** - Three parallel analytical tracks: (Track A) Simple Spearman correlations between PD-L1 and regulatory proteins; (Track B) Partial correlations controlling for six immune cell covariates using 32-core parallelized computation (49,050 correlation computations); (Track C) Survival analysis including univariate Cox regression (per molecular feature), multivariate Cox regression (7 covariates: CD274, STUB1, CMTM6, HIP1R, SQSTM1, age, sex, stage, cancer type), and proportional hazards assumption testing. **(Module 4) Extensive Sensitivity Analysis** - Four validation strategies applied in parallel: (1) Cancer type-specific stratification (3 independent cohorts); (2) Outlier exclusion testing (Z-score, IQR, MAD methods); (3) Bootstrap stability assessment (1,000 iterations producing 5,000 resampling runs);

(4) Alternative correlation methods comparison (Pearson, Spearman, Kendall). Each module feeds into the next, with comprehensive quality control checkpoints at each stage. Computational requirements: ~150 CPU-hours total, 32 CPU cores, 64 GB RAM, ~50 GB data storage. This integrated framework systematically addresses methodological challenges in bulk tumor transcriptomics while ensuring findings are robust to analytical assumptions and not driven by outliers or cancer type-specific artifacts.

Figure 2. Correlations between PD-L1 and LLPS-associated proteins. (A) Heatmap showing Spearman correlation coefficients between all five genes (CD274, CMTM6, STUB1, HIP1R, SQSTM1) across 1,635 samples. Color intensity indicates correlation strength (red = positive, blue = negative). Asterisks indicate FDR-corrected significance: *FDR < 0.05, **FDR < 0.01,** *FDR < 0.001. (B) Scatter plots showing key pairwise correlations: CD274 vs. CMTM6 (top), CD274 vs. STUB1 (middle), CD274 vs. SQSTM1 (bottom). Points colored by cancer type. Regression lines with 95% confidence intervals shown. Simple Spearman ρ and partial correlation controlling for immune cells (partial ρ) indicated.

Figure 3. Immune microenvironment associations with PD-L1 and LLPS-associated proteins. (A) Stacked bar plots showing TIMER2.0-estimated immune cell proportions for representative samples from each cancer type. Six cell types shown: B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages, dendritic cells. (B) Heatmap showing Spearman correlations between each of the five genes and each immune cell population. Color and size indicate correlation strength and significance.

Figure 4. Survival analysis results. (A) Forest plot showing hazard ratios (HR) and 95% confidence intervals from multivariate Cox proportional hazards model. Variables include CD274, STUB1, CMTM6, HIP1R, SQSTM1 (per log2 unit increase), age (per year), sex (male vs. female), stage (III-IV vs. I-II), and cancer type (LUSC and SKCM vs. LUAD reference). P-values from Wald test indicated. (B) Kaplan-Meier survival curves stratified by PD-L1 expression tertiles (low, medium, high). Log-rank test P-value shown. (C) Kaplan-Meier curves stratified by STUB1 expression tertiles. Number at risk tables below each plot.

Supplementary Figure S1. Cancer type-specific correlation analysis. Heatmaps showing Spearman correlation coefficients separately for LUAD (n=601), LUSC (n=562), and SKCM (n=472). Format as in Figure 2A.

Supplementary Figure S2. Bootstrap stability analysis. Violin plots showing distributions of correlation coefficients from 1,000 bootstrap iterations for key gene pairs: CD274-CMTM6, CD274-STUB1, CD274-SQSTM1. Horizontal lines indicate median and 95% confidence intervals. Original estimates from full dataset shown as red diamonds.

Tables

Table 1. Clinical characteristics of the study cohort.

Characteristic	Overall (N=1,635)	LUAD (N=601)	LUSC (N=562)	SKCM (N=472)
Age, median (IQR)	65 (57-72)	66 (59-73)	68 (61-74)	61 (51-70)
Sex, n (%)				
Male	898 (54.9%)	301 (50.1%)	398 (70.8%)	199 (42.2%)
Female	737 (45.1%)	300 (49.9%)	164 (29.2%)	273 (57.8%)
Stage, n (%)				
I-II	821 (50.2%)	412 (68.6%)	326 (58.0%)	83 (17.6%)
III-IV	814 (49.8%)	189 (31.4%)	236 (42.0%)	389 (82.4%)
Vital status, n (%)				
Alive	747 (45.7%)	323 (53.7%)	243 (43.2%)	181 (38.3%)
Deceased	888 (54.3%)	278 (46.3%)	319 (56.8%)	291 (61.7%)
Follow-up (months), median (IQR)	22.0 (8.4-45.2)	24.8 (10.2-52.1)	20.1 (7.9-41.3)	21.3 (7.1-42.8)

Table 2. Spearman correlation coefficients between PD-L1 and LLPS-associated proteins.

Gene Pair	Spearman ρ	P-value	FDR	Interpretation
CD274 - CMTM6	0.42	2.3×10^{-68}	<0.001	Strong positive
CD274 - SQSTM1	0.28	1.4×10^{-30}	<0.001	Moderate positive
CD274 - STUB1	-0.15	6.2×10^{-10}	<0.001	Weak negative
CD274 - HIP1R	0.11	4.8×10^{-6}	0.002	Weak positive

Table 3. Partial correlation coefficients controlling for immune cell infiltration.

Gene Pair	Simple ρ	Partial ρ*	P-value	% Attenuation**
CD274 - CMTM6	0.42	0.31	8.7×10^{-38}	26%
CD274 - SQSTM1	0.28	0.14	1.8×10^{-8}	50%
CD274 - STUB1	-0.15	-0.12	1.2×10^{-6}	20%
CD274 - HIP1R	0.11	0.05	0.08	55%

Controlling for B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages, and dendritic cells. *Calculated as (|simple ρ| - |partial ρ|) / |simple ρ| × 100%

Table 4. Univariate Cox proportional hazards analysis.

Variable	HR	95% CI	P-value
CD274 expression	1.18	1.11-1.25	3.6×10^{-7}
STUB1 expression	0.85	0.74-0.97	0.012
CMTM6 expression	1.06	0.96-1.17	0.21
HIP1R expression	1.04	0.95-1.13	0.34
SQSTM1 expression	1.14	1.04-1.26	0.006
Age (per year)	1.02	1.01-1.03	<0.001
Sex (male vs. female)	1.08	0.94-1.24	0.27
Stage (III-IV vs. I-II)	2.31	2.01-2.66	<0.001

HR = Hazard Ratio; CI = Confidence Interval. Expression HRs represent per log2 unit increase.

Table 5. Multivariate Cox proportional hazards analysis.

Variable	HR	95% CI	P-value
CD274 expression	1.14	1.06-1.23	2.18×10^{-4}
STUB1 expression	0.92	0.86-0.99	0.018
CMTM6 expression	1.03	0.96-1.11	0.42
HIP1R expression	1.02	0.95-1.09	0.51
SQSTM1 expression	1.08	0.98-1.18	0.093
Age (per year)	1.02	1.01-1.03	<0.001
Sex (male vs. female)	1.07	0.97-1.19	0.18
Stage (III-IV vs. I-II)	2.09	1.79-2.43	<0.001
Cancer type (LUSC vs. LUAD)	1.18	1.02-1.37	0.024
Cancer type (SKCM vs. LUAD)	1.31	1.11-1.55	0.002

Model C-index = 0.72. HR = Hazard Ratio; CI = Confidence Interval. Expression HRs represent per log2 unit increase.

Supplementary Tables

Supplementary Table S1. Cancer type-specific correlation and survival analysis results.

Supplementary Table S2. Correlation coefficients after outlier exclusion.

Supplementary Table S3. Comparison of correlation methods.

[Detailed supplementary tables to be provided as separate Excel/CSV files]

Note: This manuscript is a computational study using publicly available data. All findings are predictive and require experimental validation. The clinical data used in this study are simulated for proof-of-concept; application to real TCGA clinical data is required before clinical interpretation.

Corresponding Author Contact: Hsiu-Chi Tsai National Yang Ming Chiao Tung University Hsinchu, Taiwan ctsai1006@cs.nctu.edu.tw

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