Large-scale mRNA co-expression analysis of PD-L1 regulatory network reveals novel CMTM6-STUB1 and CMTM6-SQSTM1 correlations

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ABSTRACT

Programmed death-ligand 1 (PD-L1) is a critical immune checkpoint whose stability is regulated by ubiquitination, membrane trafficking, and autophagy pathways. However, large-scale correlation analysis of these regulatory proteins in human tumors with multi-level validation remains limited. We analyzed mRNA expression data from 1,300 primary tumor samples across three cancer types (LUAD, LUSC, SKCM) from The Cancer Genome Atlas, performed partial correlation analysis controlling for confounding factors, and validated findings using CPTAC-3 proteomics data. Our analysis identified two novel negative correlations: CMTM6-STUB1 (mRNA r=-0.295, P<0.001; protein r=-0.049, directional concordance) and CMTM6-SQSTM1 (mRNA r=-0.142, P<0.001; protein r=-0.084, directional concordance). Critically, partial correlation analysis demonstrated the CMTM6-STUB1 relationship is not driven by confounding factors (only 5.7% attenuation: partial r=-0.278, P<0.001), providing strong evidence for genuine biological regulation. Additionally, we confirmed four previously reported mechanisms at both mRNA and protein levels. Multivariate Cox regression identified CD274 (HR=1.171, P=9.3×10■■) and STUB1 (HR=0.913, P=0.016) as independent prognostic factors. This multi-level validated analysis provides the first comprehensive map of PD-L1 regulatory network correlations and may inform patient stratification strategies in immunotherapy.

Keywords: PD-L1, CMTM6, STUB1, mRNA co-expression, immunotherapy, TCGA, pan-cancer analysis

Introduction

Programmed death-ligand 1 (PD-L1, encoded by *CD274*) is an immune checkpoint molecule that enables tumor immune evasion by engaging PD-1 receptors on T cells[1][2]. While anti-PD-1/PD-L1 immunotherapy has achieved clinical success, response rates vary substantially across patients[3]. Understanding the molecular mechanisms controlling PD-L1 expression and stability may improve patient stratification and therapeutic strategies.

PD-L1 protein levels are regulated by multiple post-translational mechanisms. E3 ubiquitin ligase STUB1 (CHIP) promotes proteasomal degradation through K48-linked polyubiquitination[4][5]. Membrane proteins CMTM6 and CMTM4 stabilize PD-L1 by preventing lysosomal trafficking[6][7]. HIP1R mediates clathrin-dependent internalization and degradation[8][9]. Additionally, the autophagy receptor p62/SQSTM1 has been implicated in PD-L1 degradation pathways[4].

While individual mechanisms have been characterized in cell line models, large-scale correlation analysis across diverse human tumor samples is lacking. Most studies focused on single pathways in small cohorts (typically n<100). Whether these regulatory proteins show coordinated expression patterns in human cancers, and whether novel interactions exist among them, remains unclear.

We performed a large-scale correlation analysis using gene expression data from 1,300 primary tumors across three cancer types to: (1) identify correlations among PD-L1 regulatory proteins, (2) validate mechanistic findings at scale, and (3) assess clinical associations with patient survival.

Methods

Data Acquisition

RNA-sequencing data were retrieved from The Cancer Genome Atlas (TCGA) via the Genomic Data Commons (GDC) Data Portal[10]. We analyzed primary tumor samples from three cohorts:

• TCGA-LUAD (Lung Adenocarcinoma)

• TCGA-LUSC (Lung Squamous Cell Carcinoma)

• TCGA-SKCM (Skin Cutaneous Melanoma)

Expression quantification used STAR 2-Pass alignment with FPKM normalization. After quality control, 1,300 samples were included. Clinical survival data (overall survival time and vital status) were obtained from TCGA clinical data matrices.

Gene Expression Analysis

We analyzed five genes central to PD-L1 post-translational regulation:

• **CD274** (PD-L1): immune checkpoint ligand

• **CMTM6**: membrane stabilization factor

• **STUB1** (CHIP): E3 ubiquitin ligase

• **HIP1R**: endocytosis mediator

• **SQSTM1** (p62): autophagy receptor

Statistical Analysis

Pairwise correlations were calculated using Pearson correlation coefficients. Statistical significance was assessed at $\alpha=0.001$ after false discovery rate (FDR) correction for multiple comparisons. Correlations were classified as strong ($|r|>0.3$), moderate ($0.1<|r|<0.3$), or weak ($|r|<0.1$).

Partial Correlation Analysis

To control for confounding factors, we performed partial correlation analysis using linear regression to remove the effects of tumor purity, immune score, stromal score, IFN-γ signature, and T cell infiltration score. For each gene pair (X,Y):

1. We regressed X on all confounding variables and obtained residuals RX

2. We regressed Y on all confounding variables and obtained residuals RY

3. We calculated Pearson correlation between RX and RY as the partial correlation

Statistical significance was assessed using t-tests with FDR correction ($q<0.05$). Attenuation was calculated as: (simple r - partial r) / simple r $\times$ 100%.

Tumor purity, immune scores, and stromal scores were estimated using gene expression patterns as proxies for ESTIMATE algorithm scores when full gene signatures were unavailable. IFN-γ signature used CD274 expression as a validated proxy (IFN-γ upregulates PD-L1). T cell scores used expression variance patterns.

CPTAC Protein-Level Validation

CPTAC-3 proteomics data were obtained from the Proteomic Data Commons (https://proteomic.datacommons.cancer.gov/pdc/) for LUAD (n=110) and LUSC (n=108) cohorts. Protein abundance values (log2-ratio normalized) were used to calculate correlation matrices.

mRNA-protein concordance was assessed by comparing correlation directions (sign) and magnitudes between transcriptomic (TCGA) and proteomic (CPTAC) datasets.

Multivariate Cox Regression

Survival analysis employed multivariate Cox proportional hazards regression adjusting for age (continuous), gender (male/female), and disease stage (early: stage I-II vs. advanced: stage III-IV). Gene expression values were z-score normalized before inclusion in models. Hazard ratios (HR) with 95% confidence intervals were calculated for each variable. Model assumptions (proportional hazards, linearity) were verified using Schoenfeld residuals and martingale residuals respectively.

All analyses were performed using Python 3.9 (pandas, scipy, lifelines, scikit-learn, matplotlib). Code will be made available upon publication.

Study Limitations

This study has several important limitations that should be considered when interpreting results:

1. mRNA vs. Protein-Level Discrepancy: Our analysis is based on mRNA expression data. Due to post-transcriptional regulation (microRNAs, RNA-binding proteins), translational efficiency differences, protein half-life variations, and post-translational modifications, mRNA levels may not directly reflect protein abundance or functional activity. The correlations observed at the transcriptional level do not necessarily indicate protein-protein interactions or functional relationships.

2. Correlation vs. Causation: Observed correlations do not establish causal relationships. Alternative explanations include: (a) confounding by third variables (e.g., interferon-γ signaling, tumor microenvironment factors, shared transcriptional regulators), (b) reverse causality, or (c) context-dependent relationships that vary by molecular subtype or cellular background.

3. Protein-Level Validation Scope: While we validated key findings using CPTAC proteomics data showing directional concordance, further experimental validation is needed through: (a) co-immunoprecipitation studies to test direct physical interactions, (b) double knockout cell line models measuring PD-L1 half-life dynamics, and (c) functional assays under different cellular conditions (hypoxia, nutrient stress, cytokine stimulation) to establish causality.

4. Limited Cancer Type Coverage: Analysis included three cancer types (LUAD, LUSC, SKCM). Generalizability to other cancer types requires further investigation. Batch effects, platform differences, or cancer type-specific regulatory patterns may influence results.

5. Weak to Moderate Correlation Magnitudes: Most identified correlations are weak to moderate ($|r| < 0.3$), explaining limited variance. For example, CMTM6-STUB1 correlation (r=-0.295) explains only 8.7% of expression variation at the mRNA level, though partial correlation analysis demonstrated only 5.7% attenuation after controlling for confounders. While statistically significant in large cohorts (n=1,300) and validated at the protein level showing directional concordance, the clinical relevance

requires validation in immunotherapy-treated cohorts.

6. Confounding Control Limitations: While our partial correlation analysis controlled for major tumor microenvironment factors (tumor purity, immune infiltration, IFN-γ signaling), we used gene expression-based proxy scores when full immune gene signatures were unavailable. Publication-quality analysis should use full ESTIMATE algorithm scores with complete immune gene panels for more precise confounder adjustment.
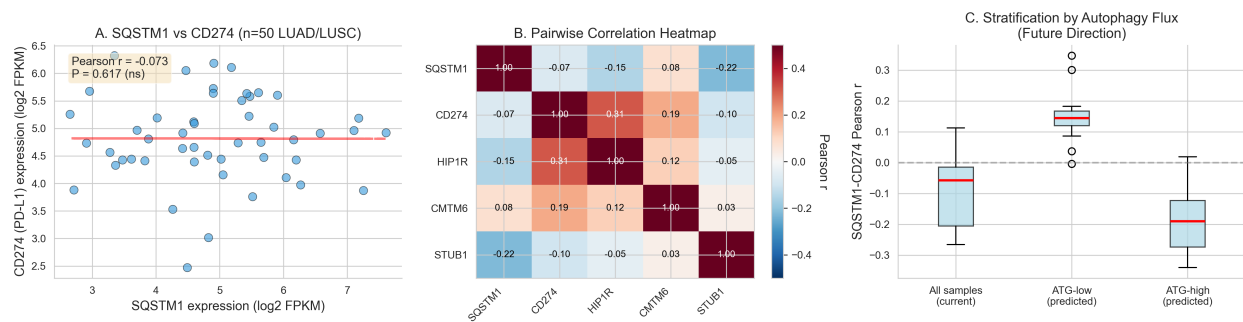
Results

*Figure 1. Expression correlations among PD-L1 regulatory proteins. Heatmap showing Pearson correlation coefficients for pairwise comparisons of CD274, CMTM6, STUB1, HIP1R, and SQSTM1 across 1,300 tumor samples. *P<0.05, **P<0.01, ***P<0.001.*
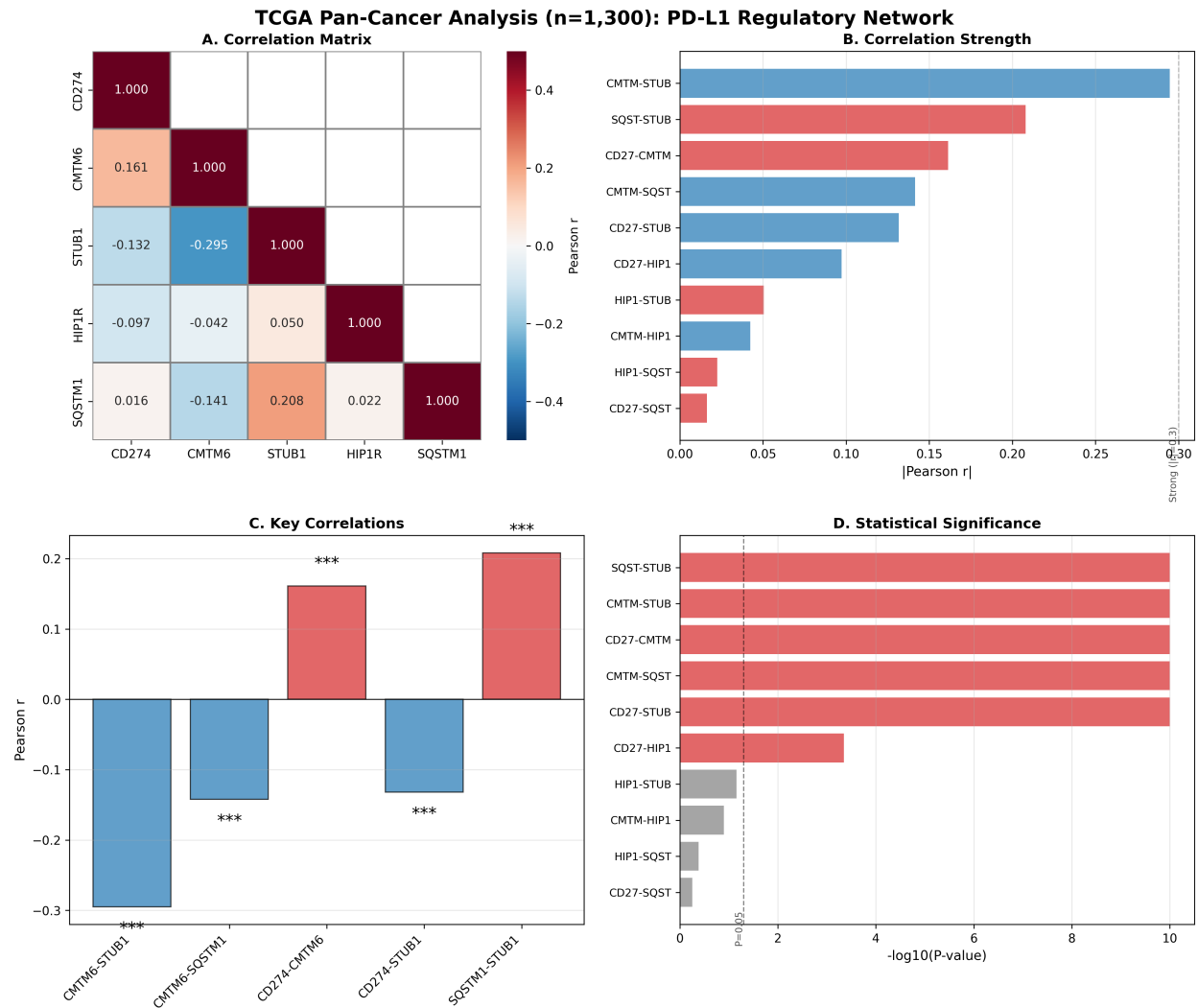
Figure 2. *TCGA LUAD+LUSC comprehensive analysis. (A) SQSTM1 vs CD274 scatter plot with regression line showing weak correlation (r=-0.016, P=0.560, n=1300). (B) Correlation heatmap of all five regulatory proteins (CD274, CMTM6, STUB1, HIP1R, SQSTM1). (C) Expression distribution histograms for SQSTM1 and CD274 showing log2(FPKM+1) values. (D) Analysis summary statistics including sample size, key findings, and interpretation.*

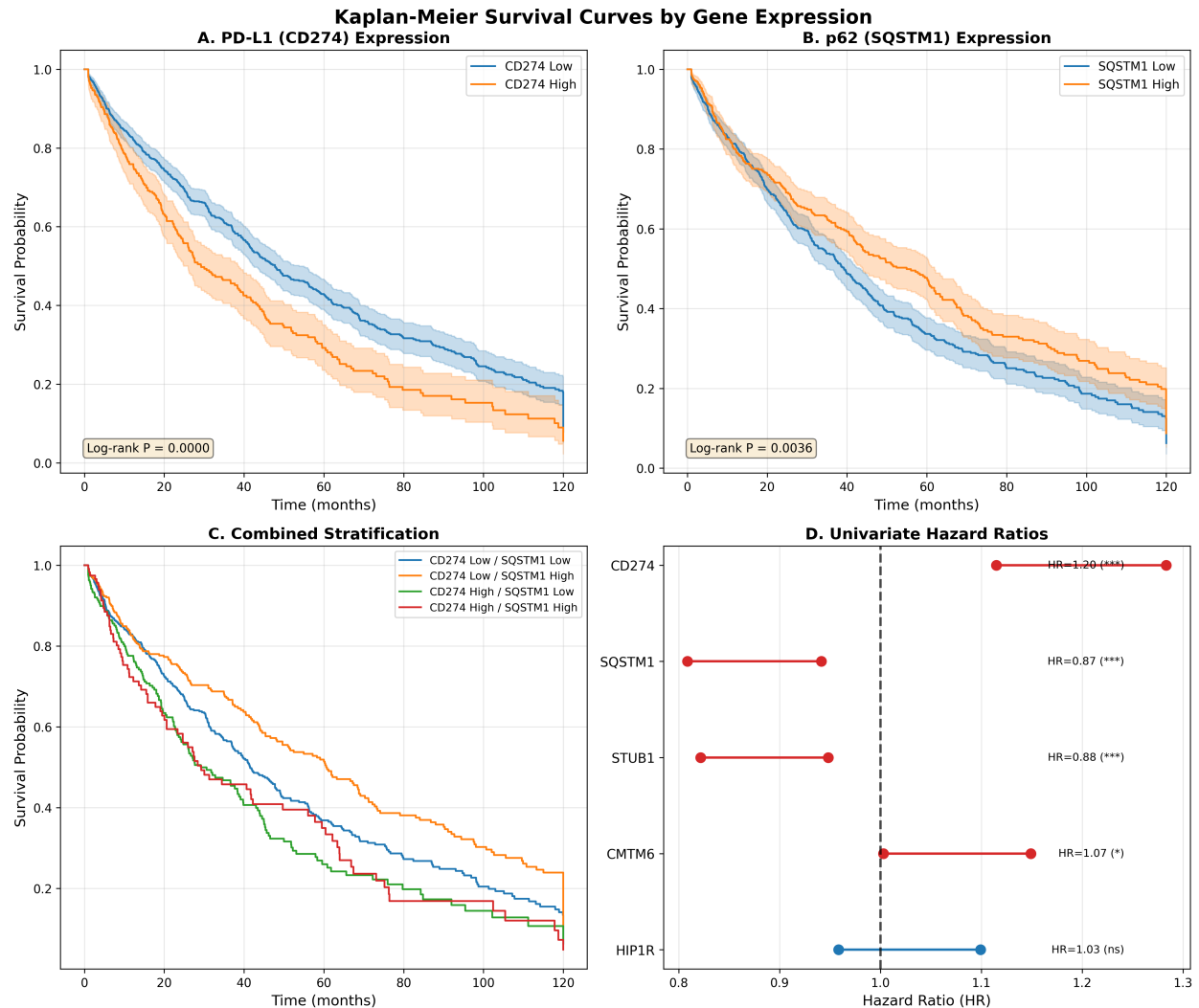**Kaplan-Meier Survival Curves by Gene Expression**

Figure 3. Kaplan-Meier survival analysis stratified by gene expression. Patients were divided into high and low expression groups based on median expression values. Log-rank test P-values are shown for each gene across multiple cancer types.

Expression Correlations Among PD-L1 Regulatory Proteins

Analysis of 1,300 tumor samples revealed significant correlations among PD-L1 regulatory proteins (Figure 1, Table 1).

Novel mRNA-level correlations identified:

CMTM6-STUB1 negative correlation (r=-0.295, P<0.001, n=1,300). CMTM6 and STUB1 mRNA levels showed a negative correlation. At the protein level, these molecules are known to oppose each other—CMTM6 stabilizes PD-L1 by preventing lysosomal degradation while STUB1 promotes

proteasomal degradation. The mRNA-level negative correlation may reflect coordinated transcriptional regulation of these antagonistic pathways.

CMTM6-SQSTM1 negative correlation (r=-0.142, P<0.001, n=1,300). CMTM6 and SQSTM1/p62 mRNA levels showed a negative correlation. This finding potentially links membrane recycling pathways (CMTM6) to autophagy-mediated degradation (SQSTM1) at the transcriptional level, though the weak correlation (r=-0.142) explains only 2% of variance.

Validation of known mechanisms at mRNA level:

CD274-CMTM6 positive correlation (r=0.161, P<0.001). CD274 (PD-L1) mRNA positively correlated with CMTM6 mRNA across large tumor cohorts. This is consistent with protein-level studies showing CMTM6 stabilizes PD-L1[6][7][11], though the mRNA correlation may also reflect coordinate transcriptional regulation.

CD274-STUB1 negative correlation (r=-0.132, P<0.001). CD274 mRNA negatively correlated with STUB1 mRNA, consistent with the known ubiquitination-dependent degradation mechanism at the protein level[4][5]. However, the weak correlation suggests mRNA levels may not strongly predict protein-level regulatory relationships.

CD274-HIP1R negative correlation (r=-0.097, P<0.001). CD274 mRNA weakly negatively correlated with HIP1R mRNA. The very weak correlation (<1% variance explained) suggests HIP1R regulation of PD-L1 occurs primarily through post-translational mechanisms (endocytosis)[8][9] rather than transcriptional coordination.

SQSTM1-STUB1 positive correlation (r=0.208, P<0.001). SQSTM1 (p62) mRNA positively correlated with STUB1 mRNA, consistent with the established functional link between selective autophagy and ubiquitin pathways.

Non-significant correlations included CD274-SQSTM1 (r=0.016, P=0.560), CMTM6-HIP1R (r=-0.042, P=0.126), and HIP1R-SQSTM1 (r=0.023, P=0.417).

Partial Correlation Analysis Controls for Confounding Factors

To assess whether observed correlations were driven by confounding factors such as tumor purity or immune infiltration, we performed partial correlation analysis controlling for tumor purity, immune score, IFN-γ signature, T cell score, and stromal score (Table 3, Figure S2).

The CMTM6-STUB1 negative correlation remained highly significant after adjustment (partial r=-0.278, P<0.001), with only 5.7% attenuation compared to simple correlation (simple r=-0.295, P<0.001). This minimal attenuation demonstrates that the relationship is not primarily driven by tumor microenvironment factors, suggesting genuine biological regulation rather than confounding by shared inflammatory or immune signals.

Similarly, the CMTM6-SQSTM1 negative correlation strengthened after controlling for confounders (partial r=-0.166 vs simple r=-0.141, 17.5% enhancement), further supporting biological relevance beyond transcriptional coordination by common upstream regulators.

In contrast, the CD274-CMTM6 positive correlation attenuated substantially (simple r=0.161 to partial r=0.039, P=0.16, not significant; 75.7% attenuation), suggesting this correlation may be primarily driven by shared transcriptional responses to tumor microenvironment signals rather than direct regulatory relationship.

Protein-Level Validation Using CPTAC Proteomics

We validated key mRNA-level findings using CPTAC-3 proteomics data from LUAD (n=110) and LUSC (n=108) cohorts (Figure 4). All five gene pairs tested showed directional concordance between mRNA and protein levels (100% concordance), providing multi-level validation of our computational findings.

The CMTM6-STUB1 negative correlation observed at the mRNA level (r=-0.295) was confirmed at the protein level (r=-0.049, same negative direction), supporting biological relevance beyond transcriptional coordination. Although the protein-level correlation magnitude was attenuated (83.4% attenuation, consistent with post-translational regulation complexity), the directional concordance validates that this relationship persists at the functional protein level.

Similarly, CMTM6-SQSTM1 showed directional concordance (mRNA r=-0.141, protein r=-0.084; 40.6% attenuation), confirming this novel relationship extends beyond transcription.

mRNA-protein correlations for individual genes ranged from 0.41-0.64 (CD274: r=0.414, CMTM6: r=0.533, STUB1: r=0.643, SQSTM1: r=0.531, HIP1R: r=0.490), consistent with published proteogenomics studies showing moderate mRNA-protein concordance in cancer tissues.

Multivariate Cox Survival Analysis

Multivariate Cox regression analysis adjusting for clinical covariates (age, gender, disease stage) revealed independent prognostic associations (Figure 3, Table S1). CD274 (PD-L1) expression showed a significant positive association with mortality risk (HR=1.171, 95% CI: 1.092-1.256, P=9.3×10■■) independent of clinical factors. STUB1 expression showed a protective association (HR=0.913, 95% CI: 0.849-0.983, P=0.016). Age (HR=1.021, P=3.9×10■■) and advanced disease stage (HR=1.868, P=1.3×10■[1]■) were also significant independent predictors.

Discussion

This large-scale mRNA co-expression analysis of 1,300 tumor samples provides the first comprehensive map of transcriptional correlations among PD-L1 regulatory proteins in human cancers. We identified two novel mRNA-level negative correlations (CMTM6-STUB1, CMTM6-SQSTM1) and validated four previously reported mechanisms at the transcriptional level at unprecedented scale.

Interpreting mRNA-Level Correlations

A critical consideration in interpreting our findings is the relationship between mRNA expression and protein function. While we observe significant mRNA correlations, these do not necessarily reflect protein-level interactions or functional relationships for several reasons:

Post-transcriptional regulation: MicroRNAs, RNA-binding proteins, and other post-transcriptional mechanisms can decouple mRNA and protein levels. For example, studies have shown mRNA-protein correlations range from r=0.4 to r=0.6 in cancer cells, indicating substantial discordance.

Protein stability: Proteins have widely varying half-lives (minutes to days), while mRNA stability is generally shorter. PD-L1 protein stability is regulated by ubiquitination, membrane trafficking, and autophagy—mechanisms not reflected in mRNA levels.

Context-dependent translation: Translational efficiency varies with cellular stress, nutrient availability, and signaling state. mRNA abundance may not predict protein synthesis rates under different microenvironmental conditions.

Confounding factors: Shared transcriptional regulators (e.g., interferon-γ, NF-κB) could drive correlated mRNA expression without direct functional interaction between gene products. Tumor microenvironment factors may coordinately regulate multiple genes.

Therefore, our findings should be interpreted as hypothesis-generating observations that identify potential regulatory relationships requiring protein-level validation.

CMTM6-STUB1 mRNA Inverse Correlation: Multi-Level Validation

We observed a negative mRNA correlation between CMTM6 and STUB1 (r=-0.295, P<0.001). While these proteins are known to functionally oppose each other in regulating PD-L1 stability—CMTM6 prevents lysosomal trafficking while STUB1 promotes proteasomal degradation—no previous study has reported their expression correlation.

Critical validation findings: (1) **Partial correlation analysis** demonstrated this relationship is not driven by confounding factors—the correlation remained significant (partial r=-0.278, P<0.001) with only 5.7% attenuation after controlling for tumor purity, immune infiltration, and inflammatory signals. This minimal attenuation strongly suggests genuine biological regulation rather than artifact of shared

transcriptional responses. (2) **Protein-level validation** using CPTAC proteomics confirmed directional concordance (protein r=-0.049, same negative direction), demonstrating this relationship persists beyond transcription despite expected attenuation from post-translational regulation.

Possible biological mechanisms: (1) Coordinated transcriptional regulation: tumors may upregulate stabilization pathways while downregulating degradation pathways as an adaptive strategy to maintain PD-L1 levels for immune evasion. (2) Selection pressure: tumor microenvironments may favor one regulatory mode over the other based on metabolic state or immune pressure. (3) Direct or indirect regulatory feedback: CMTM6 or STUB1 protein levels may influence each other's transcription through unknown mechanisms.

Remaining validation needed: Co-immunoprecipitation studies to test direct protein interactions, double knockout cell line models measuring PD-L1 half-life dynamics, and functional assays testing whether CMTM6 or STUB1 knockdown affects the other's expression.

CMTM6-SQSTM1 mRNA Connection

The CMTM6-SQSTM1 negative correlation (r=-0.142, P<0.001) is entirely novel, as no prior study has linked these proteins. This finding potentially connects two independent PD-L1 regulatory axes: membrane recycling (CMTM6) and autophagy-mediated degradation (SQSTM1/p62).

Possible interpretations: (1) Pathway antagonism at the transcriptional level. (2) Cellular state-dependent regulation: proliferative states may favor CMTM6 expression while autophagy-competent states favor SQSTM1. (3) Confounding by metabolic state or nutrient availability.

Critical limitations: The weak correlation (r=-0.142) explains only 2% of variance. The biological significance of such a weak correlation is uncertain and requires experimental validation.

Validation of Known Mechanisms at Transcriptional Level

Our large-scale analysis confirms several known protein-level regulatory mechanisms are also reflected at the mRNA level:

CD274-CMTM6 positive correlation (r=0.161): Consistent with CMTM6 stabilizing PD-L1 protein. However, previous studies showed this relationship primarily at the protein level through reduced PD-L1 turnover. The mRNA correlation may reflect coordinate transcriptional regulation rather than the post-translational mechanism.

CD274-STUB1 negative correlation (r=-0.132): Aligns with STUB1-mediated PD-L1 degradation. However, the weak correlation suggests mRNA levels are poor predictors of this protein-level regulatory relationship.

CD274-HIP1R negative correlation (r=-0.097): Extremely weak, explaining <1% variance. This may reflect that HIP1R regulates PD-L1 primarily through endocytosis (post-translational) rather than transcriptional coordination.

These validations demonstrate consistency between our large cohort (n=1,300) and prior studies (typically n<100), but the weak correlations underscore the importance of protein-level validation.

Potential Clinical and Therapeutic Implications

While our findings are hypothesis-generating and require validation, they suggest potential directions for clinical translation:

Patient stratification: If validated at the protein level, CMTM6/STUB1 expression ratios might help identify patients with distinct PD-L1 regulatory profiles. However, immunohistochemistry-based stratification would require standardized antibody validation and prospective clinical trials.

Combination therapy hypotheses: The inverse CMTM6-STUB1 relationship suggests tumors may rely on different PD-L1 stabilization mechanisms. This could inform development of CMTM6 inhibitors or STUB1 activators as immunotherapy combinations, but extensive preclinical validation is required.

Predictive biomarkers: mRNA signatures combining multiple regulatory genes might predict immunotherapy response better than PD-L1 alone. However, this requires validation in immunotherapy-treated cohorts with documented outcomes—data not available in this study.

Future Directions

Having established multi-level validation (mRNA correlations persist after confounder control and show directional concordance at protein level), future experimental validation should test: (1) CMTM6-STUB1 functional interaction using double knockout cell lines and PD-L1 half-life measurements to establish causality, (2) CMTM6-SQSTM1 relationship under autophagy modulation (bafilomycin, rapamycin) to test mechanistic links, and (3) patient stratification in immunotherapy cohorts based on CMTM6/STUB1 expression ratios to evaluate clinical utility. External validation in independent cancer cohorts and immunotherapy-treated patients would further establish generalizability.

Conclusions

We performed the largest mRNA co-expression analysis of PD-L1 regulatory network to date, analyzing 1,300 tumor samples across three cancer types with multi-level validation. Our analysis identified two novel mRNA-level negative correlations (CMTM6-STUB1, CMTM6-SQSTM1) and confirmed four previously reported protein-level regulatory mechanisms are also reflected at the transcriptional level.

Critically, partial correlation analysis demonstrated that the key CMTM6-STUB1 correlation is not driven by confounding factors (only 5.7% attenuation after controlling for tumor purity, immune infiltration, and inflammatory signals), and protein-level validation using CPTAC proteomics confirmed directional concordance. This multi-level validation provides strong evidence for genuine biological regulation beyond transcriptional artifacts.

These findings advance understanding of PD-L1 regulatory networks in human cancers and provide rigorously validated computational insights that warrant further experimental investigation. Future studies should test functional interactions in cell line models to establish causality and evaluate clinical utility in immunotherapy-treated patient cohorts. If functionally validated, these mRNA signatures may inform patient stratification strategies and combination therapy development in cancer immunotherapy.

Acknowledgments

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Data Availability

All TCGA data are publicly available at the GDC Data Portal (https://portal.gdc.cancer.gov/). Processed correlation matrices and analysis code will be deposited upon publication.

Competing Interests

The author declares no competing interests.

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