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BIOINFORMATIC ANALYSIS OF PROTEOMIC AND GENOMIC DATA FROM NSCLC TUMORS ON PROGNOSTIC AND PREDICTIVE FACTORS OF IMMUNOTHERAPY TREATMENT

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THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Pharmacy
at the University of Kentucky

By

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ABSTRACT OF THESIS

BIOINFORMATIC ANALYSIS OF PROTEOMIC AND GENOMIC DATA FROM NSCLC TUMORS ON PROGNOSTIC AND PREDICTIVE FACTORS OF IMMUNOTHERAPY TREATMENT

Recent lung cancer research has led to advancements in molecular immunology, resulting in development of small molecule inhibitors, or immune checkpoint inhibitors, that propagate an anti-tumor T cell response. Despite increased overall and progression-free survival with reduced adverse effects compared to traditional chemotherapy, treating advanced stage lung adenocarcinoma patients remains non-curative, and evidence of non-responders or tumor recurrence to immune checkpoint inhibitor therapy is growing. Also, compared to traditional chemotherapy, there is a lower percentage of patients who respond to small molecule inhibitors. In this analysis of proteomic and genomic data from The Cancer Proteome Atlas and Global Data Commons cancer databases, as well as clinical outcomes data from Phase II POPLAR and Phase III OAK clinical trials, we discuss possible prognostic and predictive factors of immunotherapy in the treatment of advanced non-small-cell lung carcinoma.

KEYWORDS: Non-Small-Cell Lung Carcinoma, Programmed Cell Death 1 Receptor, Immune Checkpoint Inhibitors, Overall Survival, Bioinformatics

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11/22/2022

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CHAPTER 1. BACKGROUND

1.1 Introduction

Deceptively simple, cancer is a preventable disease that occurs when normal cell division propagates out of control, thus creating a tumor. Tumors can be benign, and not spread, or malignant, which invades other tissues. Lung cancer occurs when the tumor originates from lung tissue (1). There are two main categories of lung cancer, small-cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). NSCLC is the major histological subtype, comprising 85% of all lung cancer cases (1) and will be my focus.

Abnormal cell growth and division in lung cancer stems from genetic cell damage, or mutations. In 2022, lung cancer is the 3rd most common cancer behind breast and prostate cancers respectively, but the deadliest cancer in the United States (6). According to the American Cancer Society, the 5-year survival rates for locally, or least, invasive non-small cell lung carcinoma (NSCLC) tumors is 59% for males and 70% for females (2). When lung cancer metastasizes, or moves to another organ system, the 5-year survival rate is 7% for males and 11% for females.

The most common cause of lung cancer is the use of cigarettes, tobacco, and alternative inhaled nicotine products. The use of tobacco and inhaled nicotine products is why lung cancer is preventable. In the U.S., smoking is linked up to 90% of deaths due to lung cancer (3). In response, local and state governments designed public health initiatives to reduce smoking to stymy the incidence of lung cancer. In the U.S. in 1999, the age-adjusted rate of lung and bronchus cancer cases was 70.8 per 100,000 people. In 2019, this rate dropped to 52.9 per 100,000 people (3). As a clinician, we do not treat lung cancer

prophylactically, which led me to believe that public smoking cessation initiatives hold value in the United States.

Kentucky has led the United States in the age-adjusted rate of lung and bronchus cancer cases, having the highest incidence of all 50 states in 14 of the past 20 years. Since 1996, there are restrictions on municipalities and city governments for imposing stricter requirements for use of tobacco and alternative nicotine products, which now include e-cigarettes and vape products, than the applicable state law (4). From 2020 Cancer Mortality data available from the CDC, Kentucky has a 20.5% higher mortality rate than the overall United States average in lung cancer.

1.2 Aspects of Lung Cancer Pathology

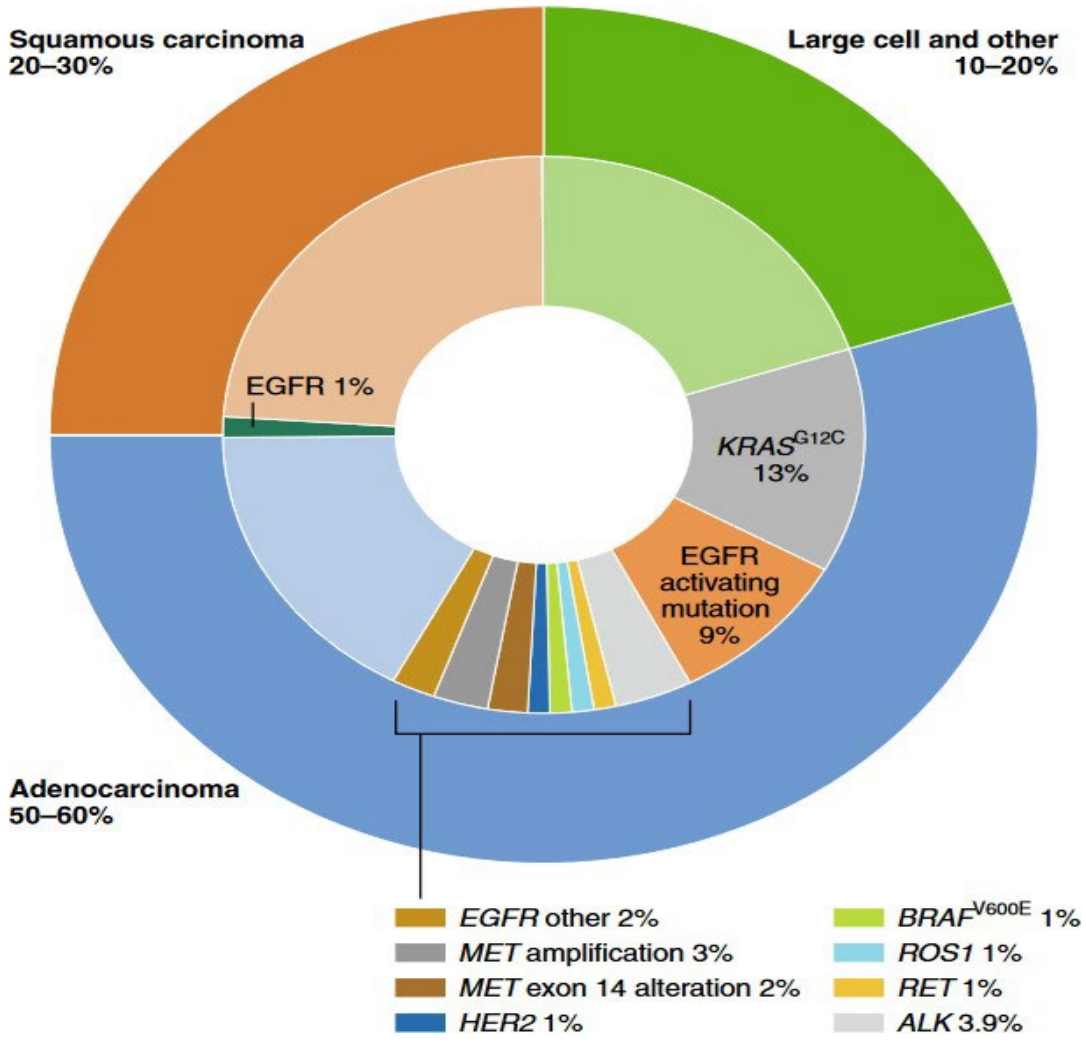
The treatment evolution of NSCLC has developed as mechanisms of resistance have been uncovered, and this is reflected in the National Comprehensive Cancer Network (NCCN) NSCLC treatment guidelines (4). When a patient presents and is diagnosed with NSCLC, the tumor undergoes a CT scan and is staged according to the American Joint Committee on Cancer (AJCC) TNM system (5). The TNM system scores based on the primary **t**umor size, lymph **n**ode involvement, and **m**etastasis. Based on the TNM score, the tumor is assigned a stage, I-IV, with stage IV being the most advanced. A locally advanced tumor, which is not resectable, is denoted as T3-4, N2+, M0 or T1+, N3, M0 is given Stage IIIb/III while any malignant cancer, no matter the size of the tumors or number of lymph nodes involved, is assigned Stage IV (M1). Clinical trials for targeted therapies begin with patients with at least Stage IIIb cancer.

Most primary tumors are resectable, unless they are too big (> 7cm), or grew into a blood vessel, the mediastinum, the windpipe, the heart, or the spine; these tumors are assigned late Stage III, or Stage IIIb/IIIc. A retrospective study found that the 3-year overall

survival (OS) rate for patients with biopsy proven stage I-II NSCLC was 92.8% when the patients underwent only a lobectomy procedure (6). A lobectomy is a surgery to remove one of the lobes of the lungs with the right lung having 3 lobes and the left lung having 2 lobes The main drawback with surgical treatments is making sure the entire tumor is removed, otherwise the tumor will recur when remaining cancerous cells (7). Despite the recurrence drawback, surgery remains our best, potentially curative, treatment for NSCLC.

Radiation and chemotherapy (chemoradiation) combined have traditionally been first line for early-Stage III NSCLC. With regional tissue and lymph node involvement, the main treatment issues are treating both regional (with radiation) and distant micrometastatic disease (with chemotherapy) (8). The drawback of chemoradiation is twofold: poor 5-year survival outcomes under 25% (9), and subjecting the patient to systemic adverse effects from nonspecific cell death exposure.

If a patient is not a candidate for surgery, and the tumor is locally advanced or malignant despite chemoradiation, the next step for treating NSCLC is molecular profiling the patient for biomarkers and oncogenic driver mutations. The major oncogenic driver mutations and biomarkers include epidermal growth factor receptor (EGFR) gene mutations, Kirsten rat sarcoma (KRAS) G12 mutations, anaplastic lymphoma kinase (ALK) gene rearrangements, ROS proto-oncogene receptor tyrosine kinase 1 (ROS1) rearrangements, BRAF V600E mutations, neurotrophic receptor tyrosine kinase (NTRK) gene fusions, and MET exon 14 skipping mutation (METΔex14). Programmed cell death ligand 1 (PD-L1) threshold status is concurrently tested with driver mutations and will be discussed later. Figure 1.1 depicts the current molecular landscape for NSCLC. Our current clinical treatment guidelines, from both the National Comprehensive Cancer Network (NCCN) and the European Society For Medical Oncology (ESMO), include a molecular biomarker workup testing for the presence of actionable driver mutations that have an associated, targeted therapy (src), (src), in addition to the immunohistochemistry (IHC) level of PD-L1.



[Figure 1.1 Molecular Landscape of Lung Cancer.]

[Adapted from Wang, M., Herbst, R. S., & Boshoff, C. (2021). Incidence of oncogenic driver mutations and biomarkers for lung cancer based on histology of tumor cells. For all areas of adenocarcinoma and squamous carcinoma not linked to a biomarker, and the entirety of large cell and other lung cancers, PD-L1 IHC expression is used determine preferred treatment.].

Small molecule inhibitors are the treatments of choice for oncogenic biomarkers and driver mutations and has been reflected in NCCN guidelines for patients with locally advanced or metastatic NSCLC due to favorable patient outcomes. A small molecule inhibitor is a low molecular weight chemical compound that can easily enter cells to disrupt activity or the function of their target, commonly a protein (10). Historically, protein kinase

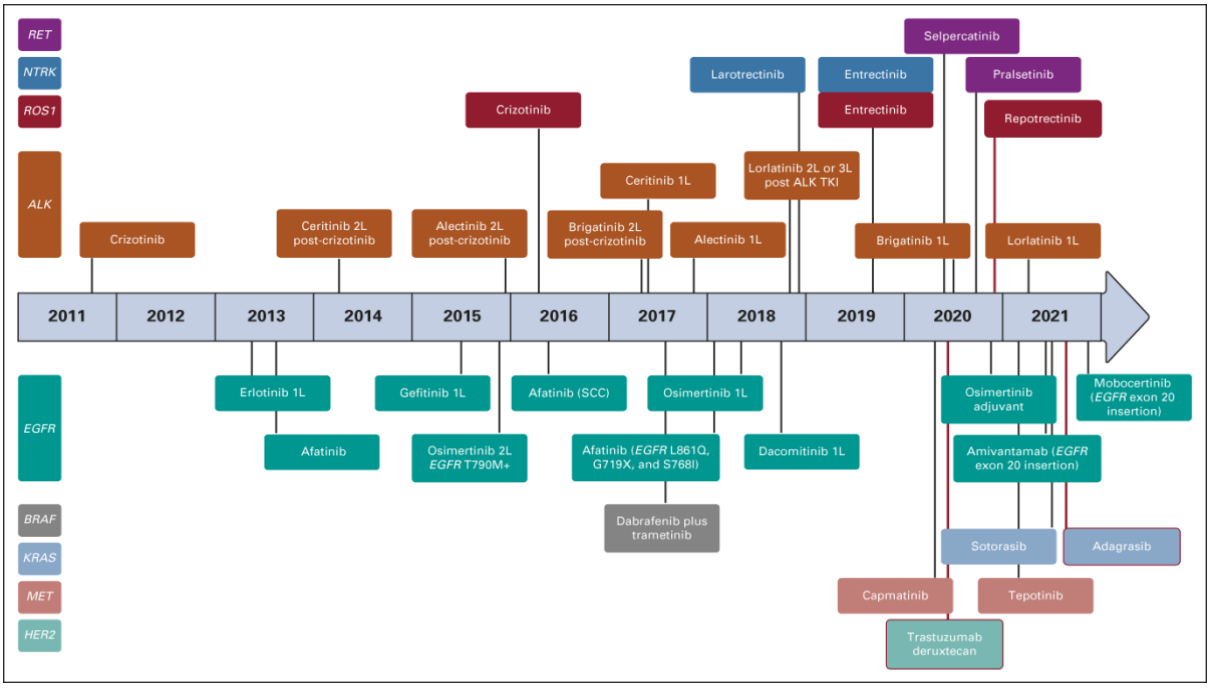
dysregulation is common in tumor pathogenesis due to genetic translocations and mutations (11). The first FDA approved protein kinase inhibitor, imatinib, revolutionized treatment for chronic myeloid leukemia (CML) by inhibiting a dysregulated fusion protein, called BCR–ABL, which is present in most cases of CML (12). Two years later, gefitinib, (Iressa) was approved for locally advanced or metastatic NSCLC by selectively inhibiting epidermal growth factor receptor's (EGFR) tyrosine kinase domain.

Small molecule inhibitors are not limited to receptor tyrosine kinase inhibitors (RTKs) but have expanded to include serine/threonine-specific kinase (STK) inhibitors, and phosphatidylinositol 3-kinase (PI-3K) inhibitors. Each of these types of kinase inhibitor has at least one FDA approved drug for treatment of a driver mutation or biomarker in a NSCLC setting.

Approximately 5% of NSCLC tumors harbor ALK gene rearrangements. In the ongoing ALEX phase III trial, alectinib (a second-generation ALK tyrosine kinase inhibitor [TKI]) to crizotinib (a first generation ALK TKI) showed incredible improvement in PFS (35 months vs. 11 months, HR 0.43), reduced of CNS progression (HR 0.16, 95% CI 0.10–0.28), and lower toxicities. Despite alectinib being a newer therapy, patients have an ORR of 82.9% (95% confidence interval 75.95 to 88.51). Nearly 1 in 5 patients will have stable disease (SD) or progressive disease (PD) and will need treatment with immunotherapy such as PD-(L)1 inhibitors with optional, adjuvant chemotherapy.

EGFR and KRAS mutations are the most common oncogenic driver mutations in NSCLC, representing 10-15% of adenocarcinoma cases and 20–25% of cases, respectively. Less common oncogenic mutations are BRAF V600E mutations, MET exon 14 alterations, NTRK rearrangements, and RET rearrangements, with each molecular alteration representing around 2% of lung adenocarcinomas (13). Small molecule inhibitors have exploded into all types of cancer treatment, autoimmune diseases, and transplant rejection treatment, with 76 FDA approved small molecule inhibitors as of

March 2021 (12). Figure 1.2 depicts the timeline of FDA approved small molecule therapies for the previously mentioned oncogene-driven NSCLC.



[Figure 1.2 Timeline of FDA-approved targeted therapies for oncogene-driven NSCLC]

[The red lines indicate breakthrough therapy designation. 1L, first-line; 2L, second-line; FDA, US Food and Drug Administration; NSCLC, non–small-cell lung cancer; TKI, tyrosine kinase. Tan, A. & Tan, D. (2022). Targeted Therapies for Lung Cancer Patients With Oncogenic Driver Molecular Alterations. Journal of Clinical Oncology, 40 (6), 611-625. doi: 10.1200/JCO.21.01626.]

In contrast with low molecular weight therapies like RTKs, immune checkpoint inhibitors (ICIs) targeting programmed cell death protein 1 (PD-1) receptors and PD-L1 were designed to induce T cell activity and have been found to have promising efficacy in locally advanced or metastatic NSCLC (14). The goal of immunotherapy is to inhibit tumor evasion of the immune system.

The difference between driver mutations and PD-L1 molecular testing is that driver mutations are much more consistent because it's a binary outcome; either the patient

expressed a $KRAS^{G12C}$ mutation, or they did not express that mutation. PD-L1 expression is quite the opposite, as it exists as a continuous variable, and thresholds are used to determine if a patient is PD-L1 positive or negative (15).

PD-(L)1 inhibitors are not low molecular weight small molecules, but instead are biological agents, specifically called monoclonal antibodies (mAbs) (16). MAbs have much higher specificity than small molecules due to binding surface antigens on tumor cells, in cancer settings. The immune response to antigens are very specific compared to cells with an EGFR receptor, which are known to be expressed on neurons, stem cells, and epithelial cells (17).

Two major differences between small molecules and mAbs are route of administration and elimination half-life, both of which constitute many of the differences in adverse reactions patients experience. Small molecules are mostly administered orally and have many gastrointestinal related adverse events, while mAbs are administered through injection, leading to injection, infusion, and immune mediated related reactions. Secondly, mAbs are proteins themselves, and therefore have extremely long half-lives compared to small molecules; pembrolizumab has a half-life of 22 days (18) versus 17.1 days for adults taking imatinib orally (19).

For patients with advanced lung cancer and without an oncogenic driver mutation or biomarker, PD-L1 threshold status directs first line therapy for patients. PD-L1 tumor proportion score (TPS) is important because around 30% of NSCLC tumors do not have an oncogenic biomarker and the TPS score of an individual tumor remains our only other guideline for treatment of locally advanced or metastatic lung cancer. Patients with high, >50% PD-L1 immunohistochemistry (IHC) expression, are treated with a PD-1 or PD-L1 inhibitor, such as pembrolizumab or atezolizumab, respectively. All of patients with positive PD-L1 status, 1-49% IHC expression, receive pembrolizumab with platinum-based chemotherapy. Atezolizumab added on to chemotherapy is less preferred in this patient population.

While it is difficult to compare outcomes across clinical trials due to heterogeneity of patient populations, the results of the KEYNOTE-021 (20) and IMpower130 (21) trials illustrate the rationale for PD-1 inhibitor preference over PD-L1 inhibitors in patients with 1-49% PD-L1 IHC expression. Both trials compared an ICI monotherapy against ICI plus chemotherapy against chemotherapy with no ICI. The ICI in KEYNOTE-021 was pembrolizumab, while the ICI in IMpower130 was atezolizumab. From the results of KEYNOTE-021, median PFS was 8.8 months in the pembrolizumab-combination group and 4.9 months in the placebo-combination group ($p<0.001$). From IMpower130 results, median PFS was 7.0 months in the atezolizumab plus chemotherapy group and 5.5 months in the chemotherapy group ($p<0.0001$). Both trial populations allowed, but did not require, patients to be previously treated for Stage I-III lung cancer with surgery, radiation, or chemotherapy, but excluded patients treated with an immunotherapy for Stage IV lung cancer.

Since immunotherapies are newer, there is a lack of data surrounding the length of response following treatment. In the landmark KEYNOTE-010 trial, pembrolizumab was compared to docetaxel in previously treated, PD-L1 positive advanced NSCLC (22). The 5-year OS rates were 25.0% (pembrolizumab) versus 8.2% (docetaxel) in patients with PD-L1 TPS ≥50% and 15.6% (pembrolizumab) versus 6.5% (docetaxel) with PD-L1 TPS ≥1%. This is a marked improvement from the 5-year OS survival rate of 8.5% for NSCLC in the U.S. population from 2012-2018 (23). Despite pembrolizumab's promising data, less than half of patients (43.0%) had an ongoing response at 5 years from randomization in patients with PD-L1 TPS ≥50%. This is due to tumor resistance to immunotherapies, such as tumor mutational burden and heterogeneity of the disease. It's critical to explore new strategies to improve patient survival.

A new strategy to improve NSCLC prognosis is to exploit advances in genetic engineering that specifically bind to antigens on tumor cells, very analogous to

immunotherapy. Instead of using monoclonal antibodies as in immuntherapy, modified T-cells, called chimeric antigen receptor (CAR)-T cells, has attracted scientific attention in cancers, especially lung cancer (24) (25)
(26). CAR-T cell therapies are already used in B-cell malignancies with promising results from clinical trials (27). In NSCLC, targetable antigens include EGFR, mesothelin (MSLN), mucin 1 (MUC1), prostate stem cell antigen (PSCA), carcinoembryonic antigen (CEA), PD-L1, CD80/CD86, inactive tyrosine-protein kinase transmembrane receptor (ROR1), and human epidermal growth factor receptor 2 (HER2) (28). Several CAR-T cell therapies are being explored in ongoing phase I clinical trials (29) (30) (31).

Newly approved therapeutics improve patient outcomes; however, chemotherapy and ICIs are not a cure for lung cancer. The RECIST criteria is a standardized way to measure how well patients respond to treatment using imaging techniques, such as magnetic resonance imaging (MRI) to check if the tumor became smaller, larger, or no change (32). Most patients reach a partial response (PR) or stable disease (SD) as opposed to a complete response (CR), or the absence of the signs of cancer (33)

A CR does not necessarily mean a cure, and this is because of tumor resistance mechanisms. There are three main types of tumor resistance with respect to therapies for NSCLC, acquired, adaptive, and inherited resistance (34).

1.2.1 Inherited Resistance

Inherited, or intrinsic, resistance is defined as tumor cells having resistance to therapy due to an innate aspect of the cells genetic code or protein structure (34), but before therapy is started. A common outcome of inherited resistance is failure of initial therapy due to a targetable protein defect that makes tumor cells insensitive to small molecule inhibitors. A study from Memorial Sloan Kettering (MSK) Cancer Center examined the

variable response of EGFR mutations to erlotinib, a TKI approved to treat EGFR mutations in NSCLC, in lung cancer cell lines predicted to be sensitive to erlotinib (35). They found overexpression of two proteins, caspase-8 or IKBKB, resulted in increased NF-kB activation, leading to erlotinib resistance.

1.2.2 Adaptive Resistance

Adaptive resistance is defined as a change in cell signaling mechanisms during therapy that promote resistance and make patient outcomes less favorable (34). Another research group from MSK Cancer Center, showed KRAS mutated tumors gain adaptive resistance to trametinib, a MEK inhibitor, through compensatory upregulation of fibroblast growth factor receptor 1 (FGFR1), a receptor tyrosine kinase (RTK), in NSCLC and pancreatic cell lines (36). Adaptive resistance can be overcome by inhibiting the compensatory mechanism, as shown in a study done from NYU's Perlmutter Cancer Center (37). In their study, a SHP-2 inhibitor inhibited increased RAS and RTK activation in a KRAS mutated NSCLC cell line treated with sotorasib, a $KRAS^{G12C}$ inhibitor.

1.2.3 Acquired Resistance

Acquired resistance is a molecular alteration in gene or protein structure that resist targeted therapy after an initial sensitivity (src). In a joint report from Massachusetts General Hospital Cancer Center and Harvard Medical School, researchers use cells from a NSCLC patient with a known $KRAS^{G12C}$ patient pretreated with adagrasib, found four genes in the RAS-MAPK signal transduction pathway that were altered as a result of treatment using a $KRAS^{G12C}$ inhibitor, causing increased incidence of other KRAS mutations, or reduced binding affinity of a $KRAS^{G12C}$ inhibitor (38).

1.2.4 Intratumoral and Intertumoral Heterogeneity

Intra- and intertumoral heterogeneity are important, and research is dedicated to understanding it because they may allow insight into new predictive factors of treatment. A concerted effort to understand intratumor heterogeneity in NSCLC is currently being studied an ongoing longitudinal observational study TRACERx (Tracking Cancer Evolution through Therapy) Lung (39). The patient population is diagnosed Stage I-IIIA NSCLC, and the goal is to prospectively elucidate how intratumoral heterogeneity affects clinical outcomes following surgical resection with appropriate adjuvant therapy. The study intends to follow patients from diagnosis to relapse.

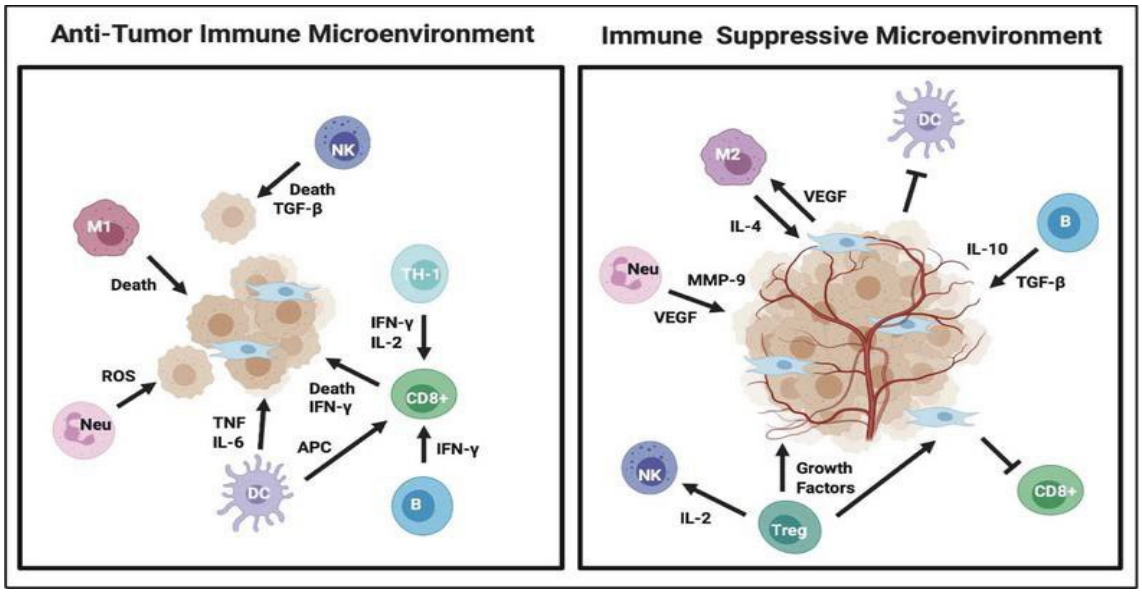
While TRACERx is trying to understand the relationship between intratumoral heterogeneity and clinical outcome, DARWIN II is investigating the role on intratumoral heterogeneity and response to therapy (40), including anti-PD-L1 inhibitors for patients without a driver mutation, and 3 other arms with patients having a BRAF mutation, ALK rearrangement, or HER2 amplification, and receiving appropriate first-line therapies for each mutation (41).

Tumor tissue is genomically heterogeneous, with varying tumor mutation burden (TMB). TMB is defined as the total number of somatic/acquired mutations per coding area of a tumor genome (Mut/Mb) (42). Tumors with a higher mutation burden have more potential to generate a larger number of neoantigens, making them more immunogenic.

1.2.5 The Tumor Microenvironment (TME)

The tumor microenvironment (TME) is the collection of cells and extracellular matrix around a tumor, including T cells, B cells, cytokines, macrophages, stromal cells, and blood vessels (43). Interaction and secretory molecules between tumor cells and

immune cells in the TME helps control tumor proliferation, survival, and metastasis (44). Recent research indicates tumors and their surrounding TME forms a reciprocal relationship that can either suppress or stimulate the host's immune system, as shown in Figure 1.3 (44).



[Figure 1.3 Example Effect of a TME on Immune Cells. Adapted from Anderson, N. M., & Simon, M. C. (2020)

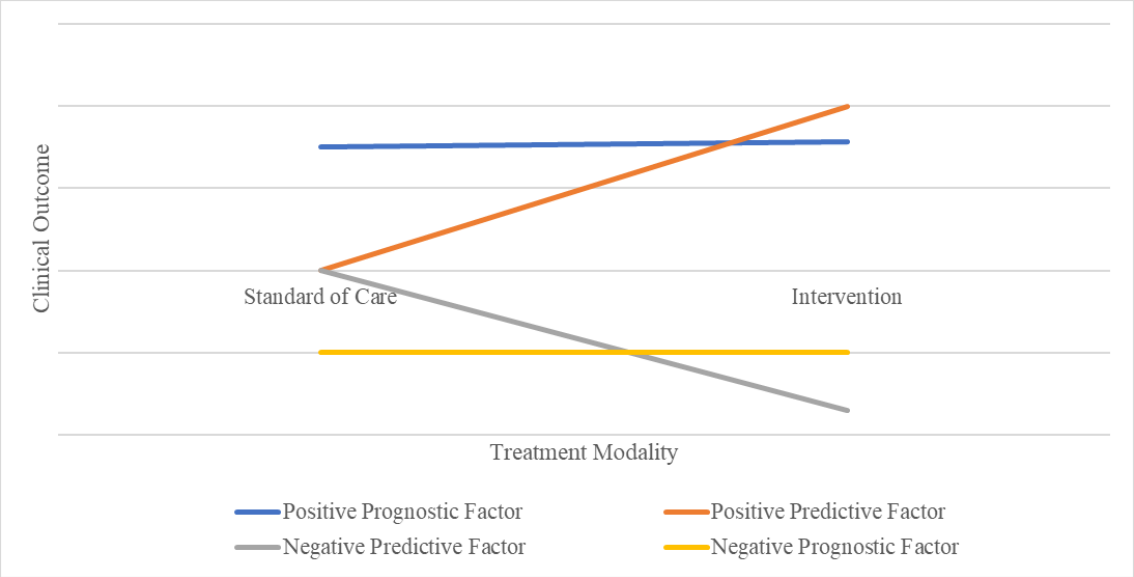
[In an anti-tumor environment, a pro-inflammatory tumor microenvironment results in B cells and TH-1 cells releasing interferon-gamma (IFN-γ) helps upregulate cytotoxic T cells (CD8+) and M1 macrophages that attack tumor cells directly. In an anti-inflammatory tumor microenvironment, TH-2 cells are dominant, releasing anti-inflammatory cytokines, such as interleukin-2 (IL-2) that inhibits natural killer (NK) cells and interleukin-10 (IL-10) that simultaneously downregulates TH-1 and upregulates M2 macrophages.]

Since investigating the compositions of different types of TME, new strategies to target and promote an anti-tumor microenvironment have surfaced. Part of the rationale for the effectiveness of immunotherapy is to help the host immune system identify tumor cells to fight tumor cell proliferation. In KEYNOTE-001, researchers gave patients pembrolizumab, a PD-1 inhibitor, and assessed overall response rate PFS based on PD-L1 threshold status (45). Interestingly, the overall response rate in all participants was 19.4%,

while the overall response rate for participants with a PD-L1 threshold status was 45.2%. A possible reason, and limitation of this study, is that the T cells from the tumor biopsy could have been depleted, thus lowering the apparent response of therapy. Research has since shown in locally advanced or metastatic NSCLC, a high PD-L1 threshold expression is possible with low tumor infiltrating cell expression (including but not limited to T cells) (46).

1.3 Predictive and Prognostic Factors of Treatment Outcomes

Prognostic and predictive factors are important to clinical decision making but are susceptible to being used imprecisely and interchangeably despite being separate things (47). Well studied prognostic and predictive factors can identify new avenues of treatment and lessen patient exposure to unnecessary (immuno)chemotherapy. A prognostic factor is an element measured before treatment that impacts patient outcomes independently of a proposed treatment, because it is representative of innate tumor behavior (48). A predictive factor is an indicator of patients that will have a different outcome to a specific treatment, due to an interaction between the predictive biomarker and intervention on patient outcome (48). Figure 1.4 illustrates that difference between hypothetical prognostic and predictive factors.



[Figure 1.4 Hypothetical Predictive and Prognostic Factors in a Clinical Decision Setting]

[Hypothetical Predictive and Prognostic Factors in a Clinical Decision Setting. The center line is the patient's baseline at beginning of treatment. A negative prognostic factor would be tumor size. Oncogenic driver mutations before small molecule inhibitors were on the market would be negative predictive factors. Now, EGFR, $KRAS^{G12C}$, and others are all positive predictive factors with small molecule inhibitors targeting their respective driver mutation protein. PD-L1 expression >50% is also a positive predictive factor with PD-L1 inhibitors. To make the case that PD-L1 expression is a positive prognostic factor, then irrespective of whether a PD-L1 inhibitor or chemotherapy was used, those patient's survival outcomes would be longer, or would have a higher objective response rate, depending on the outcomes measured in the study.]

In NSCLC, oncogenic driver mutations, like EGFR or KRASG12C, are examples of predictive factors; if the protein with a mutation is inhibited, the patient's outcome has been shown to be favorable. PD-L1 threshold expression is another example of a predictive biomarker because PD-(L)1 inhibitors, such as pembrolizumab and atezolizumab, improve outcomes when PD-L1 expression is higher.

A challenge of lung cancer pathology is that the tumor microenvironment and intratumoral and intertumoral heterogeneity all affect the prognostic and predictive biomarker landscape in NSCLC. As mentioned previously, intratumoral heterogeneity can impact the composition of the tumor microenvironment. Using clinical outcomes data from the POPLAR and OAK clinical trials, researchers found a statistically significant overall survival benefit to patients on atezolizumab based on the intratumoral composition of plasma B cells, as compared to patients on chemotherapy (docetaxel) (49).

There are several signaling pathways involved in NSCLC TME, including the SHP-2, interferon gamma (IFN-γ), JAK2, and STAT3 pathways. SHP-2, a protein tyrosine phosphatase, plays a crucial role in cell growth, differentiation, and survival by regulating various signaling pathways. Dysregulation of SHP-2 has been shown to promote NSCLC cell proliferation and survival in the TME. IFN-γ is a cytokine that regulates immune responses and has antitumor activity. However, IFN-γ can also promote tumor growth by activating the JAK2-STAT3 pathway in the TME. JAK2, a non-receptor tyrosine kinase, is involved in the activation of downstream signaling pathways, including STAT3. Dysregulation of the JAK2-STAT3 pathway has been linked to various cancers, including NSCLC, by altering the TME.

In NSCLC, dysregulation of SHP-2, IFN-γ, JAK2, and STAT3 in the TME has been shown to promote pro-tumorigenic signaling cascades. Understanding the molecular mechanisms underlying their dysregulation in the TME is essential for developing effective therapies. Therefore, I aim to investigate the roles of SHP-2, IFN-γ, JAK2, and STAT3 in the NSCLC TM by examining their expression levels in various cell types in the TME and investigate the mechanisms underlying their dysregulation.

1.4 Hypothesis

From results of the phase III OAK trial, we determined there is greater benefit from using PD-L1 inhibitors in patients with a higher PD-L1 expression. Therefore, my hypothesis is that increasing PD-L1 expression in all patients receiving immune checkpoint inhibitors will have a greater survival benefit. Specifically, I believe modulation of the JAK2/STAT3 signaling pathway will improve patient survival outcomes.

To address this hypothesis, I first analyzed publicly available protein and gene expression data from NSCLC patient tumors to find candidate genes that are upregulated when PD-L1 is highly expressed. Then I confirmed previously published literature that upregulation of the JAK2/STAT3 pathway increases PD-L1 expression *in vitro* also holds true in real world NSCLC patients (Chapter 2). Second, I acquired data sets from two recently published clinical trials and retrospectively analyzed outcomes data on pretreated NSCLC patients receiving immunotherapy to see if survival outcomes changed based on the protein or gene expression of PD-L1 (Chapter 3).

CHAPTER 2. AN INFORMATICS ANALYSIS OF THE INTERACTIONS BETWEEN CANDIDATE GENES

2.1 Introduction

Lung cancers remain a leading cause of cancer morbidity and mortality worldwide despite increased efforts toward drug discovery and implementation of personalized medicine approaches (51). Perhaps the most significant advance in therapy for many cancer types was the entry of immune checkpoint inhibitors (ICI) as a standard of care therapy for melanomas in 2014 (52). For non-small cell lung cancers (NSCLC), specifically those without targetable mutations in the epidermal growth factor receptor (EGFR) or anaplastic lymphoma kinase (ALK), immune checkpoint inhibitors, specifically the antibodies that target programmed cell death 1 (PD-1) or programmed death ligand 1 (PD-L1), have revolutionized cancer therapy even though response rates are relatively low (53). Both pembrolizumab and atezolizumab are approved ICI for frontline lung adenocarcinoma therapy for patients with high levels of PD-L1 expression on tumor cells (54). Durvalumab, an anti-PD-1 agent, is approved as maintenance therapy (55). Decisions to implement ICI therapy is often dependent on the PD-L1 tumor proportion score using evidence from the KEYNOTE-024 and -042 trials (56, 57). Importantly, PD-L1 expression may not be the optimal biomarker of response as suggested in pivotal clinical studies (e.g. KEYNOTE and OAK), but it is clear that patients with high levels of tumoral PD-L1 are likely to experience a robust response to checkpoint inhibition (58). While many research groups have searched for improved biomarkers of response for checkpoint inhibitors, others have focused on identification of therapies that might be combined with ICI to improve patient outcomes. The work presented herein falls into the latter category (59).

Our group found that inhibition of the tyrosine phosphatase, SHP-2, increased gene and cell surface protein expression of PD-L1 in KRAS-active NSCLC cell lines (manuscript submitted). PD-L1 is normally expressed on the surface of antigen presenting cells while PD-1 is expressed on T cells. It is the abnormal expression of PD-L1 on tumor cells, and the subsequent engagement with PD-1 on T cells, that causes tumors to be masked from an immune response (60). Inhibiting this interaction with antibodies against either PD-1 or PD-L1 can release a potent immune response toward the tumor.

We hypothesized that because SHP-2 provides some control of expression of PD-L1 on NSCLC cells that inhibition of SHP-2 would increase PD-L1 expression and synergize with ICI therapy. Supportive of our hypothesis is recently published data by Chen and colleagues showed in a NSCLC model system that combined SHP2 and PD-L1 inhibition, with accompanying radiation, can overcome resistance to PD-1 inhibitors (61). Other groups have suggested that SHP-2 activity may be more important in T cells, that infiltrate the tumor, to carry out signaling events downstream of PD-1 stimulation (62). Uncovering the precise mechanism of SHP-2 action on PD-L1 expression consumes many research groups, the model systems are expensive, and experimental time is long to get a drug to the clinic. In this study, we chose to go straight to real world data to determine whether SHP-2 activity is related to PD-L1 expression and thereby focus our research efforts.

We took advantage of three publicly-available data sets to assess whether moving forward with wet lab experimentation to determine if exploring the combination of ICI and SHP-2 inhibition is warranted. First, The Cancer Genome Atlas, now known as the NCI Genetic Data Portal (NCI-GDC), holds well-annotated expression and functional proteomic data (The Cancer Proteome Atlas (TCPA)) for patient tumors. However, most

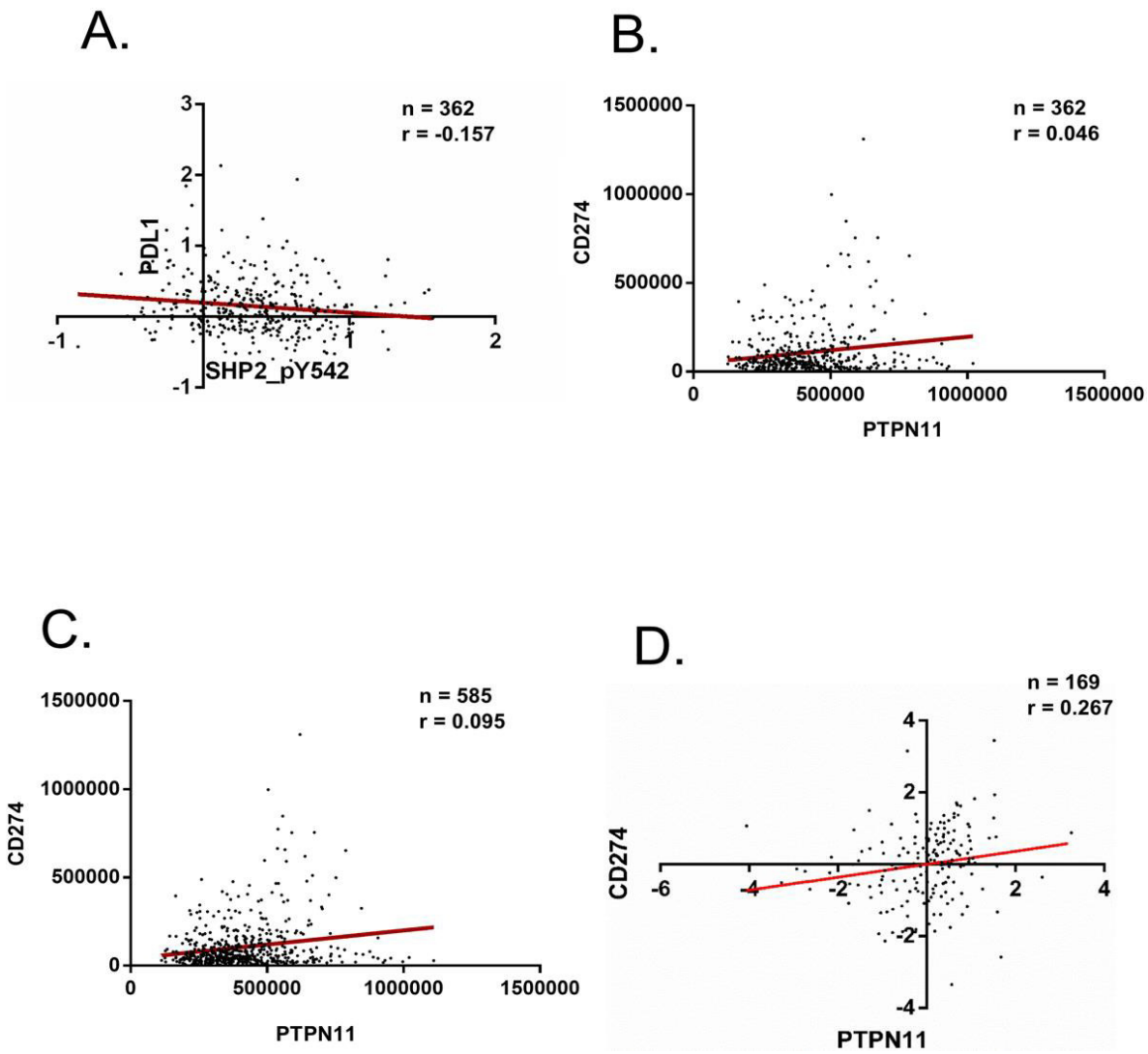
samples were collected prior to FDA approvals for ICI therapy, so no response data for ICI treatment is available (https://portal.gdc.cancer.gov/projects/TCGA-LUAD). Unfortunately, larger, industry-sponsored trials are still open (e.g. KEYNOTE and OAK), and full genomic and patient response datasets are not yet published. Therefore, in order to link expression of SHP-2 and PD-L1 with response to ICI, we uncovered two small studies: one in NSCLC and one in melanoma patients [63, 64]. Using real world data from the three studies identified, we believe that inhibition of SHP-2 activity is likely to improve response to PD-L1/PD-1 inhibitors and justifies continue wet-lab characterization of the mechanism(s) of activity.

2.2 Methods

First, using TCPA (https://tcpaportal.org/tcpa/index.html), a functional proteomics database which contains reverse phase protein array (RPPA) data from a wide variety of clinical tumor samples, we identified a lung adenocarcinoma (TCGA-LUAD-L4) dataset containing RPPA data from 362 individual patient samples. These data contain quantitative protein expression levels of 237 unique proteins for each subject.

From the TCPA data, SHP-2_ pY542, the phosphorylated and active form of SHP-2, and PD-L1 were compared from 362 patient tumors for relative protein expression levels using a two-tailed, non-parametric Spearman correlation analysis with 95% confidence intervals. The analysis revealed that levels of SHP-2_pY542 negatively correlate with PD-L1 expression ($r = -0.157$, p-value = 0.0028**) in these subjects, suggesting that inhibition of SHP-2 activity may increase PD-L1 protein expression (Figs 2.1A and 2.2A). Data capture and analysis was automated, and the annotated code in Python is linked here:

(https://github.com/mwu228/Summer2021/blob/main/Correlate%20proteins%20of%20interest.ipynb).

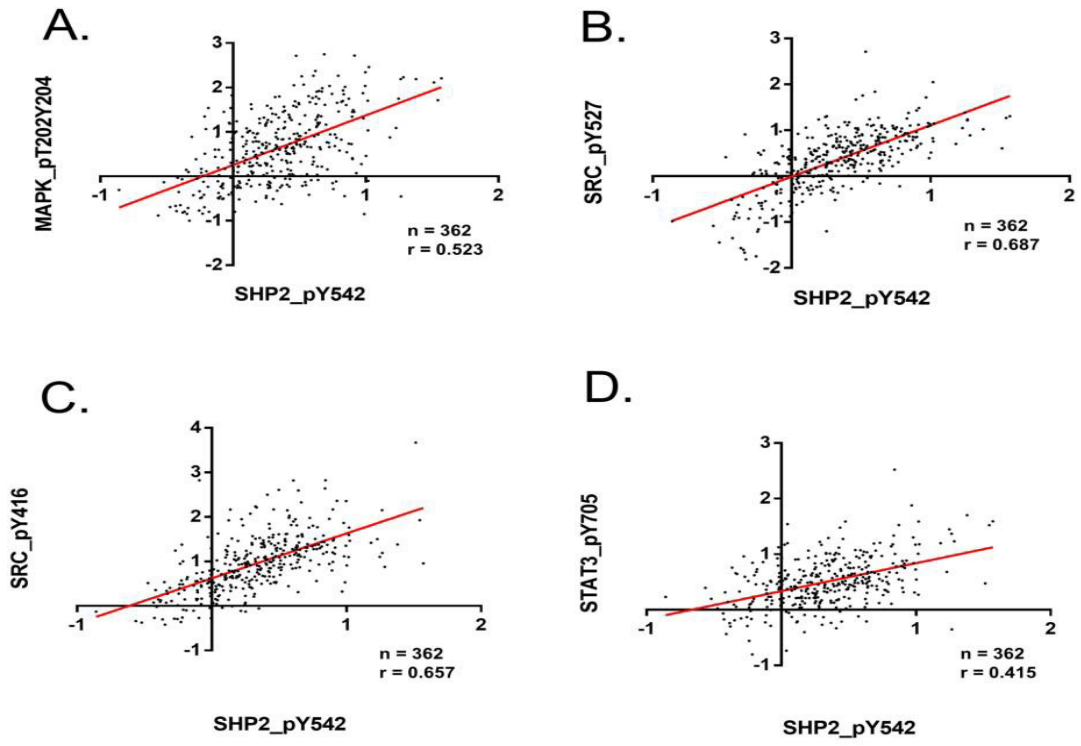


[Figure 2.1 SHP-2 activity and expression correlates with expression of PD-L1 in NSCLC adenocarcinomas.]

[**A**. Two-tailed non-parametric Spearman correlation analysis of RPPA protein expression data for Y542 phosphorylated SHP-2 and PD-L1 from 362 adenocarcinomas taken from The Cancer Proteome Atlas (TCPA: https://gdc.cancer.gov/about-data/publications/pancanatlas) LUAD-L4 dataset. **B**. Two-tailed non-parametric Spearman correlation analysis of bulk RNA-seq FPKM-UQ values taken from TCGA (GDC) for the 362 patients that had corresponding RPPA protein expression data from TCPA. **C**. Two-tailed non-parametric Spearman correlation analysis of bulk RNA-seq FPKM-UQ values taken from TCGA (GDC) for all 585 patients in the TCGA-LUAD dataset. **D**. Two-tailed

non-parametric Spearman correlation analysis of mRNA z-scores taken from cBioPortal (PMID:32015526) for 169 lung adenocarcinoma tumors. The red line in each panel represents a linear regression line of best fit. doi: https://doi.org/10.1371/journal.pone.0256416.g001]

The data contained in the TCPA contained only expression levels of the active, phosphorylated SHP-2, so we were unable to compare interactions with the unphosphorylated form. Thus, we looked to proteins in the TCPA dataset known to be in signaling cascades controlled by SHP-2 activity as internal controls, specifically Src, STAT3, and MAPK. We conducted the same correlation analysis between SHP-2_pY542 and either Src_pY527, Src_pY416, STAT3_pY705, or MAPK_pT202Y204. We found that the levels of active SHP-2 maintain strong ($r > 0.4$) and statistically significant ($p < 1x10\text{-}15$) positive correlations with each of these four proteins, providing additional support that the Y542 phosphorylation of SHP-2 correlation with PD-L1 protein expression is a meaningful interaction (Fig 2.2).



[Figure 2.2 SHP2_pY542 significantly correlates with phosphorylated proteins found in pathways that are SHP-2 targets.]

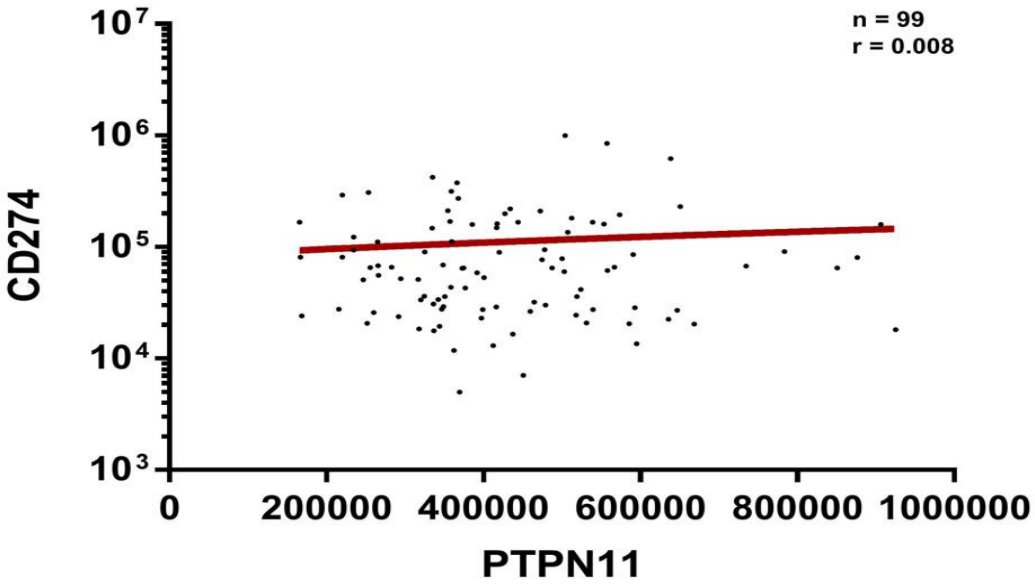
[A. Two-tailed non-parametric Spearman correlation analysis of RPPA protein expression data for Y542 phosphorylated SHP-2 and T202/Y204 phosphorylated MAPK B. Y527 phosphorylated Src kinase C. Y416 phosphorylated Src kinase D. Y705 phosphorylated STAT3 from 362 adenocarcinomas taken from The Cancer Proteome Atlas (TCPA: https://gdc.cancer.gov/about-data/publications/pancanatlas) LUAD-L4 dataset. The red line represents a linear regression line of best fit. https://doi.org/10.1371/journal.pone.0256416.s001]

Next, to better understand the relationship between expression of SHP-2 (PTPN11) and PD-L1 (CD274) mRNA in these patient tumors, we acquired corresponding RNA-sequencing data from TCGA, now known as the NCI-GDC (https://portal.gdc.cancer.gov). In this database, the TCGA-LUAD dataset contained 585 tumor samples, 223 more than the TCPA data. To look at only RNA-sequencing data that matched the previously-queried RPPA data, the 362 patient identifiers provided by the TCPA database were used to identify the corresponding RNA-sequencing data deposited into the GDC. We utilized fragments per kilobase-upper quartile (FPKM-UQ) values. The FKPM-UQ values for the genes PTPN11 and CD274 for each tumor were subjected to the same Spearman correlation analysis as previously described. Data capture and analysis was automated, and the annotated code in Python is linked here: (https://github.com/mwu228/Summer2021/blob/main/RNAseq%20FPKM%20correlation%20and%20pvalue.ipynb).

2.3 Results

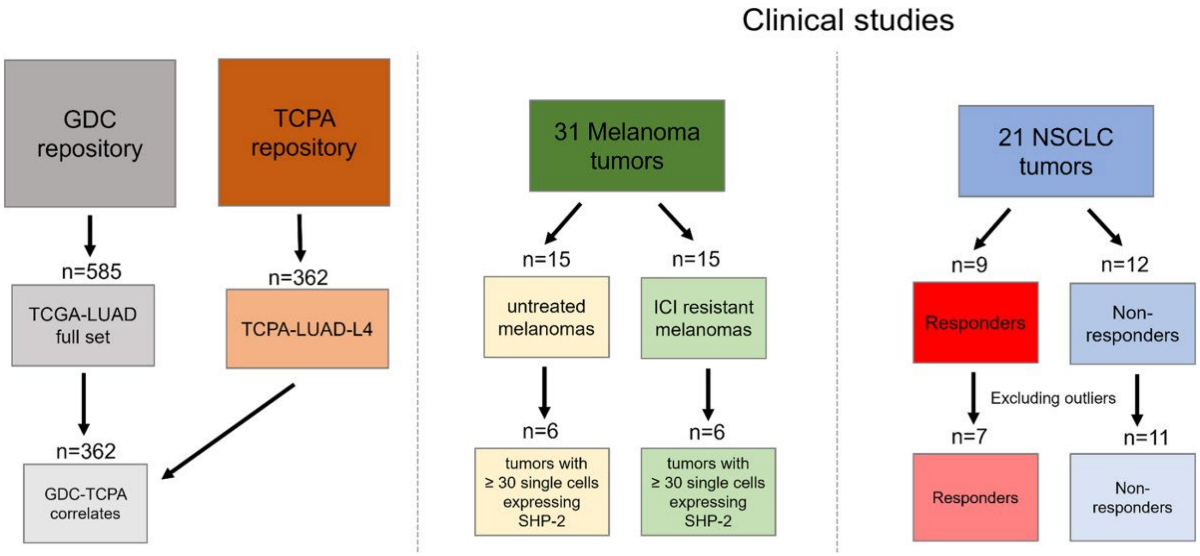
Interestingly, this analysis revealed no significant correlation (p-value = 0.3488) between PTPN11 and CD274 mRNA expression levels (Fig 2.1B). Following this observation, we wanted to know if any relationship between PTPN11 and CD274

expression was found using the entire TCGA-LUAD RNA-sequencing dataset (n = 585). We found a slight positive correlation existed (r = 0.095, p-value = 0.0211*) between PTPN11 and CD274 mRNA levels (Fig 2.1C). Because we are most interested in the role of SHP-2 in KRAS-active LUAD, we sub-grouped tumors with variants of KRAS known to be active from this dataset. No significant relationship was found in KRAS-active lung adenocarcinomas between PTPN11 and CD274 levels (Fig). Extending our observations from the aforementioned data that suggest a relationship between SHP-2 activity and PD-L1 expression, we identified another data warehouse (cBioPortal: cbioportal.org) that contains gene expression data from clinical cancer studies. Specifically, we located a study sought to characterize the genomic landscape of lung adenocarcinomas in East Asians (65). This study contains RNAseq data for 169 patients, from which we conducted a two-tailed, non-parametric Spearman correlation analysis with 95% confidence intervals between PTPN11 and CD274 mRNA levels (normalization method: z-score). The analysis revealed a positive (r = 0.267) and significant (p-value = 0.0005***) correlation between PTPN11 and CD274 mRNA, again suggesting that SHP-2 and PD-L1 protein are coexpressed in LUAD tumors (Fig 2.1D). Together, these TCPA and RNA seq data suggest that SHP-2 and PD-L1 protein are co-expressed in LUAD tumor tissue and that activation of SHP-2, not simply expression, may control levels of PD-L1. However, without knowing the expression levels of inactive SHP-2, we cannot state with certainty that SHP-2 activity is the primary role by which SHP-2 regulates PD-L1 expression.



[Figure 2.3 KRAS mutation status had no impact on PTPN11 and CD274 relationship.]

[Two-tailed non-parametric Spearman correlation analysis of bulk RNA-seq FPKM-UQ values taken from TCGA (GDC: https://gdc.cancer.gov/about-data/publications/pancanatlas) for 99 patients harboring mutations in the KRAS gene found in the TCGA-LUAD dataset. The red line represents a linear regression line of best fit. https://doi.org/10.1371/journal.pone.0256416.s002]



[Figure 2.4 Workflow scheme for evaluation of SHP2 and PD-L1 relationships]

[Reverse-phase protein array (RPPA) was collected from the TCPA data repository (https://gdc.cancer.gov/about-data/publications/pancanatlas) for 362 total patients labeled as the TCPA-LUAD-L4 data set. RNAseq data was collected from GDC for the full TCGA-LUAD dataset (n = 585). These data were parsed to include only patients for which there was matching RPPA data on TCPA (n = 362). Single-cell RNAseq reads for 31 melanoma tumors were collected and separated into two groups based on ICI treatment status. Only single-cell reads for 'malignant melanoma cells' were retained for analysis. Tumors which had ≥ 30 unique malignant cells with non-zero PTPN11 values were included in the analysis. NSCLC tumors (n = 21) with sequence data were first separated into two groups based on response to ICI treatment. Average TPM values were calculated for PTPN11 and CD274, and tumors that had PTPN11 TPM value >2 standard deviations from the mean were excluded from the analysis. doi: https://doi.org/10.1371/journal.pone.0256416.g002]

Having established a connection between tumoral SHP-2 activity and PD-L1 expression, but not corresponding gene expression levels, in lung adenocarcinomas, we sought to understand whether PTPN11 and CD274 expression levels associate with response of patient tumors treated with ICIs. A study was identified that analyzed single-cell RNA-sequencing (scRNAseq) data from 31 melanoma tumors that were either not treated with ICIs or became resistant to ICIs following treatment. Importantly, the authors of the study were interested in characteristics of the melanoma cells that lead to immune evasion (63). We used the R-studio Bioconductor GEOquery package to download the raw scRNA-seq transcript-per-million (TPM) values, cell counts, and annotations from this study (GSE115978). TPM values were calculated as described in Jerby-Arnon L., et al. (63). We sought to answer two main questions using these data: 1) does PTPN11 mRNA expression correlate with CD274 mRNA levels and 2) does PTPN11 expression correlate with poor response to PD-1 inhibition? The scheme for the analysis workflow is found in Fig 2.4. To address the first question, we identified the tumors which were not treated with ICI (n = 15). For each of these samples, we established that scRNA-seq reads were

available for several cell types, including immune cell types and malignant cells. Cell types were detected by fluorescence activated cell sorting using cell-type specific proteins. Because we are only interested in associations between PTPN11 and CD274 in tumor cells, we selected only single cells determined to be malignant melanoma cells. Of the 15 untreated tumors, the analysis was narrowed to include patient tumors that have at least 30 unique malignant cells (n = 6) resulting in an average of 108 (range, 91–487) single-cells per tumor. To understand the proportion of single cells in an individual tumor that expressed PTPN11, the percentage of cells with nonzero TPM scores for PTPN11 for each tumor was calculated (Fig 2.5). This processing uncovered that six untreated tumors (Mel71, Mel79, Mel103, Mel80, Mel81, Mel89) demonstrated ≥50% of single malignant cells (mean = 69%; range, 50–83%) expressed PTPN11. The TPM values for PTPN11 and CD274 for all single malignant cells in these six tumors were then assessed together, resulting in mean/standard deviation TPM values for PTPN11(1.40, 0.22) and CD274 (0.11, 0.06). We observed a similar trend similar to that of the TCPA/NCI-GDC analysis above that elevated expression of PTPN11 associated with lower expression of CD274 in treatment naïve tumors. Finally for this dataset, we wanted to understand the relationship of PTPN11 and CD274 expression and response to therapy. We used the patient tumors which acquired resistance to ICI therapy (n = 15) to ask whether the relative levels of PTPN11 and CD274 levels were different than the treatment-naïve tumors. Again, the data were processed to include only tumors with ≥ 30 unique malignant cells (n = 6) resulting in an average of 79 single cells (range, 96–169) per tumor. We applied the methods used above to calculate the proportion of single cells expressing PTPN11 for each tumor, and the average TPM values for PTPN11 and CD274 when the single cells of all six tumors

were evaluated together. We found that these six ICI resistant tumors (Mel78, Mel88, Mel98, Mel102, 196 Mel110, Mel94) again showed ≥50% single malignant cells (mean = 67%; range, 57–82%) expressed PTPN11, and the combined mean/standard deviation TPM values for PTPN11 (1.29, 0.12) and CD274 (0.09, 0.06). Here, similar expression patterns of PTPN11 and CD274 were observed compared with treatment-naïve tumors, and again CD274 levels remain low when PTPN11 is expressed. Importantly, we were unable to observe any relationship between PTPN11 or CD274 expression and acquired resistance to ICI in this dataset.

A

Treatment	Tumor	% cells expressing PTPN11	Tumoral PTPN11 TPM	Tumoral CD274 TPM	Average PTPN11 TPM	Average CD274 TPM
None	Mel80	80.4	1.35	0.07	1.40	0.14
None	Mel81	81.7	1.57	0.02		
None	Mel89	83.0	1.78	0.18		
None	Mel71	56.5	1.24	0.07		
None	Mel79	58.8	1.40	0.18		
None	Mel103	50.4	1.08	0.12		

B

Treatment	Tumor	% cells expressing PTPN11	Tumoral PTPN11 TPM	Tumoral CD274 TPM	Average PTPN11 TPM	Average CD274 TPM
Ipilimumab+nivolumab	Mel78	75.8	1.49	0.15	1.29	0.09
Ipilimumab+pembrolizumab	Mel110	82.1	1.39	0.05		
Tremlimumab	Mel88	66.9	1.16	0.17		
Ipilimumab	Mel98	61.8	1.19	0.14		
Ipilimumab+nivolumab	Mel102	56.8	1.20	0.03		
Ipilimumab+pembrolizumab +nivolumab	Mel194	59.4	1.29	0.03		

A. Six tumors had ≥ 30 unique malignant cells that had non-zero PTPN11 TPM values and are ICI therapy naïve [13]. Shown are the percentage of single cells that expressed PTPN11, the combined TPM values for all the single cells within each individual tumor, and the average TPM values for all six untreated tumors combined. **B**. The same details as **(A)** but for tumors that did not respond to ICI therapy. There was no significant difference in average PTPN11 and CD274 TPM values between treatment naïve and treatment resistant groups.

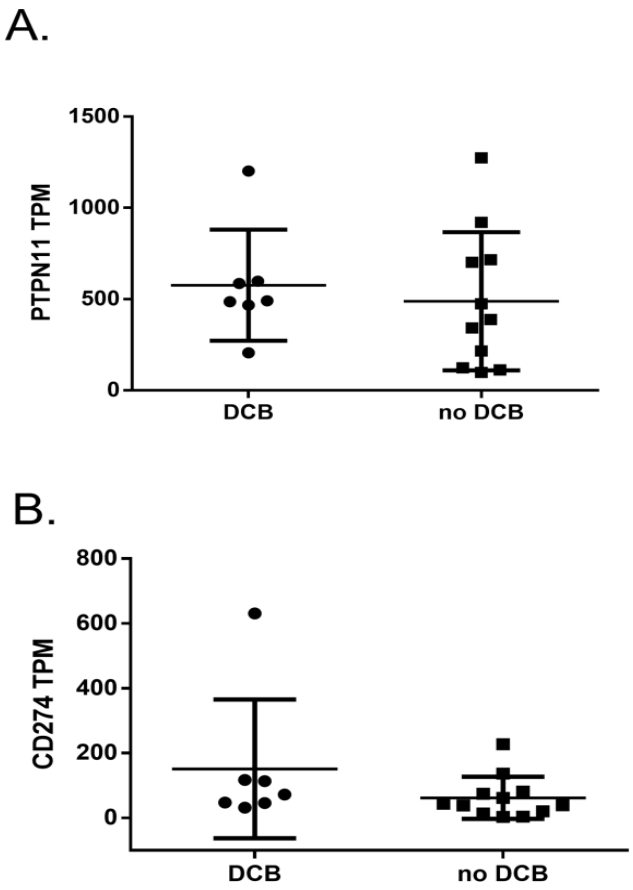
https://doi.org/10.1371/journal.pone.0256416.t001

[Figure 2.5 A. PTPN11 mRNA expression weakly associated with reduced CD274 mRNA expression in melanoma tumors regardless of ICI exposure. B. PTPN11 mRNA expression weakly associated with reduced CD274 mRNA expression in melanoma tumors regardless of ICI exposure. doi: https://doi.org/10.1371/journal.pone.0256416.t001]

Using the data from the third study, we asked whether expression of PTPN11 and CD274 mRNA associates with response to ICI therapy in NSCLC. The investigators in this report aimed to find immune signatures predictive of response to anti- PD-1 inhibitors in

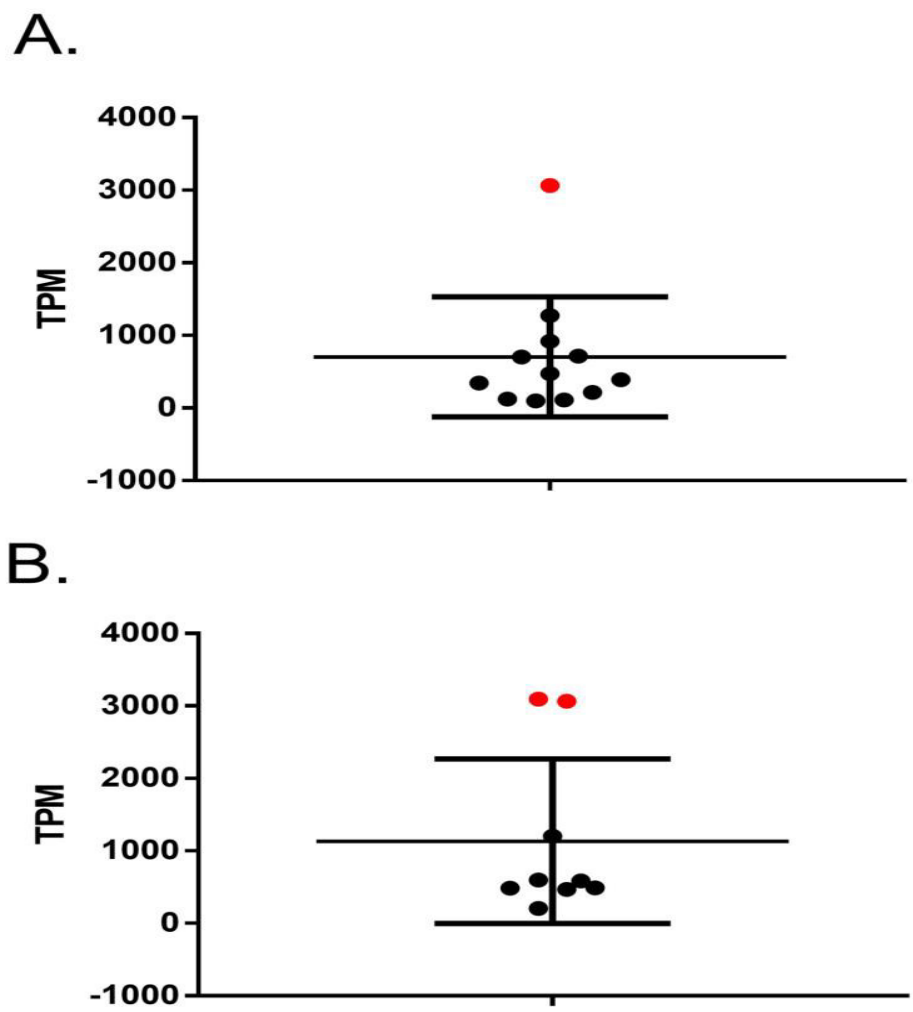
NSCLC. The dataset contained bulk tumor RNA-sequencing data and clinical response data for 21 NSCLC subjects treated with single agent anti-PD-1 therapy [64]. As before, the R-studio Bioconductor GEOquery package was used to capture raw RNA-sequencing TPM values from this study (GSE136961). Patients demonstrating progression of disease or stable disease that lasted less than 24 weeks were deemed by the authors to have no durable clinical benefit (DCB) to anti-PD-1 therapy. Patients showing partial or complete response by Response Evaluation Criteria in Solid Tumor (RECIST) v1.1 or stable disease for more than 24 weeks were defined as receiving DCB. The analysis of these data followed a workflow scheme like that in Fig 2.4. Of the 21 NSCLC patients in this study, nine demonstrated a DCB to ICI therapy and twelve showed no DCB. We separated the data by DCB status and then averaged all TPM values for PTPN11 and CD274 for each patient tumor to generate one average TPM score for each group. An outlier analysis was performed on the PTPN11 TPM values for both responders and non-responders (Fig 2.7), resulting in final groups of 7 responders (n = 7) and 11 nonresponders (n = 11). Our analysis revealed no significant difference in the expression of PTPN11 mRNA between subjects with DCB from those that did not respond to anti-PD-1 therapy (Fig 2.6). Specifically, the mean/standard deviation PTPN11 TPM scores were (576.15, 281.78) and (487.73, 361.24) for responders and non-responders, respectively. Importantly, the mean expression of CD274 mRNA was nearly 3-fold higher in patients who responded to therapy (151.16, 198.33) compared to those who did not (61.96, 64.54). We note the standard deviation was large for the last two groups assessed. Together, these data showed that PTPN11 expression does not associate with CD274 expression or response therapy in NSCLC patients. These findings are consistent with the results from the first study that suggested that SHP-2

activity, not expression, correlates with PD-L1 expression. In contrast, these data demonstrated a positive relationship between PD-L1 expression and response to ICI which was not observed in the melanoma study.



[Figure 2.6 CD274, but not PTPN11, mRNA expression is associated with response to ICI in NSCLC tumors.]

[A. PTPN11 TPM values for patients who did or did not demonstrate a durable clinical benefit (DCB) from ICI therapy, as determined by RECIST criteria [14]. There was no significant difference between groups, as measured by a student's t-test. B. TPM values for CD274 in patients who did or did not demonstrate a durable clinical benefit (DCB) from ICI therapy, as determined by RECIST criteria. There was no significant difference between groups, as measured by a student's t-test. doi: https://doi.org/10.1371/journal.pone.0256416.g003]



[Figure 2.7 Outlier analysis of PTPN11 TPM values for NSCLC response study.]

[Box and whisker plot of PTPN11 TPM values for NSCLC patients who did not respond to ICI therapy (A) or patients who did respond (B). Outliers, highlighted in red, were determined by the 1.5 interquartile range (IQR) method which adds 1.5 times the IQR to the third quartile and excludes data points that fall above that value, and subtracts 1.5 times the IQR from the first quartile and excludes data points that fall below that value. https://doi.org/10.1371/journal.pone.0256416.s003]

2.4 Discussion

In this study, we applied information obtained from publicly-available protein and gene expression datasets to gain further insight into our overarching research question:

does SHP-2 activity or expression influence PD-L1 mRNA and protein levels and subsequent response to anti-PD-1 or PD-L1 therapies in NSCLC? We used this approach because we believe that the utilization of real-world datasets can inform and direct wet-lab experimentation. The design and execution of pre-clinical and clinical studies is expensive, time-consuming, and labor-intensive. Here, we present a quick and efficient process that, when combined with bench-side techniques, can offer substantial insight into the clinical translatability of commonly-used, highly-controlled model systems designed for drug discovery applications. Through the analysis of two major cancer data repositories and two smaller clinical studies, we were able to take further steps towards establishing a connection between the activity of SHP-2 and PD-L1 expression in human tumors without carrying out a study de novo.

Of the datasets chosen for this study, the most statistically-powerful and revealing information arose from the composite analyses of the TCPA and GDC data repositories. Using genomic and protein information from a large cohort of NSCLC patients, our most important observation was the strong negative correlation ($r = -0.157$, p-value = 0.0028**) between the active, tyrosyl-phosphorylated form of SHP-2 and PD-L1 protein expression (Fig 2.2A). A limitation of these data was that the RPPA data did not include expression levels of the unphosphorylated and inactive form of SHP-2 which would have been a useful control as informed by our wet-lab studies. However, to address this limitation, we investigated the relationship of SHP-2 at Y542 with known targets. We found strong positive correlations in the expression levels of SHP2_pY542 and three proteins (Src, MAPK, STAT3) whose activity is dependent on phosphorylation status and known to be regulated by SHP-2 activity (66–68). While this is not a perfect control to confirm that the

activity of SHP-2 predominates total SHP-2 expression as they relate to PD-L1 expression, it does provide additional evidence that quantifies association of SHP2_pY542 with known substrates.

The conformational changes induced by phosphorylation of SHP-2 could alter protein-protein interactions among signaling components and intracellular signaling cascades that impact PD-L1 expression (69). Following from that hypothesis, we observed no statistically significant correlation between the levels of SHP-2 and PD-L1 mRNA in the patients in the NCI-GDC dataset that were initially studied in the TCPA dataset, again highlighting the potential importance of molecular interactions of SHP-2 dependent on its activated state. Interestingly, when we conducted the same analysis on the entire cohort of LUAD patients in the NCI-GDC repository, a weak, but inverse correlation, ($r = 0.095$, p-value = 0.0211*) between PTPN11 and CD274 mRNA was observed, suggesting that SHP-2 and PD-L1 are co-expressed in LUAD tumors. It is then plausible that SHP-2 activation may function to finetune PD-L1 expression levels. In immune cells, SHP-2 functions downstream of the PD-1:PD-L1 interaction by facilitating the internalization of the PD-1 receptor which ultimately results in the deactivation of the immune cell (70). Likewise, it is conceivable that SHP-2 functions in a similar manner with regard to tumoral PD-L1 expression. The significance of SHP-2 co-expression with PD-L1 mRNA may be in a negative feedback loop, reducing PD-L1 levels once its expression is no longer necessary. Mutation of SHP-2 in malignant cells may alter SHP-2 activity or expression to disrupt this negative feedback loop, resulting in the aberrant constitutive expression of PD-L1 protein and continuous T-cell deactivation.

2.5 Conclusion

When we embarked on our studies, we most desired to understand how SHP-2 influences response to ICI therapy in KRAS-active tumors in order to direct our drug discovery efforts in a wet-lab setting. A limitation of the data deposited in the NCI-GDC is that the clinical data are often incomplete and lacking details on drug treatment and associated response or perhaps pre-date a particular therapy, like ICI in this case. However, we were able to address the expression of SHP-2 and PD-L1 in KRAS-active LUAD (~26% of the tumors). KRAS status did not change the outcome of the analysis. We identified other studies in which RNAseq data was collected from tumors treated with ICIs, one in melanoma and one in NSCLC (63, 64). PLOS ONE Tumor genomic data informs benchtop experimental design (59-63). While the focus of our study is on NSCLC, treatment of melanoma using ICIs was approved a few years prior to use in NSCLC, and thus the data available in this cancer with respect to ICI treatment is more mature. It should be noted that melanomas rarely harbor KRAS mutations and more often HRAS mutations. Neither of the two small studies made the mutation status of Ras available.

The melanoma study was embarked by Regev and colleagues (63) and sought identify a gene expression profile that is associated with immune evasion that might predict response to ICI treatment. They conducted scRNAseq on melanoma tumors that were either untreated at the time of sequencing, or had acquired resistance to ICI therapy. These data allowed us to determine whether PTPN11 and CD274 gene expression associated with response to therapy. The authors of the study were more interested with defining signatures of resistance that could be used to screen patients prior to ICI therapy, so the experimental design was not ideal and the sample size was small. Importantly, PTPN11 mRNA levels were roughly equivalent between the treatment naïve and ICI resistant tumors. While this

analysis provides some insight into the landscape of SHP-2 and PD-L1 coexpression, it is important to acknowledge that these tumors did not originate from the lung, have differing oncogenic mutations, and sample sizes were relatively low.

Finally, we used data from the NSCLC study carried out by Hwang and colleagues in which they sought to identify immune gene signatures that may predict clinical response to anti-PD-1 therapy (64). The authors performed RNAseq on 21 NSCLC tumors that were divided by response to ICI therapy. For our analysis, we used average TPM values for PTPN11 and CD274 and compared tumors based on response to therapy. Expression of PTPN11 did not associate with DCB, but the tumors from patients who experienced DCB displayed increased expression of CD274 mRNA, consistent with other studies (71–73). Taken together, these two studies do not suggest that the expression of PTPN11 mRNA is associated with to response to ICI therapy. Given these analyses considered alongside the TCPA analysis, it is likely that SHP-2 activity, not expression, bears more importance to PD-L1 expression, and subsequently response to ICI therapy, in NSCLC. Further, our findings suggest that reducing SHP-2 activity by pharmacological means would increase tumoral PD-L1 expression. Patients with PD-L1 expression >50% respond better to ICI therapy, supporting the potential for synergy of the coinhibition of SHP-2 and PD-L1 in NSCLC (73–75).

This study outlines the significance of using of simple and efficient methods in real-world data analysis to further discovery efforts at the benchtop. Each study from which we gathered data had limitations that we have noted. The take-home message is that there is likely value in combining the use of molecules that inhibit the activity of SHP-2 and ICI in

lung tumors and convince us that continued exploration into the role of SHP-2 on both PD-L1 expression is clinically important.

CHAPTER 3. A RETROSPECTIVE ANALYSIS OF POPLAR/OAK TRIALS TO DETERMINE WHETHER SHP2/PTPN11 OR PD-L1/CD274 LEVELS PREDICT RESPONSE TO THERAPY

3.1 Introduction

In NSCLC, there is evidence that atezolizumab, a PD-L1 inhibitor, provides short-term OS and PFS benefit compared to docetaxel for previously treated NSCLC patients from the phase II POPLAR and phase III OAK clinical trials (76), (77). Overall survival is defined as the length of time from the randomization of treatment to death from any cause (78). Progression free survival is defined as the length of time from randomization of treatment to tumor progression or death (79). For the POPLAR and OAK trials, tumor progression was defined as an independent investigator assessing the tumor as progressive disease (PD) using response evaluation criteria in solid tumors (RECIST) v1.1. An unanswered question from those trials, is what happens to survival outcomes and adverse effect incidence if we follow these patients past the primary endpoint of 28 months?

The published data of the combined POPLAR and OAK trials is one of the first studies linking immunotherapy vs chemotherapy to survival outcomes combined with PD-L1 threshold expression and an extensive gene expression profile for every participant (80).

3.1.1 POPLAR Trial

The POPLAR Trial was an open label Phase II randomized controlled trial primarily exploring superiority of atezolizumab against docetaxel in patients with locally advanced, metastatic, or recurrent NSCLC and were previously treated with, at minimum, a single platinum agent containing regimen (76). Patients were stratified by PD-L1 tumor-infiltrating immune cell (TIC) status, histology, and previous lines of therapy. An important

factor in the study design is patients must be ICI-treatment naïve to be included in the POPLAR trial. excluded patients included those previously treated with CTLA-4 inhibitors, PD-1 and PD-L1 inhibitors, or a CD137 inhibitor. Therefore, the study design controlled for acquired resistance to atezolizumab.

The POPLAR trial was also significant for its contribution to future exploratory analysis, with a full biomarker profile, mRNA expression, complete IHC in tumor cells (as percentage of PD-L1-expressing tumor cells $TC_3 \geq 50\%$, $TC_2 \geq 5\%$ and $<50\%$, $TC_1 \geq 1\%$ and $<5\%$, and $TC_0 < 1\%$) and TICs as percentage of tumor area: $IC_3 \geq 10\%$, $IC_2 \geq 5\%$ and $<10\%$, $IC_1 \geq 1\%$ and $<5\%$, and $IC_0 < 1\%$).

The researchers found that OS significantly improved in the atezolizumab arm ($p=0.04$) and there was evidence of increasing improvement in overall survival was associated with increasing PD-L1 expression (TC_3 or IC_3 [$p=0.068$], $TC_{2/3}$ or $IC_{2/3}$ [$p=0.014$], $TC_{1/2/3}$ or $IC_{1/2/3}$ [$p=0.005$], TC_0 and IC_0 [$p=0.871$]). They concluded that atezolizumab significantly improved OS compared with docetaxel in patients with previously treated NSCLC and correlated with PD-L1 IHC expression on tumor cells and TICs, suggesting that PD-L1 expression is predictive for atezolizumab benefit.

3.1.2 OAK Trial

The OAK Trial was an open label Phase III randomized controlled trial primarily exploring superiority of atezolizumab against docetaxel in patients with locally advanced, metastatic, or recurrent NSCLC, exploring efficacy of atezolizumab against docetaxel with identical inclusion and exclusion criteria as the POPLAR trial (77). The major difference between the trials is the scale; POPLAR had 287 patients included in the intention-to-treat

analysis while OAK had 1225 patients at randomization for safety analysis and 850 patients in the primary efficacy analysis.

The primary endpoints for OAK was OS in the intention-to-treat (ITT) population and stratified PD-L1 expression population. The investigators also wanted to test a hypothesis from the results of the POPLAR trial, that PD-L1 expression on tumor cells or tumor-infiltrating immune cells independently contribute to OS. An ITT analysis analyzes patients in the group to which they were originally randomized, irrespective of the treatment the patient received (81).

In the ITT population, overall survival was improved with atezolizumab compared with docetaxel (p=0.0003). Overall survival in the $TC_{1/2/3}$ or $IC_{1/2/3}$ population was improved with atezolizumab (n=241) compared with docetaxel (n=222); median overall survival was 15.7 months with atezolizumab vs 10.3 months with docetaxel (p=0.0102). The researchers concluded that atezolizumab treatment resulting in a clinically relevant improvement of OS versus docetaxel in previously treated non-small-cell lung cancer, regardless of PD-L1 expression or histology.

3.2 Hypothesis

From the POPLAR and OAK trials, OS is improved as PD-L1 TPS is higher. I hypothesize that OS positively correlated with CD274 (PD-L1 gene) expression. To further understand the mechanism behind PD-L1 and CD274 regulation, I also hypothesize that PD-L1 protein expression and CD274 gene expression are positively correlated. Further, continuing from Chapter 2, phosphorylated SHP2 and PD-L1 are negatively correlated, therefore I hypothesize that PTPN11 (SHP-2 gene) and CD274 are negatively correlated. Finally, I will be exploring the JAK2/STAT3 signaling pathway with PD-L1 and CD274

expression. Previous literature established JAK2 and phosphorylated STAT3 are upregulated with upregulated PD-L1 expression. In parallel with PTPN11, I hypothesize that JAK2 and STAT3 genes are both positively correlated with CD274 expression.

3.3 Methods

Deidentified patient dataset (EGAF00004681873) from the linked phase 2 POPLAR and phase 3 OAK trials were utilized in this analysis and provided by Genentech. Demographics in the dataset included age, gender, and race. Clinical data included type of cancer, study arm, PD-L1 status, PD-L1 tumor cell scoring (IC), PD-L1 tumor infiltrating cell scoring (TC), and tumor mutation burden. Clinical outcomes data included overall survival (OS) defined by death from any cause, progression free survival (PFS) defined by disease progression or death, and overall response rate (ORR) defined by the RECIST criteria. Patients were censored if they did not meet the OS or PFS endpoint at study cutoff. A separate RNAseq dataset (EGAF00004943100) was also obtained from Genentech. RNA encoding PD-L1 and SHP-2 were CD274 and PTPN11, respectively.

In the POPLAR trials, patients were randomized 1:1 into atezolizumab and docetaxel arm. PD-L1 immunohistochemistry (IHC) positivity status was defined as previously reported in POPLAR (cite). Patients were stratified based on treatment arm (docetaxel or atezolizumab) and PD-L1 immunohistochemistry (IHC) expression (positive or negative).

From EGAF00004943100, the data was filtered down to match the 156 patients from EGAF00004681873 using Python Data Analysis (pandas v1.4.1) Library in Jupyter Notebook (v6.4.3) into a single pandas DataFrame. From this single DataFrame, all clinical data types, demographic information, and RNA data were indexed and could be accessed

to produce smaller DataFrames for modular data analysis. Code is available at <github link>

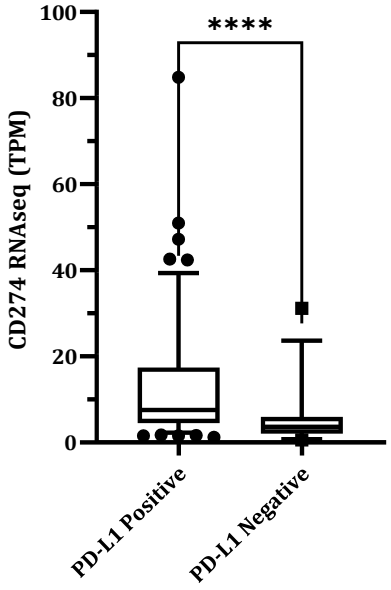
3.3.1 Data Analysis

All data analysis was performed in GraphPad Prism (v9.0.0) between patients in the atezolizumab arm using simple linear regression and a two-tailed, t-test with Welch's correction to compare means and a two-tailed F-test correlation analysis to compare variances. Both significance tests will be calculated with 95% confidence intervals. An alpha value of 0.05 was utilized to determine statistical significance. Cox regression was used for multivariable and survival analysis using R (v1.4.1103). Variables used in the multivariable analysis included gender, age (≥65 or <65), and race. PD-L1 IHC positivity, IC status, TC status, and ORR were not included in the multivariable model since these outcomes were correlated with improved PFS and OS. Therefore, these factors can contribute as confounders.

3.4 Results

From the POPLAR and OAK trials, there were 156 patients followed with previously treated NSCLC, with 119 patients PD-L1 positive and 37 patients PD-L1 negative. In PD-L1 positive patients, the mean CD274 RNAseq transcripts per million (TPM) was significantly higher at 12.57, and in PD-L1 negative patients, the mean CD274 RNAseq TPM was 5.518 [(95% C.I. 2.799 to 11.30), p-value 0.00129] (**Fig 3.1**).

CD274 RNASeq in POPLAR and OAK NSCLC Patients Treated with Atezolizumab or Docetaxel Stratified by PD-L1 Expression

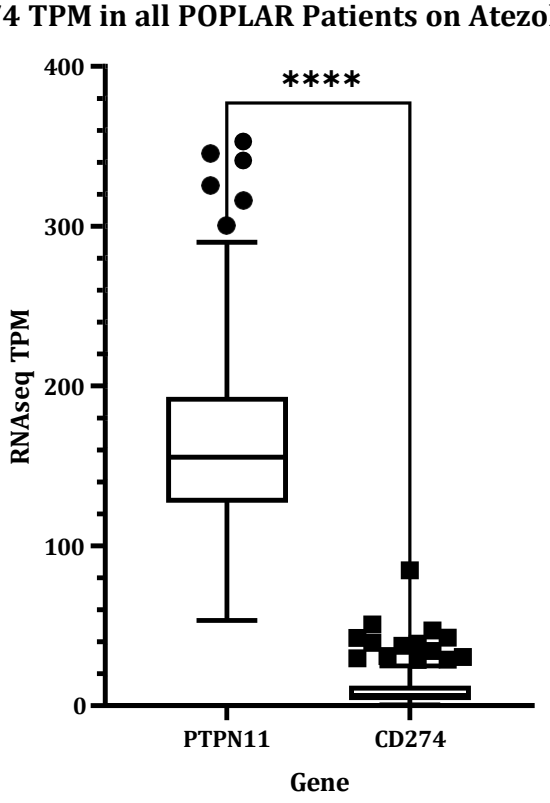


[Figure 3.1 Two-tailed unpaired t-test comparing means between PD-L1 positive and PD-L1 negative patients from POPLAR trial receiving atezolizumab or docetaxel]

[The mean CD274 TPM was significantly higher in PD-L1 positive patients than PD-L1 negative patients. **** signifies p-value < 0.001]

There were 81 patients followed that were treated with atezolizumab from the POPLAR trial. In these patients, the mean PTPN11 TPM was 41.9 and the mean CD274 TPM was 36.0. I found a significant difference in mean TPM between PTPN11 and CD274 using an unpaired t-test with Welch's correction (p-value < 0.001). A plot of the data is shown in **Fig. 3.2**. There was a weak, negative correlation between PTPN11 TPM and CD274 TPM in this population (Pearson's r = -0.067, p-value = 0.41). When stratifying by PD-L1 positive IHC status, the correlation is also not statistically significant (Pearson's r = -0.103, p-value = 0.45). PTPN11 had a positive correlation of 0.14 with OS and a

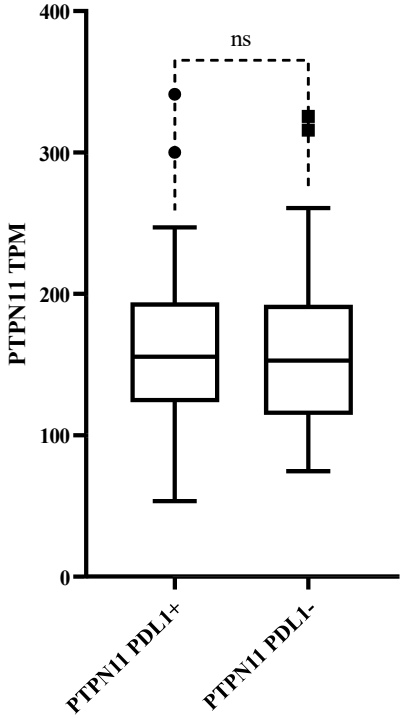
negative correlation of -0.08 with PFS. Both results were not significant. CD274 had a positive correlation of 0.11 with OS and a positive correlation of 0.01 with PFS. Both results were not significant.



[Figure 3.2 PTPN11 and CD274 TPM in POPLAR Patients on Atezolizumab Monotherapy]

[Figure 3.2. Two-tailed unpaired t-test with Welch's correction comparing mean TPM between PTPN11 and CD274 in both PD-L1 positive and PD-L1 negative patients from POPLAR trial receiving only atezolizumab monotherapy. **** signifies p-value < 0.0001]

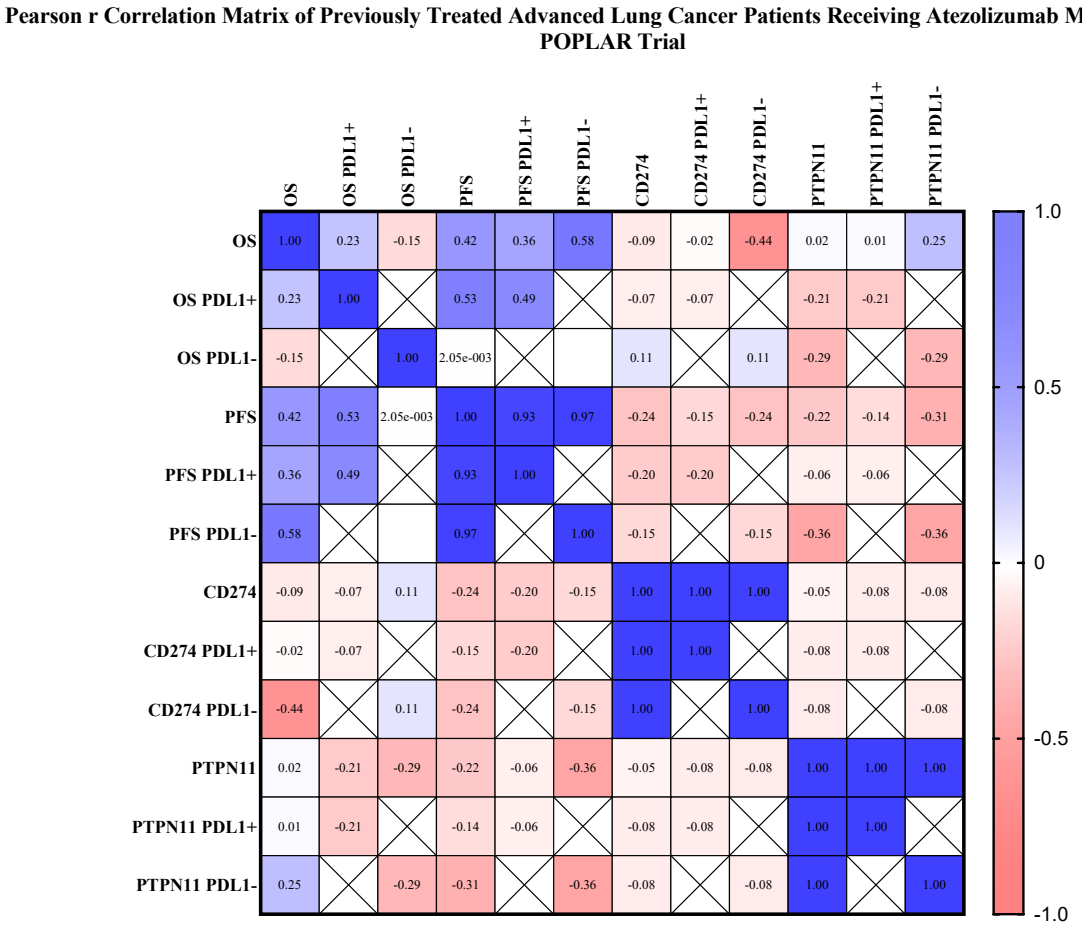
PTPN11 TPM in POPLAR Patients Stratified by PD-L1 Positive or Negative IHC Expression on Atezolizumab Monotherapy



[Figure 3.3 PTPN11 TPM in POPLAR Patients Stratified by PD-L1 Positive or Negative IHC Expression on Atezolizumab Monotherapy]

[Figure 3.3. Two-tailed unpaired t-test with Welch's correction comparing mean PTPN11 TPM in patients that are PD-L1 positive vs PD-L1 negative from POPLAR trial receiving only atezolizumab monotherapy. There was no significant interaction between these two variables. ns signifies not significant.]

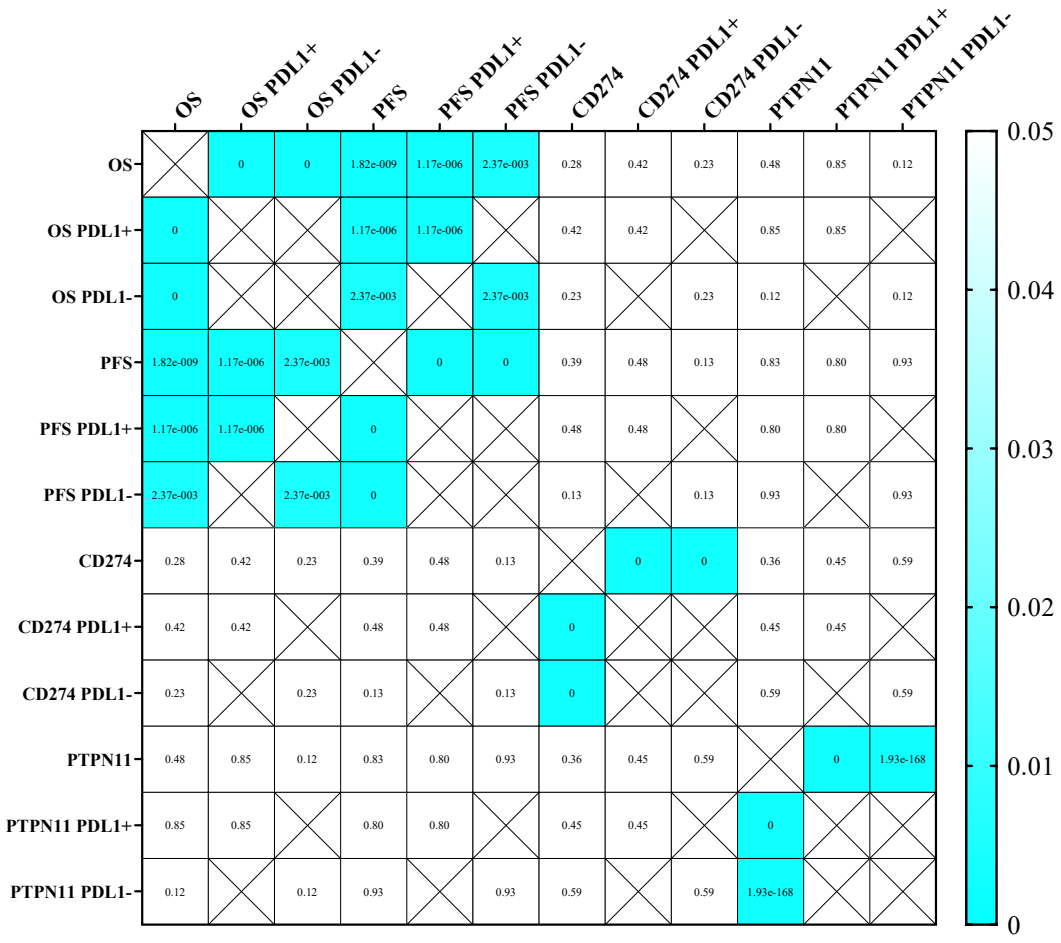
-1.0



[Figure 3.4 Pear r Correlation Matrix of Previously Treated Advanced Lung Cancer Patients Receiving Atezolizumab Monotherapy in POPLAR Trial]

[Figure 3.4. Pearson's r calculated for each independent variable on the X and Y axes. From the Upper Left Y Axis, Overall Survival is correlated with all 12 independent variables. OS is then stratified by patients with positive PD-L1 IHC expression (PDL1+) or negative PD-L1 IHC expression (PDL1-). A correlation of 1.0, denoted as bold blue, is a perfect positive correlation, while a correlation of -1.0, denoted as bold red, is a perfect inverse or negative correlation.]

P values: Correlation Matrix of Previously Treated Advanced Lung Cancer Patients Receiving Atezolizumab Monotherapy in POPLAR Trial



[Figure 3.5 P Values of Correlation Matrix of Previously Treated Advanced Lung Cancer Patients Receiving Atezolizumab Monotherapy in POPLAR Trial]

[Figure #3.5. P-values for each of Pearson's r calculated in Fig. #3.4 using a two-tailed unpaired t-test with Welch's correction. Significance was defined with alpha = 0.05. Significant results where $p < 0.05$ were highlighted in cyan.]

3.5 Discussion

In this study, I applied correlation, survival, and significance testing to gene expression and survival outcomes data provided by Genentech in order to better understand how CD274 (PD-L1 gene) and PTPN11 (SHP-2 gene) affects overall and progression-free survival outcomes when treated with an immune checkpoint inhibitor (atezolizumab).

From Figure 3.1, CD274 TPM was significantly higher in patients stratified to PD-L1 positive groups, or patients with a minimum of PD-L1-expressing tumor cells ≥ 1% or a minimum of PD-L1 expressing tumor-infiltrating immune cells ≥ 1%. I used this approach to validate if PD-L1 protein expression is higher, then CD274 gene expression is higher, which it is in this population of patients with advanced lung cancer.

In Chapter 2, it was shown that active SHP-2 affects PD-L1 expression. I wanted to test if there was a connection between gene expression of those two proteins. While there was a significant difference in gene expression of PTPN11 and CD274 (Figure 3.2), there was not a significant correlation between the TPM of those two genes. There also was not a significant difference in mean PTPN11 TPM between patients that were PD-L1 expression positive and patients that were PD-L1 expression negative (Figure 3.3). Fig. 3.4 and Fig. 3.5 answer the questions, are CD274 (PD-L1 coding gene) or PTPN11 (SHP-2 coding gene) correlated with each other, or with OS or PFS, and are the correlations statistically significant? I expected CD274 to be positively correlated with OS and PFS, since PD-L1 expression is associated with improved OS and PFS. CD274 was positively correlated with OS ($r = 0.12$) but inversely correlated with PFS ($r = -0.27$), even when stratified to patients that are PD-L1 positive ($r = -0.11$), meaning as CD274 expression increases, the PFS duration is expected to decrease. PTPN11 on the other hand, was slightly correlated with both OS ($r = 0.08$) and very slightly with PFS ($r = 0.03$).

While phosphorylated SHP-2 negatively regulates PD-L1 expression, it may not necessarily be true that PD-L1 up- or down-regulates PTPN11 expression, which this data supports. PTPN11 has interactions with many other pro-inflammatory and signaling genes, such as STAT3, that could cause of the significant difference in mean TPM level. Further,

PTPN11 could be upstream or downstream of regulatory molecules directly affecting PD-L1 expression. A limitation of the data is the lack of protein expression; neither active SHP-2 nor total SHP-2 levels were measured in this patient population. Since phosphorylated SHP-2 downregulates PD-L1, regulatory molecules could affect the amount of translation of PTPN11 mRNA to SHP-2, or a phosphatase could be overexpressed and deactivated SHP-2 in PD-L1 positive patients.

Analyzing patient populations based on PD-L1 IHC expression scores were considered, however the patient sample sizes were too small, with 8 patients being randomized to $TC_{1,}$ 9 patients to TC_2, and 8 patients to TC_3 in the atezolizumab arm of the POPLAR trial. The small sample sizes would make it hard to generalize predictions to the larger patient population of advanced stage lung cancer.

3.6 Conclusion

The overarching question this analysis answered is, do protein coding genes of oncogenic predict overall and progression free survival? With respect to CD274, the protein coding gene for PD-L1, the answer is no. CD274 was positively correlated with OS but negatively correlated with PFS, and neither was significant. Furthermore, while PTPN11, the SHP-2 protein coding gene, was inversely correlated with CD274, it was not statistically significant, nor was it statistically significant in predictive value for OS or PFS. For both CD274 and PTPN11, stratification based on PD-L1 positive or negative IHC did not play a role in changing the outcome of the analysis.

CHAPTER 4. CONCLUSION

As lung cancer continues to be a Top 10 cause of death in the United States, the vastness of publicly available data will increase, as will the informatics approaches to analyze the enormous clinical and patient datasets produced from clinical trials. The work here describes two similar informatics-based approaches to logically analyze predictive biomarkers in a patient population with a very poor prognosis. Cell signaling pathways, such as JAK2 and STAT3, have been shown to be upregulated in NSCLC tumors, as well as oncogenic proteins such as SHP-2. However, the missing link is the connection to between oncogenic gene expression and patient survival outcomes. Therefore, I hypothesized that upregulated cell signaling genes, JAK2 and STAT3, would be positively correlated with patient overall and progression free survival. I also hypothesized PTPN11 (the coding gene for the protein SHP-2) would be inversely correlated with patient survival in response to immunotherapy, since its mechanism is to inhibit PD-L1 expression.

4.1 Summary of Results

From the TCPA and GDC data repositories for NSCLC patients, the single, most important observation was the strong inverse correlation between the active ,phosphorylated form of SHP-2 and PD-L1 protein expression ($r = -0.157$, p-value $= 0.0028$**).

Next, patient data from the POPLAR trial was made available by Genentech, and I could analyze relationships between genome and NSCLC patient survival outcomes, when treated with an immune checkpoint inhibitor (ICI). While there was a statistically significant difference in PTPN11 RNAseq TPM between PD-L1 IHC positive and PD-L1 IHC negative patients (p-value < 0.001), there was no statistically significant correlation between PTPN11 and CD274 ($p = 0.36$). Despite this, gene expression of PTPN11 was

inversely correlated with CD274 expression (r = -0.30), similar to the inverse correlation of SHP-2 and PD-L1 protein expression. For each of overall and progression free survival, PTPN11, and CD274 expression, stratifying by PD-L1 subpopulations did not yield any significant correlation.

4.2 Experimental Limitations

A limitation of the data presented by Genentech in the POPLAR and OAK trials is only genomic data was collected. There was no data on concurrent protein expression (neither total nor active proteins).

Another limitation was the possibility of patients having a driver mutation that were included in the analysis but were not tested prior to the clinical trial. The preferred treatment for patients with a driver mutation is a targeted small molecule instead of an ICI or chemotherapy. Therefore, patients with driver mutations may have experienced worse outcomes in these trials.

A final limitation is the relatively small sample size of 156 patients with advanced lung cancer. Meaningful conclusions would be lost when further subdividing the patient population by TC or IC status. For example, the number of patients labeled as TC1, TC2, and TC3 in the atezolizumab arm of the POPLAR trial was 8, 9, and 8 patients, respectively.

4.3 Conclusions and Future Directions

The work described here suggests tumor progression is due to protein-protein interactions rather than changes in gene expression levels in patients with advanced NSCLC. However, PD-L1 IHC expression may not be as clinically valuable as once thought, as there were no significant differences in its correlation, with IHC positive or

IHC negative, with overall or progression free survival when looking at CD274 in patients treated with an immune checkpoint inhibitor. The inclusion of PD-L1 IHC biomarker testing was largely the result of the data published from the POPLAR and OAK trials described earlier, because there was a significant difference in outcomes with patients treated with an immune checkpoint inhibitor when compared to traditional chemotherapy. While the authors of the POPLAR trial reported "Improvement correlated with PD-L1 immunohistochemistry expression on tumor cells and tumor-infiltrating immune cells…", it is not a linear correlation, suggesting that there is other mechanism(s) affecting tumor pathology.

The next steps of this project would be to ask, is PD-L1 expression still a clinically useful predictive factor for treatment of advanced NSCLC in the absence of driver mutations? Or, are there better predictive factors than PD-L1 IHC expression for survival outcomes? Currently, even if patients with advanced NSCLC have PD-L1 IHC expression < 1%, they are still treated with ICIs, the only difference being how long until chemotherapy is added to the regimen, and that is at the clinician's discretion.

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PUBLICATIONS

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