

In-Silico Analysis of The Correlation Between PD-L1 and Pro-Inflammatory Type Interleukins and The Distribution of Their Potential Primary Sources in KRAS-Mutated Non-Small Cell Lung Carcinoma

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ABSTRACT

Objective: Non-Small Cell Lung Cancer has a high incidence and great clinical importance as the cancer subtype with the highest mortality. It is necessary to investigate cytokines associated with the Programmed death-ligand 1, one of the immunotherapeutic target molecules, in KRas mutant lung cancer cells.

Materials and Methods: In this study, the expression of Programmed death-ligand 1 as well as pro-inflammatory interleukins was evaluated in 44 lung cancer cell lines harboring KRas mutations and RNAseq expression data of lung adenocarcinoma patients and correlation analyses were performed. Macrophages and dendritic cells, the major immune cells associated with Interleukin-1, Interleukin-6, Interleukin-12 and Interleukin-23, were also evaluated.

Results: In KRas mutant lung cancer cells and lung adenocarcinoma tissues, expression of cytokines Interleukin-1A, Interleukin-6, Interleukin-12 and Interleukin-23 showed a positive correlation with Programmed death-ligand 1 expression ($p \leq 0.05$). The quantity of M1 macrophages and dendritic cells, both of which are cytokine-producing immune cells, is less in KRas mutant lung cancer tissues than non-mutants.

Conclusion: Detailed studies in clinical samples, especially in blood, primary, and metastatic tissues, will help to create and validate cytokine panels that can be used in therapeutic targeting of KRas mutant subtype lung cancer with high Programmed death-ligand 1 expression.

Keywords: Lung cancer, programmed death-ligand 1, KRas, pro-inflammatory interleukins

INTRODUCTION

Lung cancer is the most prominent cause of cancer-related mortality worldwide. According to the European Union age-standardized mortality rates, the predicted total deaths from lung cancer in 2020 account for about 20% of all cancer deaths [1]. Lung cancer is stratified into 2 major histological categories: small cell lung carcinoma (SCLC, which accounts for 15% of lung cancers) and non-small cell lung carcinoma (NSCLC, which accounts for 85% of lung cancers) [2].

KRas is a frequent oncogenic driver in NSCLC associated with shortened survival [3]. Comprehensive molecular research including in-depth analysis of genomic profiling, genetic alterations, and co-occurring mutations detected in KRas-related NSCLC plays a critical role in elucidating tumor susceptibility to anti-cancer strategy regimens [4]. While the incidence of KRAS mutation in small cell lung cancer (SCLC) varies between 1% and 16%, the frequency of KRAS mutation is between 12% and 36% in lung adenocarcinomas [5].

PD-L1, also known as CD274 or B7 homolog 1 (B7-H1), is expressed on the surface of immune cells including dendritic cells, macrophages, and T and B lymphocytes as well as on tumor cells enhancing escape from immune surveillance [6,7]. Targeting PD-L1 and its receptor with immunotherapy regimens has enhanced the survival of lung cancer patients [8].

The signaling axis of PD-L1 and its receptor PD-1 is responsible for immune suppression in the tumor microenvironment and can be regulated by cytokines and growth hormones. PD-L1 expression can be constitutive or induced by a variety of cytokines [9]. It has been reported that the expression of PD-L1 is increased through the presence of cytokines such as Interleukin-1 α , Interleukin-10, Interleukin-27, and Interleukin-32 γ in addition to Interferon- γ [10].

Clinical trials have suggested that lung cancer patients with KRas mutations are more sensitive to PD-1/PD-L1 inhibitors than those with wild-type KRas [11]. Mutant KRas proteins and infiltrating immune cells have been studied in lung cancer patients and the application of immunotherapy

strategies mutant KRas variants expressed by cancer cells draw attention to the classification of lung cancer patients [12]. In lung adenocarcinoma, KRAS mutation may promote PD-L1 expression via p-ERK signaling [13].

Inflammatory cytokines such as IL-6 have been shown to promote RAS-associated tumorigenesis in the lung, pancreas, and other cancer types [14]. Additionally, genetic ablation of the IL6 gene or treatment with an IL6 neutralizing antibody has been demonstrated to delay Ras-driven tumorigenesis [15]. Although the mechanistic studies on how pro-inflammatory cytokines regulate PD-L1 expression are ongoing, a compilation of the extensive in-silico data obtained will contribute to the discovery of targeted therapy tailored to mutant KRas lung cancer with high PD-L1 expression.

In this study, the expression of PD-L1 and pro-inflammatory interleukins along with the correlation between PD-L1 and IL-1, IL-6, IL-12 (IL-12A) or IL-23 was evaluated in KRas mutated lung cancer cell lines. Also, the distribution of the immune cells, which are the potential source of these pro-inflammatory cytokines, supports the notion that high PD-L1 expression may contribute to the suppressive immune environment via changes in the expression of pro-inflammatory cytokines driven by KRas mutation.

MATERIALS and METHODS

Rnaseq Analysis of PD-L1 and Pro-Inflammatory Interleukin Type Cytokines in NSCLC Cells

Lung cancer cells in the Cancer Cell Line Encyclopedia (CCLE) database were filtered and categorized according to whether they carry a KRas mutation and their histological characteristics. In addition, the Catalogue Of Somatic Mutations In Cancer (COSMIC) database was utilized in order to identify and classify KRas mutated lung cancer cells. 44 cell lines were described as KRas mutated out of 142 NSCLC cell lines expressing PD-L1. The expression of PD-L1 and pro-inflammatory interleukins IL-1 (IL-1A), IL-6, IL-12 (IL-12A), IL-17, IL-18, IL-22 or IL-23 (IL-23A) were explored and sorted in the 44 NSCLC cell lines identified using the CCLE database at <https://portals.broadinstitute.org/ccle>.

Correlation Analysis between PD-L1 and IL1/IL6/IL-12/IL23 Cytokines in Non-Small Cell Lung Adenocarcinoma

The RNAseq expression data of the PD-L1 gene from lung adenocarcinoma patients using The Cancer Genome Atlas (TCGA, PanCancer Atlas; n=510) were downloaded. The correlations between PD-L1 and pro-inflammatory IL-1 (IL-1A), IL-6, IL-12 (IL-12A) or IL-23 (IL-23A) were evaluated using the cBioportal database at <https://www.cbioportal.org>.

Tumor IMMune Estimation Resource (TIMER Analysis) Assessment

The correlation between KRas mutation and the infiltration of macrophages and dendritic cells, the main immune cells associated with IL-1, IL-6, IL-12 and IL-23, were analyzed with Spearman correlation analysis using the TCGA lung adenocarcinoma patient cohort (n=515) in TIMER database [16-18] at <http://cistrome.org/TIMER/>. P-value of 0.05 was used as a threshold for determining significance. CIBERSORT algorithm [19] was used to assess the relative proportions of M1 macrophage profiling. On the other hand, TIMER algorithm [18] was utilized for the analysis of myeloid dendritic cells.

Statistical Analysis

Significant correlations between PD-L1 and pro-inflammatory Interleukin expression levels were examined using the Pearson correlation. Statistical significance was determined using a p-value of 0.05 or less based on a two-tailed test. Wilcoxon, a non-parametric test, was used to compare distribution of immune infiltration in KRas mutant versus wild-type lung adenocarcinoma patients. Analyses were conducted using GraphPad Prism version 8.1.1.

RESULTS

Demonstration of Gene Expressions of PD-L1 and Pro-Inflammatory Interleukins in KRas Mutated Non-Small Cell Lung Cancer Cell Lines.

Gene expression data for lung cancer cell lines were downloaded from the Cancer Cell Line Encyclopedia (CCLE) database at www.broadinstitute.org/ccle. To begin, PD-L1 expression was evaluated using the RNA-seq dataset of lung cancer cell lines and was compared between and NSCLC (n=142) and SCLC (n=48). From this analysis, PD-L1 expression was

shown remarkably higher in NSCLC versus SCLC (Figure 1, $p \leq 0.05$) according to the determined mean & 95% confidence intervals. Following the analysis of the results, 44 human NSCLC cell lines with KRas mutation were evaluated in terms of PD-L1 (Figure 2A) and pro-inflammatory type cytokine expression.

In addition to the expression of PD-L1, the mean expression of pro-inflammatory interleukins including IL-1A, IL-6, IL-12 (IL-12A), IL-18, and IL-23 are shown for that particular non-small cell lung cancer cell line with the solid black dots (Figure 2B-F, respectively). Robust Multi-array Average (RMA) Normalization was used to normalize the expression and then the data was converted to log2. This means the difference of 1 unit on the y-scale will correspond to a 2-fold expression difference between two cell-types (Figure 2A-F). IL-17 and IL-22 gene expression were found to be considerably low (data not shown). However, correlation analysis between PD-L1 and the pro-inflammatory interleukins including IL-1 (P=0.05), IL-6 (P=0.002), IL-12A (P=0.0152) and IL-23 (P=0.0411) were performed based on the CCLE data set and found to be significant (Figure 3A-D). On the other hand, IL-18 did not show a correlation with PD-L1 (P=0.76, data not shown) in KRas mutated NSCLC cells.

TCGA Data Supports The Correlation between PD-L1 and The Pro-Inflammatory Interleukins Including IL-1, IL-6, IL-12 and IL-23.

TCGA data were used to confirm the significant correlation between PD-L1 and interleukins detected in lung cancer cell lines as well as in

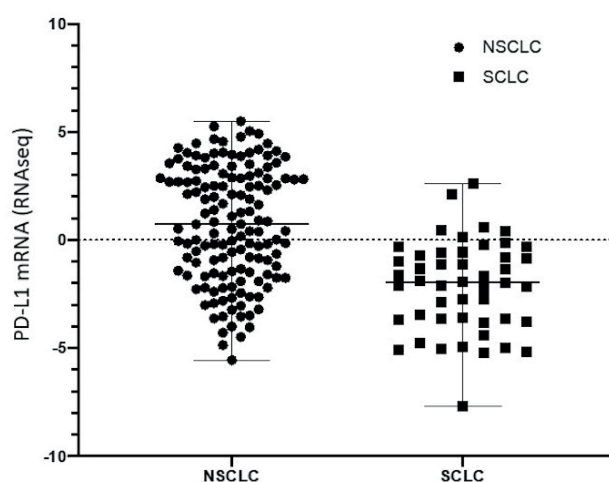


Figure 1. RNAseq data analysis of PD-L1 expression in NSCLC (n=142) and SCLC (n=48) cell lines obtained from CCLE database.

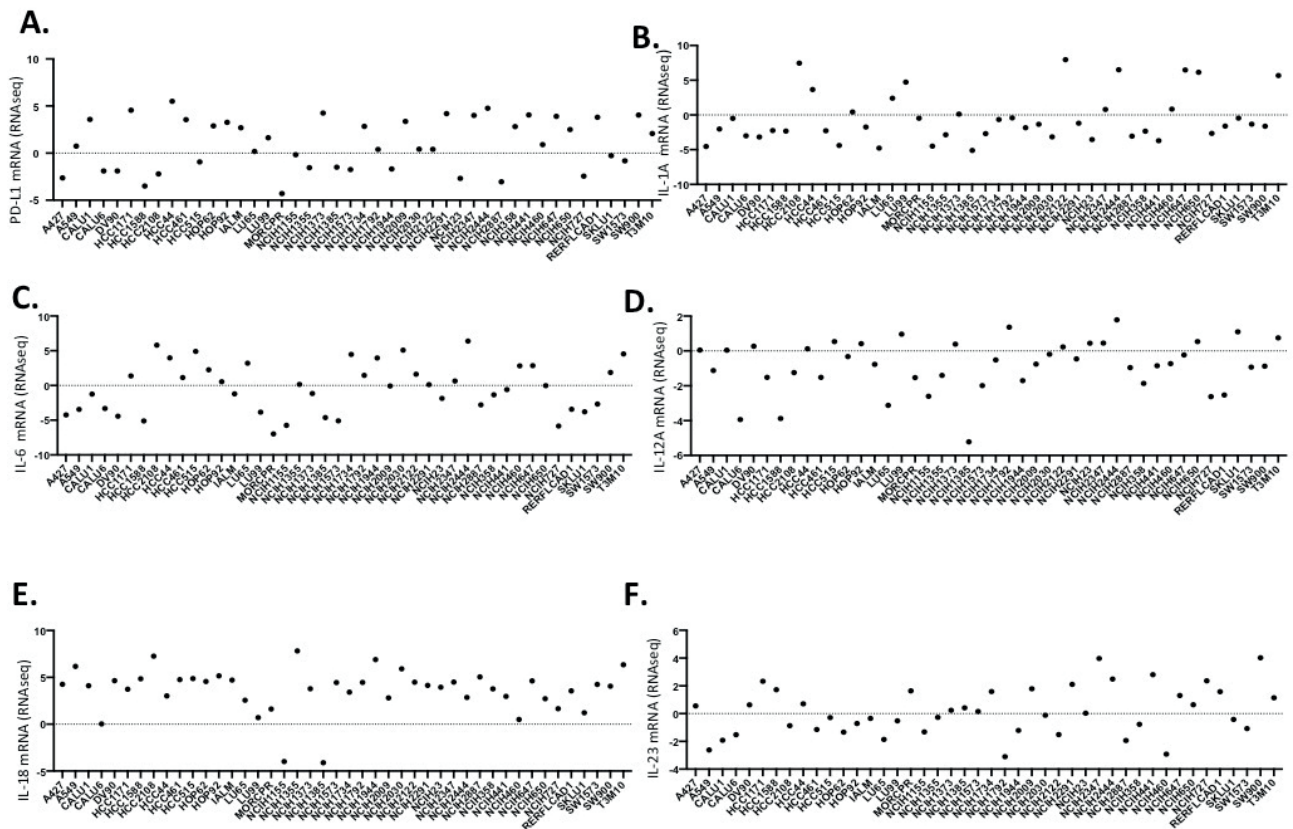


Figure 2. RNaseq data analysis of PD-L1 and pro-inflammatory interleukins in KRas mutant non-small cell lung cancer cell lines based on CCLE data (n=44). A) PD-L1 mRNA expression, B) IL-1A mRNA expression, C) IL-6 mRNA expression, D) IL-12 mRNA expression, E) IL-18 mRNA expression, F) IL-23 mRNA expression shown with the solid black dots of the mean log2-transformed values.

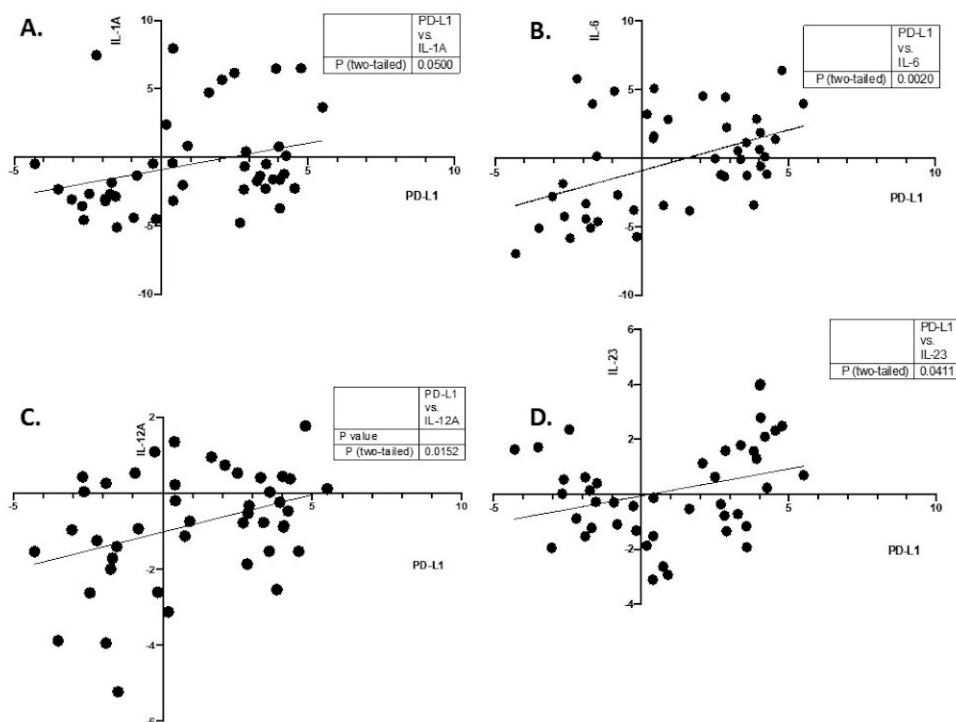


Figure 3. Correlation between PD-L1 and IL-1A/IL-6/IL-12/IL-23 transcript levels based on CCLE dataset with KRas mutant lung cancer cell lines. A) Correlation of gene expression levels between PD-L1 and IL-1A genes. B) Correlation of gene expression levels between PD-L1 and IL-6 genes. C) Correlation of gene expression levels between PD-L1 and IL-12 genes. D) Correlation of gene expression levels between PD-L1 and IL-23 genes.

the tissues of lung adenocarcinoma patients. The correlation analyses have been performed to demonstrate which interleukins have similar expression patterns with the PD-L1 gene.

According to this analysis, PD-L1 (CD274) showed a significant positive correlation with IL-1A, IL-12A and IL-23 in 510 patients with lung adenocarcinoma in the TCGA (PanCancer Atlas) dataset collected with the Illumina RNAseq platform (Figure 4A, Figure 4C-D). (Pearson correlation, IL-1A: 0.35, $P=9.42e-16$; IL-12A: 0.20, $P=4.941e-6$; IL-23: 0.26, $P=3.64e-9$). Furthermore, correlation analysis between IL-6 and PD-L1 (CD274) was shown to have the highest significance (Pearson correlation: 0.37, $P=2.68e-18$) (Figure 4B).

The Distribution of The M1 Macrophages and Dendritic Cells, Which Are The Main Source of The IL-1, IL-6, IL-12 And IL-23 Cytokines, Shows A Decreasing Trend in Kras Mutated Lung Cancer Patients.

In order to compare immune cell distribution between KRas mutant and wild-type adenocarcinoma patients, M1 type macrophages and myeloid dendritic cells were evaluated in 515 patients with lung adenocarcinoma according to TCGA. The violin plot graphs from the 'Mutation Module' displayed the difference in TIMER-estimated differential macrophage/dendritic cell infiltration levels between tumors with KRas mutant or wild-type and represented the log₂ of the fold change in number of each immune cell between KRas and wild-type lung adenocarcinoma patients.

According to the TIMER analyses, it was determined that less M1 macrophages were present in KRas mutant lung cancer patients compared to those without mutations (P value=0.041, Wilcoxon-test) (Figure 5A). Moreover, it has been demonstrated that dendritic immune cells were much fewer in the KRas mutant lung cancer patient group rather than wild-type one (P value=0.0065, Wilcoxon-test)

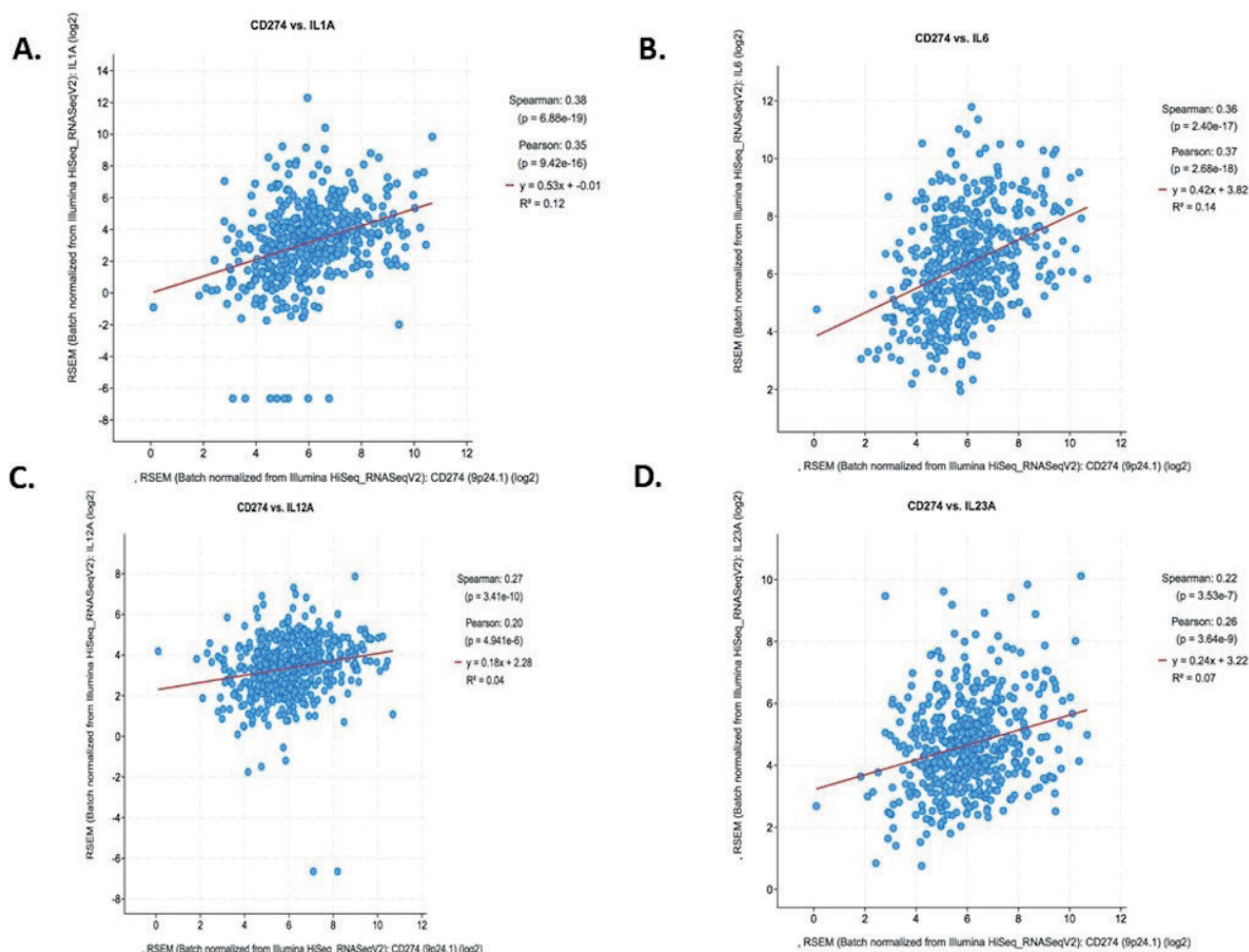


Figure 4. Correlation between PD-L1 and IL-1A/IL-6/IL-12/IL-23 transcript levels based on TCGA dataset with lung cancer adenocarcinoma patients. A) Correlation of gene expression levels between PD-L1 and IL-1A genes. B) Correlation of gene expression levels between PD-L1 and IL-6 genes. C) Correlation of gene expression levels between PD-L1 and IL-12A genes. D) Correlation of gene expression levels between PD-L1 and IL-23 genes.

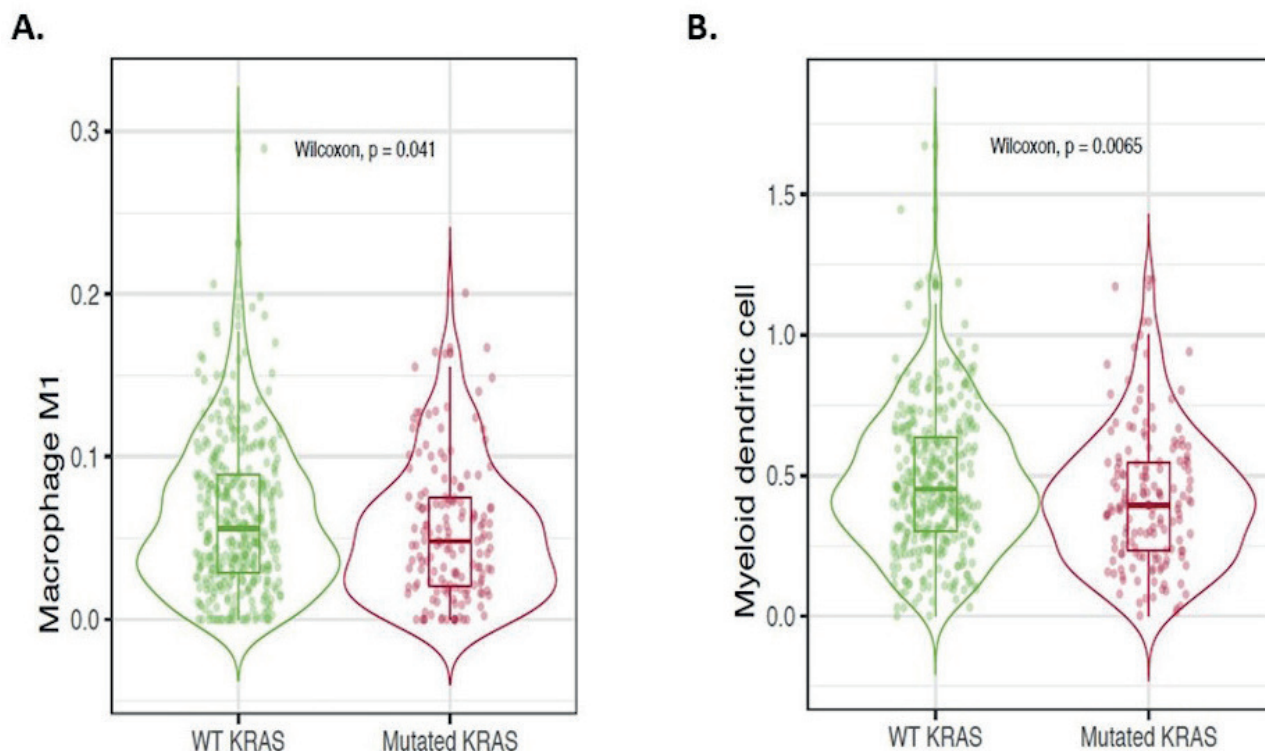


Figure 5. Violin plots graphs of macrophage and dendritic cell infiltration. A) TIMER-estimated M1 macrophage infiltration levels between tumors with mutant or wild-type KRAs in lung cancer adenocarcinoma. B) Dendritic cell infiltration levels between tumors with mutant or wild-type KRAs in lung cancer adenocarcinoma.

(Figure 5B). Collectively, decreased infiltration of both M1 macrophages and myeloid dendritic cells was presented in lung adenocarcinoma patients carrying mutant KRAs vs wild-type group.

DISCUSSION

While very rare in squamous cancer, the diversity of KRAs codon 12 mutations seen in lung adenocarcinoma, the accompanying amino acid change, and the persistent active phase of the KRAs oncoprotein account for a major part of the heterogeneity in lung cancer with distinct KRAs mutation subtypes [20]. Since mutant KRAs proteins do not show very high antigenicity, strategies are being developed to strengthen immune responses by recognizing KRAs mutants as neo-antigens [21].

PD-L1 expression is associated with KRAs mutations in patients with NSCLC [22]. It has been shown that PD-L1 expression also can be regulated with MAPK inhibitors. The increase of PD-L1 expression through the KRAs mediated p-ERK pathway contributes to the apoptosis of CD3+ T cells and thus the escape of lung adenocarcinoma cells from immune surveillance [15,23]. Therefore, shedding

light on the mechanisms of PD-L1 regulation in KRAs mutant lung cancer cells will contribute to the success of immunotherapy approaches.

Although the basal level of expression of pro-inflammatory cytokines is low in KRAs mutant lung cancer cells, IL-1A, IL-6, IL-12A and IL-23 expression were found to positively correlate with PD-L1 expression. This correlation was also observed in the TCGA data set, in which the KRAs mutation is the primary oncogenic driver.

IFN γ , a hallmark of antitumor immunity, is an inducer of PD-L1 expression [10,24]. In addition to IFN γ , IL-6 can also play a role in the stability of PD-L1 expression [25]. There are ongoing investigations in both genetically modified mouse lung cancer models and clinical samples related to IL-6, which shows a significant correlation with PD-L1 in the analyses of NSCLC cells and tissues shown in this study. Oncogenic Ras has been shown to stimulate the secretion of cytokine IL6 in different cell types. Moreover, IL-6 genetic ablation or blocking via neutralizing antibody has been reported to delay the development of Ras-induced tumors [15]. IL-6 modulates the lung cancer microenvironment by suppressing the M1-type anti-tumoral macrophage response as

demonstrated in BALF [26]. In addition, studies in a genetically modified KRas mutant mouse lung pre-malignant model showed decreased M1 macrophage infiltration in the chemopreventive condition compared to its untreated counterpart [27].

IL-1 is one of the stimulators of NF- κ B, which is associated with the Ras oncogenic pathway. IL-1 acts as a bridge between KRAS signaling and the NF- κ B pathway in pancreatic cancer and facilitates oncogenic development [14]. This supports the need for investigation of KRas mutant sub-group lung cancer at the pathway level. Characterization of the relationship between IL-23 and carcinogenesis has shown that this cytokine is released from bone marrow-derived dendritic cells and macrophages [28]. However, recent studies have reported that epithelial cell-derived IL-23 plays a role in suppressing the immune system during KRas/c-Myc-induced lung carcinogenesis [29]. Interestingly, the immune resource analysis indicates a lower presence of dendritic cells in the KRas mutant lung cancer tissues. This raises the question of which cells are releasing IL-23, as dendritic cells would be assumed to be the most likely potential source. Therefore, revealing the relationship between inflammatory cytokines and PD-L1 gives clues about how the tumor microenvironment is regulated. Immune-phenotyping and transcriptomic profiling of cancer cells and immune cells in the tumor microenvironment modulated by these cytokines will provide detailed information on the cytokine network.

In conclusion, correlation between the expression of pro-inflammatory cytokines and PD-L1 were shown in KRas mutant lung cancer cells. In addition, it was found that there was a lesser presence of the possible immune cell sources of these cytokines in NSCLC patients with KRas mutation. Future functional experiments with IL-6, IL-1, and IL-23 and investigations of the potential regulatory mechanisms between these cytokines and PD-L1 will unveil the utility of cytokine-based monoclonal antibodies in addition to the use of immune checkpoint blockers in immunotherapy. High-resolution gene expression profiling in tumor tissues would contribute to the stratification of KRas mutant lung cancer cells into clinically relevant subgroups. Insightful studies in clinical biopsy samples including different compartments will help to clarify cytokine panels that can be used in therapeutically targeting of KRas subtype of lung cancer with high PD-L1 expression.

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CONFLICT of INTEREST

There is no conflict of interest.

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