PD-L1 and PD-L2 expression correlated genes in non-small-cell lung cancer

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Abstract

Background: Programmed cell death ligand-1 (PD-L1) and ligand-2 (PD-L2) interaction with programmed cell death protein-1 (PD-1) represent an immune-inhibiting checkpoint mediating immune evasion and is, accordingly, an important target for blockade-based immunotherapy in cancer. In non-small-cell lung cancer (NSCLC), improved understanding of PD-1 checkpoint blockade-responsive biology and identification of biomarkers for prediction of a clinical response to immunotherapy is warranted. Thus, in the present study, we systematically described PD-L1 and PD-L2 expression correlated genes in NSCLC.

Methods: We performed comparative retrospective analyses to identify PD-L1 and PD-L2 mRNA expression correlated genes in NSCLC. For this, we examined available datasets from the cancer cell line encyclopedia (CCLE) project lung non-small-cell (Lung_NSC) and the cancer genome atlas (TCGA) projects lung adenocarcinoma (LUAD) and squamous cell carcinoma (LUSC).

Results: Analysis of the CCLE dataset Lung_NSC identified expression correlation between PD-L1 and PD-L2. Moreover, we identified expression correlation between 489 genes and PD-L1, 191 genes and PD-L2, and 111 genes for both. PD-L1 and PD-L2 also expression correlated in TCGA datasets LUAD and LUSC. In LUAD, we identified expression correlation between 257 genes and PD-L1, 914 genes and PD-L2, and 211 genes for both. In LUSC, we identified expression correlation between 26 genes and PD-L1, 326 genes and PD-L2, and 13 genes for both. Only a few genes expression correlated with PD-L1 and PD-L2 across the CCLE and TCGA datasets. Expression of Interferon signaling-involved genes converged in particular with the expression correlated genes for PD-L1 in Lung_NSC, for PD-L2 in LUSC, and for both PD-L1 and PD-L2 in LUAD. In LUSC, PD-L1, and to a lesser extent PD-L2, expression correlated with chromosome 9p24 localized genes, indicating a chromosome 9p24 topologically associated domain as an important driver of in particular LUSC PD-L1 expression. Expression correlation analyses of the PD-L1 and PD-L2 receptors programmed cell death protein-1 (PD-1), Cluster of differentiation 80 (CD80), and Repulsive guidance molecule B (RGMB) showed that PD-1 and CD80 expression correlated with both PD-L1 and PD-L2 in LUAD. CD80 expression correlated with PD-L2 in LUSC.

Conclusions: We present gene signatures associated with PD-L1 and PD-L2 mRNA expression in NSCLC which could possess importance in relation to understand PD-1 checkpoint blockade-responsive biology and development of gene signature based biomarkers for predicting clinical responses to immunotherapy.

Keywords: Non-small-cell lung cancer, Biomarker, Immunotherapy, Immune checkpoints, Interferon, Chr9p24, PD-L1, PD-1, PD-L2

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Background
Non-small-cell lung cancer (NSCLC), the most common form of lung cancer, is a heterogeneous disease with adenocarcinoma and squamous cell carcinoma being the predominant molecular subtypes [1]. Antibody-mediated blockade of programmed cell death protein-1 (PD-1) or cell-surface localized programmed cell death ligand-1 (PD-L1) provides a novel therapeutic paradigm for patients with advanced NSCLC [2–6]. However, only a subset of the NSCLC patients benefits from immunotherapy with PD-L1/PD-1 axis blockade [2, 6]. PD-1 is inducibly expressed on cluster of differentiation 4 positive (CD4+) T cells, cluster of differentiation 8 positive (CD8+) T cells, regulatory T cells, natural killer T cells, B cells, and activated monocytes [7–11]. PD-1 expression is induced by T cell receptor and B cell receptor signaling and augmented by tumor necrosis factor (TNF) stimulation [7–9]. Besides binding to PD-L1, PD-1 can bind to the cell-surface of localized programmed cell death ligand-2 (PD-L2). PD-L1 and PD-L2 compete for binding to PD-1. The interaction of PD-L2/PD-1 shows a 6-fold higher affinity compared to the interaction of PD-L1/PD-1, but the generally lower expression level of PD-L2 may favor PD-L1 as the primary ligand for PD-1 [12]. The binding of PD-1 ligands to PD-1 prevent a cytotoxic T cell response against the tumor cells by inhibiting kinases involved in T cell activation [9, 13–15]. Whereas PD-L1/PD-1 interaction blocks the activation of most T cell subtypes, the PD-L2/PD-1 interaction has been proposed to primarily inhibit the CD4+ T helper 2 subsets (Th2) response [16]. Additionally, PD-L1, but not PD-L2, can bind to the cluster of differentiation 80 (CD80), which can result in a bidirectional immune inhibitory response [17]. Conversely, PD-L2, but not PD-L1, can bind the repulsive guidance molecule B (RGMB) receptor, a co-receptor for bone morphogenetic protein (BMP) [18]. The PD-L2/RGMB interaction co-stimulates CD4+ T cell responses and promotes T helper 1 subset (Th1) polarization [19]. The molecular interactions involving PD-L1 and PD-L2 are illustrated schematically in Fig. 1a. The PD-L1 and PD-L2 genes are tandem localized at Chr9p24.1 with a distance of 60 kb between the cognate promoters. PD-L1 is expressed on a variety of immune cells and cancer cells, including NSCLC cells [3, 11, 15]. PD-L2 is expressed on macrophages, dendritic cells and cancer cells, including NSCLC cells [3, 11]. In general, PD-L2 expression is less prevalent than PD-L1 expression in cancer cells [16]. In the tumor microenvironment, PD-L1 and PD-L2 mRNA expression can be induced by immune cell-secreted inflammatory cytokines, such as Interferon-alpha (IFN-α), Interferon-beta (IFN-β), Interferon-gamma (IFN-γ), Interleukin 4, Interleukin 10, and granulocyte/macrophage colony-stimulating factor, resulting in adaptive immune resistance [15, 16, 20, 21]. PD-L1 and PD-L2 mRNA expression can also be upregulated due to cancer cell-autonomous mechanisms, for example, mutation depending oncogenic signaling, resulting in intrinsic immune resistance [22].

Tumor PD-L1 expression in NSCLC has been shown to be associated with poor prognosis in two meta-analyses [23, 24]. Moreover, meta-analyses of clinical trials have illustrated an overall positive response to immunotherapy with PD-L1/PD-1 axis blockade in NSCLC patients and positive correlation between the PD-L1 tumor proportion score (TPS) (the percentage of PD-L1 expressing tumor cells determined by immunohistochemistry) and the response rate [25, 26]. In the CheckMate 017 and CheckMate 057 trials corresponding to squamous and non-squamous NSCLC patients, respectively, patients receiving the PD-1 antibody, nivolumab, as a second-line of treatment showed a response rate of 20% regardless of PD-L1 TPS [4, 5]. Analysis of subgroups defined by PD-L1 expression showed that PD-L1 TPS ≥ 1%, ≥ 5%, and ≥ 10% were associated with improved response.

(See figure on next page.)

**Fig. 1** PD-L1 and PD-L2 expression correlation analyses in 114 NSCLC cell lines from the CCLE dataset (Lung_NSC). a Overview of key factors interacting with PD-L1 and PD-L2 in NSCLC. PD-L1 and PD-L2 are ligands of PD-1 and the interaction of PD-L1 or PD-L2 with PD-1 results in T cell suppression. Moreover, PD-L1 can interact with CD80 on activated T cells and inhibit T cell activity. PD-L2 has a second receptor, RGMB, and this interaction can activate T cells. b Expression correlation analysis of PD-L1 and PD-L2 in the CCLE dataset (Lung_NSC, n = 114) using Broad portal analysis of mRNA sequencing-based gene expression data. Spearman and Pearson correlation coefficients r and the corresponding P value are shown. c Gene expression correlation between PD-1, CD80, RGMB, PD-L1, and PD-L2 in CCLE dataset (Lung_NSC, n = 114) Spearman and Pearson correlation coefficients r and the corresponding P values are shown. The horizontal lines show the pairwise comparison of genes and line thickness is proportional to the degree of expression correlation. Dashed lines indicate negative expression correlation. Significant expression correlation is indicated in red. d Number of genes expressed correlated with PD-L1, PD-L2, and both in CCLE dataset (Lung_NSC, n = 114). For all panels, the criteria for significant expression correlation were Pearson correlation coefficient r ≥ 0.3 or ≤ − 0.3, Spearman correlation coefficient r ≥ 0.4 or ≤ − 0.4, and P values < 0.05. The analysis was performed using the GenomicScape portal.
rates (31%, 36%, and 37%, respectively) in the CheckMate 057, whereas, an association between PD-L1 TPS and response rate was not evident from the CheckMate 017 [4, 5]. In the KEYNOTE-001 and KEYNOTE-24 trials, which were the first trials to use first- and second-line therapies with PD-1 antibody, pembrolizumab, in advanced NSCLC patients, PD-L1 TPS ≥ 50% resulted in response rates of 52% and 45%, respectively [27–29]. The PD-L1 antibodies, atezolizumab, durvalumab, and avelumab, also showed promising response rates as immunotherapy in NSCLC even with low PD-L1 TPS [6, 30]. Unlike the PD-1 antibodies, the PD-L1 antibodies also block the PD-L1/CD80 axis but leave the PD-L2/PD-1 axis unaffected. The response rate in NSCLC was similar for PD-1 and PD-L1 antibody immunotherapy but less immune-related adverse effects were observed from
the latter [31]. The efficient stratification of immu-


therapy responders and non-responders among NSCLC

patients remains a major challenge [2, 3, 6, 32]. Given the

potential overlapping involvement of PD-L1 and PD-L2

for defining immune checkpoint inhibition, we per-

formed this retrospective expression correlation study

to identify genes in NSCLC having expression profiles

closely associated with the PD-L1 and PD-L2 expression

profiles. The presented study illustrates novel aspects

of PD-L1 and PD-L2 regulation, with potential biologi-

cal relevance, as well as relevance for immunotherapy

response stratification.

Methods and materials

Datasets

The following datasets were used for retrospective gene

expression analyses. The cancer cell line encyclopedia

(CCLE) project dataset is a compilation of gene expres-
sion data from human cancer cell lines [33]. The CCLE
subset, lung_non-small-cell (Lung_NSC), contains gene
expression data from NSCLC cell lines, were obtained
from the CCLE portal (https://portals.broadinstitute.org/
ccele/home). The Cancer Genome Atlas (TCGA) project

dataset is a comprehensive atlas of gene expression and

gene regulation across human cancers [34]. The TCGA

data subsets, lung adenocarcinoma (LUAD) and lung

squamous cell carcinoma (LUSC), contain gene expres-
sion data from dissected NSCLC tumors, were obtained
from the TCGA data portal (https://tcga-data.nci.nih.
gov). The Genotype-Tissue Expression (GTEx) pro-
duct dataset is a comprehensive atlas database of gene

expression in human tissues [35]. The GTEx lung subset,

contains RNA sequencing-based expression data from

normal lung tissue, were obtained from the GTEx portal

(http://www.gtexportal.org/home/).

Broad portal analysis

The Broad portal (https://portals.broadinstitute.org/ccele)
was used to generate scatter plots from CCLE Lung_NSC
expression data for PD-L1 and PD-L2 using Illumina
RNA sequencing data (processed with normalization fol-
lowed by Log2 transformation).

GenomicScape portal analysis

The portal GenomicScape (http://www.genomicsdale.
com/) was used to analyze CCLE Lung_NSC expres-
sion data based on deposited microarray data from a
robust multi-array average (RMA) normalized Affymet-
rix Human Genome U133 Plus 2.0 Arrays. The Genom-
icScape co-expression module was used to calculate
pairwise expression correlations between PD-L1, PD-L2,
IFNG, IFNGR1, IFNGR2, STAT1, JAK1, JAK2, IRF1, IRF9,
TYK2, STAT2, STAT3, IFNAR1, IFNAR2, PD-1, CD80,
and RGMB, as well as to generate gene lists composed of
expression correlated genes for PD-L1, PD-L2, IRF1 and
IRF9.

Wanderer portal analysis

The Maplab tool Wanderer (http://maplab.imppc.org/
wanderer/) was used to calculate the mRNA expres-
sion levels of PD-L1, PD-L2, PD-1, CD80, and RGMB
with corresponding Wilcoxon P values in GTEx and
TCGA datasets using RNA sequencing data (in format
Log2(normalized_RSEM+1)).

cBioPortal analysis

TCGA RNA sequencing data (in format V2 RSEM
Z-score) were in the cBioPortal for Cancer Genomics
interface (http://www.cbioportal.org/) used to gener-
ate gene lists composed of expression correlated genes
for PD-L1, PD-L2, IRF1, and IRF9. Correlation coeffi-
cients were calculated before the Log2 transformation
of RNA sequencing data. Log2 transformed TCGA RNA
sequencing data were used for heat map analyses and
hierarchical clustering. Finally, the plot function in the
cBioPortal interface was used to calculate correlation
coefficients between PD-L1 protein expression (from
reverse-phase protein arrays) and mRNA expression of
identified PD-L1 and PD-L2 expression correlated genes.

Xena portal analysis

UCSC Xena browser (https://xenabrowser.net/) was used
to calculate pairwise expression correlation using the
GTEx lung and TCGA RNA sequencing data (in format
Log2(normalized_counts+1)). Expression correlation
coefficients and corresponding P values were calculated
before Log2 transformation using the browser http://
gepia.cancer-pku.cn/ which analyses data present in the
Xena portal.

Gene set enrichment analysis (GSEA)

The molecular signatures database (MSigDB) is a col-
lection of annotated gene sets [36, 37]. GSEA was used
to compute overlaps between gene sets representing
expression correlated genes for PD-L1, PD-L2, Interferon
response factor 1 (IRF1), and Interferon response factor
9 (IRF9) and available gene sets in MSigDB (http://softw
are.broadinstitute.org/gsea/). We analyzed the MSigDB
gene set collection named hallmark gene sets, which
summarized and represented specific well-defined bio-
logical states or processes [36, 37]. Moreover, we ana-
alyzed the MSigDB gene set collection named positional
gen sets, corresponding to each human chromosome
and each cytogenetic band that contained at least one
gen. Values in the tables presenting GSEA represented
the number of genes in the respective MSigDB hallmark
gene sets (K), the number of genes in the examined PD-L1, PD-L2, IRF1, and IRF9 expression correlation gene signatures overlapping with the MSigDB hallmark gene set (k), P value representing the hypergeometric distribution of overlapping genes, and the false discovery rate (FDR) q value after correction for multiple hypothesis testing. Enriched gene signatures with q value < 1E−03 were considered significant. The list of the specific gene sets analyzed and their sources are available in supplementary tables.

Statistical analysis
To decrease data overfitting, the criteria used for significant expression correlation between two genes was a Pearson correlation coefficient \( r \geq 0.3 \) or \( \leq -0.3 \), a Spearman correlation coefficient \( r \geq 0.4 \) or \( \leq -0.4 \), and all corresponding \( P \) values < 0.05. Notably, cBioPortal and Xena portal analyses included gene expression data for different numbers of TCGA LUAD and LUSC patients resulting in minor differences in obtained correlation coefficients and resulting statistics.

Results
Analysis of PD-L1 and PD-L2 expression correlated genes in CCLE dataset
We first addressed PD-L1 and PD-L2 mRNA expression in 114 NSCLC cell lines from the CCLE project Lung_NSC. Retrospective data analyses showed that PD-L1 and PD-L2 mRNA expression was correlated (Fig. 1b, c). PD-L2 mRNA expression was in general 2- to 4-fold lower than PD-L1 mRNA expression (Fig. 1b). Neither PD-L1 nor PD-L2 expression correlated with their respective receptors PD-1, CD80, and RGMB with the criteria for significant expression correlation being a Spearman correlation coefficient \( r \geq 0.4 \) or \( \leq -0.4 \), a Pearson correlation coefficient \( r \geq 0.3 \) or \( \leq -0.3 \), and all \( P \) values < 0.05 (Fig. 1c). Notably, correlation between RGMB and PD-L1 expression, as well as between RGMB and PD-L2 expression, was near significance (Fig. 1c). We next identified PD-L1 and PD-L2 expression correlated genes in the CCLE dataset Lung_NSC. 489 genes expression correlated with PD-L1, 191 genes expression correlated with PD-L2, and 111 genes expression correlated with both PD-L1 and PD-L2 (Fig. 1d and Additional file 1: Table S1). Interestingly, GSEA revealed that PD-L1 and PD-L2 expression correlated genes were enriched to the hallmark gene sets TNFα signaling via Nuclear factor kappa B (NFκB) (genes regulated by NF-kB in response to TNF), Kirsten rat sarcoma viral oncogene homolog (KRAS) signaling (genes up-regulated by KRAS activation), and Transforming growth factor β (TGFβ) signaling (genes up-regulated in response to TGFβ1) (Additional file 2: Fig. S1B and Additional file 3: Table S2). GSEA also showed that PD-L1 and PD-L2 expression correlated genes were enriched for belonging to the epithelial-mesenchymal-transition (EMT) hallmark gene set (genes defining the epithelial-mesenchymal-transition, as in wound healing, fibrosis, and metastasis) (Additional file 2: Fig. S1B and Additional file 3: Table S2). We note a previously described association between EMT, PD-L1 expression, and immunosuppression [38, 39]. GSEA showed that among the hallmark gene sets enriched with PD-L1 expression correlated genes, but not with PD-L2 expression correlated genes, were the hallmark gene sets IFN-α response (genes up-regulated in response to alpha interferon proteins) and IFN-γ response (genes up-regulated in response to IFNγ) (Additional file 3: Table S2). We next addressed how individual genes involved in IFN signaling expression correlated with PD-L1 and PD-L2 in Lung_NSC (Additional file 4: Table S3). Genes in the canonical signaling pathway for IFN-γ include IFN-γ receptor 1 and 2 (IFNGR1 and IFNGR2), Janus kinase 1 and 2 (JAK1 and JAK2), Signal transducer and activator of transcription 1, 2, and 3 (STAT1, STAT2, and STAT3), and Interferon regulatory factor 1 (IRF1). Genes in the canonical signaling pathway for IFN-α include IFN-α receptor 1 and 2 (IFNAR1 and IFNAR2), JAK1 and Tyrosine kinase 2 (TYK2), STAT1, STAT2, and STAT3, and Interferon regulatory factor 9 (IRF9) [21]. In Lung_NSC, 152 genes expression correlated with IRF1 and 248 genes expression correlated with IRF9 (Additional file 2: Fig. S1A and Additional file 5: Table S4). Interestingly, IRF9, but not IRF1, expression correlated with PD-L1 (Additional file 4: Table S3). IRF1 and IRF9 expression correlated not with PD-L2 (Additional file 4: Table S3). We also examined whether the identified Lung_NSC PD-L1 and PD-L2 expression correlated genes (Additional file 1: Table S1) were represented among the identified Lung_NSC IRF1 and IRF9 expression correlated genes (Additional file 5: Table S4). Of the PD-L1 expression correlated genes, 10% (49/489) expression correlated with IRF1, and 17% (83/489) expression correlated with IRF9 (Additional file 2: Fig. S1A). Of the PD-L2 expression correlated genes, 6% (11/191) expression correlated with IRF1, and 5% (9/191) expression correlated with IRF9 (Additional file 2: Fig. S1A). IRF1 and IRF9 were not expression correlated with PD-1, CD80, and RGMB (Additional file 4: Table S3). GSEA showed extensive overlaps between hallmark gene sets enriched with PD-L1 and PD-L2 expression correlated genes and hallmark gene sets enriched with IRF1 and IRF9 expression correlated genes (Additional file 2: Fig. S1B, Additional file 3: Table S2, and Additional file 6: Table S5).
**PD-L1 and PD-L2 expression correlated genes are differentially associated with IFN signaling and immune cell markers in NSCLC tumor samples**

To investigate PD-L1 and PD-L2 mRNA expression in NSCLC tumor samples we analyzed available RNA sequencing data from the TCGA projects LUAD and LUSC datasets, as well as RNA sequencing data for normal lung tissue from the GTEx project. In the LUAD and LUSC datasets, PD-L1 expression was 2- to 3-fold higher than PD-L2 expression (Additional file 7: Table S6). PD-L1 and PD-L2 expression were correlated in LUSC and LUAD tumor samples, and as well as in normal lung tissue (Fig. 2a–c). In LUAD, both PD-1 and CD80 expression correlated with PD-L1, as well as with PD-L2 (Fig. 2b). In LUSC, CD80 expression correlated with PD-L2 (Fig. 2c).

In the LUAD and LUSC datasets, no expression correlation between RGMB and PD-L1 or between RGMB and PD-L2 was identified (Fig. 2b, c). We note that CD80 expression correlated with PD-1 in both the LUAD and LUSC datasets (Fig. 2b, c).

Next, we identified PD-L1 and PD-L2 expression correlated genes in LUAD and LUSC datasets. For the LUAD dataset, 257 genes and 914 genes expression correlated with PD-L1 and PD-L2, respectively (Fig. 2d and Additional file 1: Table S1). We noted that 211 genes expression correlated with both PD-L1 and PD-L2, representing 82% of the PD-L1 expression correlated genes and 23% of the PD-L2 expression correlated genes (Fig. 2d). GSEA showed that PD-L1 and PD-L2 expression correlated genes were enriched for belonging to the same hallmark gene sets, except for the EMT gene set only being significant among the PD-L2 expression correlated genes (Additional file 3: Table S2 and Additional file 8: Fig. S2B). Notably, hallmark gene sets IFN-α response and IFN-γ

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### Table A

| CD80 | PD-L1 | PD-1 | PD-L2 | RGMB |
|------|-------|------|-------|------|
| Sp, r = 0.20, P < 0.01 | Sp, r = 0.14, P < 0.02 | Sp, r = 0.24, P < 0.01 | Sp, r = 0.42, P < 0.01 |
| Pe, r = 0.24, P < 0.01 | Pe, r = 0.24, P < 0.01 |

### Table B

| CD80 | PD-L1 | PD-1 | PD-L2 | RGMB |
|------|-------|------|-------|------|
| Sp, r = 0.66, P < 0.01 | Sp, r = 0.10, P < 0.03 | Sp, r = 0.00, P < 1.00 | Sp, r = 0.76, P < 0.01 |
| Pe, r = 0.46, P < 0.01 | Pe, r = 0.00, P < 0.01 |

### Table C

| CD80 | PD-L1 | PD-1 | PD-L2 | RGMB |
|------|-------|------|-------|------|
| Sp, r = 0.54, P < 0.01 | Sp, r = 0.54, P < 0.01 | Sp, r = 0.42, P < 0.01 |
| Pe, r = 0.20, P < 0.01 | Pe, r = 0.28, P < 0.01 |

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**Fig. 2** mRNA expression correlation analysis of PD-L1 and PD-L2 in GTEx lung dataset and TCGA datasets (LUAD and LUSC) reveals major differences in mRNA expression profiles. a–c Expression correlation between PD-L1, PD-L2 and mRNA for cognate receptors CD80, PD-1, and RGMB in normal lung tissue from GTEx lung (n = 387) a, TCGA dataset LUAD (n = 708) b, and TCGA dataset LUSC (n = 626) c using Xena portal data. Spearman and Pearson correlation coefficients r and their corresponding P values are shown. The horizontal lines show the pairwise comparison of genes and line thickness is proportional to the degree of expression correlation. Dashed lines indicate negative expression correlation. Significant expression correlation is indicated in red. d, e Venn diagrams illustrating the number of genes expression correlated with PD-L1, PD-L2, and both, in TCGA dataset LUAD (n = 517) d, and LUSC (n = 501) e, using cBioPortal. For all panels, the criteria for significant expression correlation were Pearson correlation coefficient r ≥ 0.3 or ≤ −0.3, Spearman correlation coefficient r ≥ 0.4 or ≤ −0.4, and P values < 0.05. Pe Pearson, r correlation coefficient, Sp Spearman.
response were among the hallmark gene sets for both PD-L1 and PD-L2 expression correlated genes (Additional file 3: Table S2 and Additional file 8: Fig. S2B). In the LUSC dataset, 26 genes expression correlated with PD-L1, and 326 genes expression correlated with PD-L2 (Fig. 2e and Additional file 1: Table S1). Of these, 13 genes expression correlated with both PD-L1 and PD-L2, representing 50% of the PD-L1 expression correlated genes but only 4% of the PD-L2 expression correlated genes (Fig. 2e). GSEA showed that all hallmark gene sets enriched for PD-L1 expression correlated genes also were enriched for PD-L2 expression correlated genes (Additional file 3: Table S2 and Additional file 8: Fig. S2D).

We also investigated the connection between PD-L1 and PD-L2 expression correlated genes and IFN signaling, with IFN signaling being represented by the 13 genes IFNG (encoding IFN-γ), IFNGRI, IFNGR2, IFNAR1, IFNAR2, JAK1, JAK2, TYK2, STAT1, STAT2, STAT3, IRF1, and IRF9 (Additional file 9: Table S7). We performed an expression correlation analysis between these 13 genes and PD-L1 and PD-L2 (Additional file 4: Table S3). In the LUAD dataset, PD-L1 and PD-L2 expression correlated with IRF1, IFNG, STAT1, and JAK2 (Additional file 4: Table S3). In addition, PD-L2 expression correlated with IFNGRI1 (Additional file 4: Table S3). Notably, PD-L1 expression was not correlated with IRF9, a different scenario from that observed in CCLE Lung_NSC. In line with this, we noted that in the LUAD dataset the number of expression correlated genes with IRF1 (559 genes) was higher than that for IRF9 (82 genes) (Additional file 5: Table S4 and Additional file 8: Fig. S2A) which differed from the observations for CCLE Lung_NSC (Additional file 2: Fig. S1A and Additional file 5: Table S4). Prominent numbers of commonly correlated genes for IRF1 and PD-L1 (n = 141), as well as for IRF1 and PD-L2 (n = 488), were observed in LUAD (Additional file 8: Fig. S2A). This further illustrated that only 66/559 IRF1 expression correlated genes were not PD-L1 or PD-L2 expression correlated (Additional file 8: Fig. S2A). In comparison, there were fewer commonly correlated genes for IRF9 and PD-L1 (n = 19) and for IRF9 and PD-L2 (n = 44) (Additional file 8: Fig. S2A). GSEA showed extensive overlapping among hallmark gene sets enriched with PD-L1, PD-L2, IRF1, and IRF9 expression correlated genes (Additional file 3: Table S2, Additional file 6: Table S5 and Additional file 8: Fig. S2B).

In the LUSC dataset, of the 13 IFN signaling genes JAK2 and IRF1 expression correlated with PD-L1 (Additional file 4: Table S3). Moreover, 10/26 of the PD-L1 expression correlated genes were IRF1 expression correlated and 3/26 of the PD-L1 expression correlated genes were IRF9 expression correlated (Additional file 8: Fig. S2C). GSEA showed that the hallmark gene sets IFN-γ response and allograft rejection (genes up-regulated during transplant rejection) were common hallmark gene sets for both PD-L1 and IRF1 expression correlated genes, as well as for PD-L1 and IRF9 expression correlated genes (Additional file 3: Table S2, Additional file 6: Table S5 and Additional file 8: Fig. S2D). In the LUSC dataset, of the 13 IFN signaling genes IFNG, JAK2, STAT1, and IRF1 expression correlated with PD-L2 (Additional file 4: Table S3). As also observed in the LUAD dataset, in the LUSC dataset the number of IRF1 expression correlated genes (n = 751) was more pronounced than the number of IFR9 expression correlated genes (n = 90) (Additional file 5: Table S4 and Additional file 8: Fig. S2A, C). 297/326 of the PD-L2 expression correlated genes were IRF1 expression correlated and 18/326 of the PD-L2 expression correlated genes were IFR9 expression correlated (Additional file 8: Fig. S2C). GSEA showed that PD-L2, IRF1, and IFR9 expression correlated genes to a large extent belong to the same hallmark gene sets (Additional file 3: Table S2, Additional file 6: Table S5 and Additional file 8: Fig. S2D). We note that in the LUAD and LUSC datasets both PD-1 and CD80 expression correlated with IRF1, but not with IFR9, indicating the participation of IFN-γ signaling in both ligand and receptor expression during the development of immune resistance (Additional file 4: Table S3). RGMB expression was not correlated with IRF1 and IFR9 expression (Additional file 4: Table S3).

We next addressed how PD-L1 and PD-L2 expression correlated genes converged with three previously described gene signatures, IFN-γ, expanded immune, and T cell inflamed, used for predicting pembrolizumab responders (Additional file 9: Table S7) [40]. The genes belonging to these gene signatures were largely included among the PD-L2 expression correlated genes in the LUAD and LUSC datasets (Fig. 3a). In Lung_NSC the overlap was less evident as could be expected due to the lack of immune cells and cytokine stimulation in these in vitro grown cancer cells (Fig. 3a). The overlap between the three gene signatures and PD-L1 expression correlated genes was less pronounced than that for PD-L2 expression correlated genes (Fig. 3a). We also examined how previously described gene signatures representing immune cell types converged with PD-L1 and PD-L2 expression correlated genes in the LUAD, LUSC, and Lung_NSC datasets (Fig. 3a) (Additional file 9: Table S7) [21]. Again, the overlap between these gene signatures and PD-L1 expression correlated genes was less pronounced than that for PD-L2 expression correlated genes (Fig. 3a).

We analyzed the potential of PD-L1 and PD-L2 expression correlated genes to represent gene signatures with potential biomarker implications for immunotherapy with PD-1/PD-L1 axis blockade by searching for genes
whose expression were correlated across the datasets Lung_NSC, LUAD, and LUSC. Notably, only PD-L1 and PD-L2 themselves remained after selecting for expression correlated genes for both PD-L1 and PD-L2 across all three datasets (Fig. 3b). PD-L2, plasminogen receptor with a c-terminal lysine (PLGRKT), and apolipoprotein L6 (APOL6) expression correlated with PD-L1 across the Lung_NSC, LUAD and LUSC datasets (Fig. 3c and Additional file 10: Table S8). For PD-L2, the three genes PD-L1, Transmembrane protein 106A (TMEM106A), and Tripartite motif containing 22 (TRIM22) expression correlated across the Lung_NSC, LUAD, and LUSC datasets (Fig. 3d and Additional file 10: Table S8). Notably, these genes also represented the only PD-L1 and
**PD-L2** expression correlated genes across the Lung_NSC and LUSC datasets (Fig. 3c, d and Additional file 10: Table S8). The expression correlation between the identified genes, PD-L1, and PD-L2 in individual LUAD and LUSC tumor samples is illustrated in Additional file 11: Fig. S3A and B. The examination of available LUSC and LUAD PD-L1 protein expression data also revealed expression correlation with PD-L1 protein except for APOL6 mRNA (Additional file 11: Fig. S3A and B). Next, we focused on PD-L1 expression correlated genes across the Lung_NSC and LUAD datasets (Additional file 10: Table S8). Here we identified 49 genes (Fig. 3c and Additional file 10: Table S8) and their expression correlation with PD-L1 for individual LUAD tumors is illustrated in Additional file 11: Fig. S3C. The mRNA expression for most of these 49 genes correlated with the expression of PD-L1 protein (Additional file 11: Fig. S3C, upper panel). For PD-L2, 26 genes expression correlated across the Lung_NSC and LUAD datasets (Fig. 3d and Additional file 10: Table S8). The expression correlation of these 26 genes with PD-L2 for individual LUAD tumor samples is illustrated in Additional file 11: Fig. S3C. The mRNA expression for most of these 26 genes correlated with the expression of PD-L1 protein (Additional file 11: Fig. S3C, lower panel).

**In LUSC, PD-L1 expression correlated genes are enriched for being Chr9p24 localized**

We noted fewer number of genes with PD-L1 expression correlation (n = 26) as compared to the number of genes with PD-L2 expression correlation (n = 326) in LUSC (Fig. 2e). GSEA for chromosomal localization of the 26 PD-L1 expression correlated genes in LUSC identified enrichment for localization to the Chr9p24 region for 13/26 genes (Fig. 4a, b and Additional file 12: Table S9). Chr9p24 enrichment was also present among the PD-L1 expression correlated genes in LUAD (6/257 genes), but not among the PD-L1 expression correlated genes in Lung_NSC (Fig. 4b and Additional file 12: Table S9). GSEA also identified enrichment for Chr9p24 localization among the PD-L2 expression correlated genes in LUSC, but for a lower fraction of genes (6/326) than observed for PD-L1 (13/26) (Fig. 4b and Additional file 12: Table S9). Enrichment for localization to additional genomic regions, e.g., Chr12p13 and Chr19q13, was also present among the PD-L1 and PD-L2 expression correlated genes in LUAD and LUSC (Additional file 12: Table S9). In LUSC, an expression correlation between PD-L1 protein and 7/12 examinable Chr9p24-localized genes with PD-L1 expression correlation was observed (note the Chr9p24-localized gene UHRF was excluded from the analysis) (Fig. 4c). In LUAD, the expression correlation between Chr9p24-localized genes with PD-L1 expression correlation and PD-L1 protein was less pronounced (Fig. 4d). We next performed an unsorted hierarchical cluster analysis of a merged gene signature composed of genes representing the core IFN signaling, different immune cell types, and the PD-L1 expression correlated genes in LUSC with Chr9p24 localization (the genes and the merged gene signature is shown in Additional file 9: Table S7). We found that in the LUSC samples the PD-L1 expression correlated genes with Chr9p24 localization were preferentially clustering together (Additional file 13: Fig. S4A). Furthermore, this cluster of Chr9p24 localized genes included PD-L1 and PD-L2. This point to Chr9p24 localization is the major determinant for the PD-L1 and PD-L2 expression profiles in LUSC (Additional file 13: Fig. S4A). The same analysis in LUAD samples showed that PD-L1 and PD-L2 did not cluster with the other Chr9p24-localized genes, demonstrating that in LUAD, the Chr9p24 localization was less important for determining the PD-L1 and PD-L2 expression profile (Additional file 13: Fig. S4B). Altogether, we conclude that the expression analyses of Chr9p24-localized genes with PD-L1 expression correlation may possess relevance when searching for gene expression biomarkers that can predict responsiveness to immunotherapy with PD-1/PD-L1 axis blockade in lung squamous cell carcinoma.

**Discussion**

Comparison of PD-L1 and PD-L2 gene expression results from the CCLE Lung_NSC cell line dataset and the TCGA LUAD and LUSC tumor datasets include some
Expression correlation with PD-L1, PD-L2, PD-L1 and PD-L2.
putative pitfalls. Expression analyses using the CCLE dataset identified gene expression in various cancer cell genetic backgrounds each with a unique and relative homogenous profile in terms of cancer driver and passenger mutations. However, the actual cell propagation conditions only partially mimic the context of a tumor in vivo. Gene expression analyses using the TCGA datasets (LUAD and LUSC) corresponded to incisional and core tumor biopsies, which mimic tumor cells more naturally due to stimulation of the tumor cells with factors, such as cytokines, derived from the complex tumor microenvironment. However, these biopsies may not be representative for the entire tumor burden due to tumor heterogeneity and they may as well display various degrees of tumor-infiltrating immune cells and connective tissue. With such dataset differences in mind, we still found it surprising that only PD-L2, APOL6, and PLGRK6 expression were correlated with PD-L1 across the CCLE Lung_NSC and TCGA (LUAD and LUSC) datasets. Similar, only the three genes PD-L1, TRIM22, and TMEM106A expression correlated with PD-L2 across the three datasets. As a result, PD-L2 expression should in principle be among the best indirect predictors to deduce PD-L1 mRNA expression level, and vice versa. This supports the use of PD-L2 expression analyses, beyond PD-L1 expression analyses, to predict responsiveness to immunotherapy with PD-1/PD-L1 axis blockade in NSCLC.

Examining PD-L1 and PD-L2 gene expression in the NSCLC datasets separately revealed other aspects of expression regulation. In the CCLE dataset Lung_NSC, the majority of the PD-L2 expression correlated genes were also expression correlated with PD-L1, 111/191 genes, but not vice versa, 111/489 genes. In the TCGA dataset LUAD, the opposite was observed, 211/914 genes and 211/257 genes, respectively. In the TCGA dataset LUSC, we observed a low number of PD-L1 expression correlated genes (n = 26) relative to the number of genes expression correlated with PD-L2 (n = 326). Half of these PD-L1 expression correlated genes, 13/26, expression correlated with PD-L2. Thus, gene signatures representing PD-L1 and PD-L2 expression correlated genes seem to delineate common, as well as distinct, aspects of PD-L1 and PD-L2 regulation; depending on the actual NSCLC datasets analyzed.

With previous studies showing that both PD-L1 and PD-L2 genes are regulated by IFN signaling, we focused on IFN signaling as an important driving mechanism for the expression of PD-L1 and PD-L2 [21, 40]. In the CCLE dataset (Lung_NSC) the expression correlation between IFN signaling signature genes and PD-L1 and PD-L2 differed strikingly to the expression correlation observed in the TCGA datasets (LUAD and LUSC). IRF9 expression correlated with PD-L1 in Lung_NSC, but not in the LUAD and LUSC datasets. However, IRF1 expression correlated with PD-L1 and PD-L2 expression in the LUAD and LUSC datasets indicated a shift from IFN-α involved PD-L1 expression regulation in CCLE (Lung_NSC) cell lines toward IFN-γ involved PD-L1 and PD-L2 expression regulation in TCGA (LUAD and LUSC) tumors. IRF1 expression correlation with PD-L1 and PD-L2 also goes beyond lung cancer [21]. Furthermore, our presented expression correlation data indicated a stronger correlation of PD-L2 expression, relative to PD-L1 expression, with active IFN signaling in LUAD and LUSC tumors.

We found it intriguing that PD-L1 expression correlated with only a few other genes in the TCGA LUSC dataset, and that a prominent number of these genes were localized to Chr9p24. Of the genes located to Chr9p24 and expression correlated with PD-L1, only PD-L2 and JAK2 were associated with IFN-γ signaling. JAK2 is essential for intracellular signaling upon exposure of cancer cells to IFN-γ produced by T cells upon antigen recognition. In cancer, loss of function mutations in JAK2 are relatively often observed and can in principle result in decreased antigen presentation, lack of T cell infiltrates, tumor cell resistance to the anti-proliferative effects of IFN-γ, and can prevent adaptive PD-L1 and PD-L2 expression upon IFN-γ exposure [41, 42]. One consequence could be intrinsic and/or acquired resistance to immunotherapy with PD-1/PD-L1 axis blockade [41, 42]. In LUSC, Chr9p21.3 allelic deletions of the tumor suppressor gene Cyclin-dependent kinase inhibitor 2A (CDKN2A) can, in addition, include co-deletion of JAK2, PD-L1, PD-L2 [43]. Moreover, co-amplification of JAK2 and PD-L1 is observed in NSCLC and patients with JAK2 and PD-L1 co-amplification has a poorer overall survival and recurrence-free survival compared to patients with normal PD-L1 copy number [44]. Copy number variation (CNV) is a characteristic of NSCLC and drives the expression profile of underlying genes [45–49]. However, we found no evidence that Chr9p CNVs should be the driving mechanism for the distinct PD-L1 versus PD-L2 expression correlation profile in LUSC, as such CNVs should affect PD-L1 and PD-L2 expression simultaneously. The architecture of the human genome is a major determinant for controlling gene expression [50, 51]. First, local enhancer-promoter interaction at the single gene level. Second, the genome is organized into functionally distinct topologically associated domains (TADs), from 100 kilo-bases to 5 mega-bases, encompassing multiple genes and regulatory elements [50, 52, 53]. TAD boundaries act as insulators by preventing communication between regulatory elements on either side, and TADs are mostly invariant between different cell types, while
loops within TADs (sub-TADs) are more tissue-specific [50, 52, 53]. Finally, interactions between TADs organize the genome into mega-base compartments comprising open gene-rich chromatin or closed gene-poor chromatin [54]. In LUSC, the expression correlation between PD-L1 and Chr9p24 genes indicate the existence of a specific TAD architecture making PD-L1, but not PD-L2, expression largely dependent on enhancers shared with other Chr9p24 localized genes within this sub-TAD. One consequence is the possibility of PD-L1 expression in a non-IFN stimulated tumor environment or despite genetic defects in the IFN signaling cascade. Thus, gene expression profiling of Chr9p24 could have potential as an IFN-γ signaling independent predictive biomarker for PD-L1 expression with relevance for predicting responders to immunotherapy with PD-1/PD-L1 axis blockade in lung squamous cell carcinoma.

The limitations of this study are as follows. This was a retrospective study focusing on available gene expression data from TCGA NSCLC tumors and CCLE NSCLC cell lines. Gene expression data originated from different experimental platforms and raw data initially processed with different normalization strategies. Consequences related to potential data overfitting and lack of optimal data harmonization could negatively impair the validity of the subsequent comparisons. Nevertheless, the identified gene-signatures could be an important basis from which individual genes can be selected for further validation at mRNA and protein level in subsequent expression based analyses. In this line, the inclusion of further clinical data will be valuable to substantiate the usability of expression analyses of the hereby identified genes and gene-signatures as potential new clinical relevant response and resistance biomarkers for immunotherapy with PD-1/PD-L1 axis blockade in NSCLC.

Conclusions
The analyses of this study revealed genes and gene signatures associated with PD-L1 and PD-L2 mRNA expression in NSCLC. The presented findings could possess importance in relation to a better understanding of the PD-1 checkpoint blockade-responsive biology and development of gene expression profile-based biomarkers for predicting clinical responses to immunotherapy.

Additional files

Additional file 1: Table S1. Expression correlated genes for PD-L1, PD-L2, and both.

Additional file 2: Fig. S1. PD-L1 and PD-L2 expression correlated genes converge differently with IRF1 and IRF9 expression correlated genes in CCLE dataset Lung_NSC. A. Venn diagrams illustrating the number of genes with mRNA expression correlation in CCLE dataset (Lung_NSC, n = 114) using the GenomicScape portal for PD-L1, PD-L2, and IRF9 (left panel), PD-L1, PD-L2 and IRF9 (central panel), and IRF1 and IRF9 (right panel). The criteria for significant expression correlation were Pearson correlation coefficient $r \geq 0.3$ or $\leq -0.3$, Spearman correlation coefficient $r \geq 0.4$ or $\leq -0.4$, and all $P$ values $< 0.05$. B. Venn diagrams illustrating the number of significant MSigDB hallmark gene sets for the genes in the Lung_NSC dataset having mRNA expression correlation with PD-L1, PD-L2 and IRF1 (left panel), PD-L1, PD-L2 and IRF9 (central panel), and IRF1 and IRF9 (right panel). Only selected genes and MSigDB hallmark gene sets are illustrated.

Additional file 3: Table S2. GSEA of PD-L1 and PD-L2 expression correlated genes in CCLE dataset (Lung_NSC) and TCGA datasets (LUAD and LUSC).

Additional file 4: Table S3. Expression correlation between PD-L1, PD-L2, IFN signaling pathway genes, and PD-L1 and PD-L2 cognate-receptors in CCLE dataset (Lung_NSC) and TCGA datasets (LUAD and LUSC).

Additional file 5: Table S4. Expression correlated genes for IRF1 and IRF9.

Additional file 6: Table S5. GSEA of IRF1 and IRF9 expression correlated genes in CCLE dataset (Lung_NSC) and TCGA datasets (LUAD and LUSC).

Additional file 7: Table S6. Differences in expression in TCGA datasets (LUAD and LUSC) versus normal paired tissue from GTEx and TCGA.

Additional file 8: Fig. S2. PD-L1 and PD-L2 expression correlated genes converge differently with IRF1 and IRF9 expression correlated genes in TCGA datasets (LUAD and LUSC). A. Venn diagrams illustrating the number of genes in LUAD (n = 517) having mRNA expression correlation with PD-L1, PD-L2 and IRF1 (left panel), PD-L1, PD-L2 and IRF9 (central panel), and IRF1 and IRF9 (right panel). The criteria for significant expression correlation are: Pearson correlation coefficient $r \geq 0.3$ or $\leq -0.3$, Spearman correlation coefficient $r \geq 0.4$ or $\leq -0.4$, and all $P$ values $< 0.05$. The analysis was performed using cBioPortal. B. Venn diagrams illustrating the number of significant MSigDB hallmark gene sets for the genes in the LUAD dataset having mRNA expression correlation with PD-L1, PD-L2 and IRF1 (left panel), PD-L1, PD-L2 and IRF9 (central panel), and IRF1 and IRF9 (right panel). C. D. Panels C, D are similar to panels A, B except that the LUSC dataset (n = 501) is analyzed in panels C, D. Only selected genes and MSigDB hallmark gene sets are illustrated.

Additional file 9: Table S7. Gene lists used for various analyses.

Additional file 10: Table S8. Gene lists representing various subsets of PD-L1 and PD-L2 expression correlated genes in CCLE dataset (Lung_NSC) and TCGA datasets (LUAD and LUSC).

Additional file 11: Fig. S3. Heat map analyses of PD-L1 and PD-L2 expression correlation gene signatures in TCGA datasets (LUAD and LUSC). A, B Heat map analyses of mRNA expression Z-values in TCGA dataset LUSC. A and LUAD B of gene signatures representing expression correlated genes with PD-L1 and PD-L2 across Lung_NSC, LUAD, and LUSC. Heat maps are sorted relative to the PD-L1 mRNA expression level (upper panels) or PD-L2 mRNA expression level (lower panels). Spearman and Pearson correlation coefficients r and corresponding P values for mRNA expression of signature genes and PD-L1 protein expression in LUAD (n = 365) and LUSC (n = 328) are shown to the right. C. Heat map analysis of mRNA expression Z-values for gene signatures representing expression correlated genes with PD-L1 (upper panel) and PD-L2 (lower panel) across LUAD and Lung_NSC. The heat map is sorted relative to PD-L1 mRNA expression level (upper panels) or PD-L2 mRNA expression level (lower panel). Spearman and Pearson correlation coefficients r and corresponding P values for mRNA expression of signature genes and PD-L1 protein expression in LUAD (n = 365) are shown to the right. Asterisks in the lower panel indicate genes also included in the analysis in the upper panel. Correlations assigned significant are shown in red. The criteria for significant expression correlation were Pearson correlation coefficient $r \geq 0.3$ or $\leq -0.3$, Spearman correlation coefficient $r \geq 0.4$ or $\leq -0.4$, and all $P$ values $< 0.05$. Abbreviations: IRF1, IRF1 expression correlated genes; Pe, Pearson, r, correlation coefficient; Sp, Spearman.
Additional file 12: Table S9. GSEA for genomic localization of PD-L1 and PD-L2 expression correlated genes.

Additional file 13: Fig. S4. PD-L1 expression correlated genes located at Chr9p24 clusters in LUSC. A, B, Unsupervised hierarchical cluster heat map analysis of mRNA expression Z-values from TCGA dataset LUSC. A and LLUAD B with a merged gene signature (n=94) composed of PD-L1 expression correlated genes in LUSC with localization to Chr9p24, the gene lists for immune cells from Garcia_Diaz et al. [21], and the gene list IFN signaling core composed of IFIT, IFIRF, STAT1, JAK1, and JAK2. PD-L1 expression correlated genes with Chr9p24 localization are highlighted.

Abbreviations
BMP: bone morphogenic protein; BMPR I: BMP receptor type 1; BMPR II: BMP receptor type 2; C2-type: constant 2-type; CCLE: cancer cell line encyclopedia; CD80: cluster of differentiation 80; GSEA: gene set enrichment analysis; GTEx: genotype-tissue expression project; IFN: interferon; Ig-like: immunoglobulin-like; IL: interleukin; LLUAD: TCGA lung adenocarcinoma; LUSC: TCGA lung squamous cell carcinoma; NK: natural killer cell; NSCLC: non-small-cell lung cancer; PD-1: programmed cell death protein-1; PD-L1: programmed cell death ligand-1; PD-L2: programmed cell death ligand-2; Pe: pearson; r: expression correlation coefficient; RGM: repulsive guidance molecule; RMA: robust multi-array average; RPKM: transcripts per million; TPS: tumor proportion score; V-type: variable-type.

Acknowledgements
We thank Familien Erenchs Familiefond for making this study possible.

Authors’ contributions
ALN conceived, designed and performed data extraction for the study. TVL, DH, and ALN analyzed and interpreted the data. ALN prepared the figures and tables. TVL, DH, and ALN wrote and revised the manuscript. All authors read and approved the final manuscript.

Funding
This study was supported by Familien Erenchs Familiefond. TVL and DH are supported by Ph.D. fellowships from the Faculty of Health, Aarhus University, Denmark.

Ethic approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no potential conflict of interests.

Availability of data and materials
The data supporting the conclusions of this article are included in the article.

Received: 30 October 2018 Accepted: 23 May 2019 Published online: 03 June 2019

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