이학석사 학위논문

MET-mediated regulation of immune checkpoint molecules and suppression of immune cell function

MET의 면역제어분자 발현조절을 통한 면역세포 기능 억제기전

2018 년 8 월

서울대학교 대학원

협동과정 종양생물학전공

안 현 경

A Thesis of the Degree of Master of Science

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이 논문을 이학석사 학위논문으로 제출함

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MET-mediated regulation of immune checkpoint molecules and suppression of immune cell function

by

HyunKyung AHN

(Directed by Professor Yoon Kyung Jeon, M.D.,PhD)

A Thesis Submitted to the Interdisciplinary Graduate Program in partial fulfillment of the requirement of the Degree of Master of Science in Cancer Biology at Seoul National University College of Medicine

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Approved by Thesis Committee:

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ABSTRACT

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Purpose: MET is a receptor tyrosine kinase playing an important role for the development and progression of variable tumors through diverse mechanisms. However, the role of MET in the modulation of anti-tumor immune response remains unclear. Cancer immunotherapy targeting immune checkpoint pathways (co-inhibitory pathways) and co-stimulatory pathways has shown clinical and preclinical benefits. Thus, this study was intended to investigate whether MET expressed on tumor cells is involved in the regulation of immune checkpoint pathways and immune cell function.

Methods: Lung adenocarcinoma cell lines including H596 (harboring MET exon 14 skipping mutation), H1993 (harboring MET gene amplification), H23 and H522 (MET wild-type), and a gastric carcinoma cell line, Hs746T (harboring MET exon 14 skipping mutation and gene amplification) were used. Cells were treated with MET inhibitor (PHA665752 or crizotinib) or recombinant HGF (a MET ligand), or transfected with MET siRNA and subject to

oligonucleotide microarray, qRT-PCR, Western blotting and flow cytometry. Co-culture experiment using human peripheral blood mononuclear cells (PBMC) and Hs746T cells was performed to evaluate if PD-L1 expressed on tumor cells affect the immune cell function. Cancer Cell Line Encyclopedia (CCLE) and the Cancer Genome Atlas (TCGA) data were analyzed and immunohistochemistry was also performed using human cancer tissues.

Result: Microarray analysis for Hs746T cells with MET inhibition or knockdown showed that co-inhibitory molecules were down-regulated and co-stimulatory molecules and HLA class I molecules were up-regulated by MET suppression. Among the co-inhibitory molecules, PD-L1 was one of the most significantly down-regulated genes by MET suppression. CCLE analysis revealed that a significant positive correlation between MET and PD-L1, rather than PD-L2, throughout the cancer cell lines. Immunohistochemistry using lung cancer tissues also showed a significant positive correlation between MET and PD-L1 expression in tumor cells. Stimulation of H596, H23 and H522 cells with HGF increased PD-L1 expression at the mRNA and protein levels. Treatment of H1993 and Hs746T cells with MET inhibitor or MET knockdown using siRNA resulted in down-regulation of PD-L1 at the mRNA, total protein, and surface expression levels. Co-culture of PBMC with Hs746T cells suppressed IFNγ production from PBMC,

which was reversed by PD-L1 blocking antibody or crizotinib treatment. Finally, TCGA analysis showed that MET and PD-L1 expression are significantly positively correlates with each other in variable tumor tissues, including non-small cell lung cancer (NSCLC, rho=0.219 [p<0.001]; in adenocarcinoma, r=0.401 [p<0.001]; in squamous cell carcinoma, r=0.144 [p=0.001]) and stomach cancer (rho=0.137 [p=0.005]). Moreover, TCGA analysis of NSCLC exhibited that MET expression is inversely correlated with expression level of CD8 effector function-related genes, including PDCD1, granzyme A, granzyme B, perforin, IFNγ, CXCL9 and CXCL10, in CD274-high group rather than CD274-low group.

Conclusion This study demonstrates that MET signaling increases PD-L1 transcription and protein overexpression in tumor cells, which might contribute to immune escape of tumor via PD-1/PD-L1 pathway.

Keywords: MET, immune evasion, immune checkpoint, PD-1/PD-L1 pathway, PD-L1, cancer immunotherapy, lung cancer

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INTRODUCTION

MET is a receptor tyrosine kinase that is activated upon binding to the hepatocyte growth factor (HGF)/scatter factor (SF) ligand. MET plays an important role in both cancer development and progression by promoting cell survival, proliferation, angiogenesis, invasion and metastasis (1). MET alteration (e.g., protein overexpression, gene amplification and mutation) has been observed in certain types of cancer and was associated with aggressive behavior of cancer as represented by non-small cell lung cancer (NSCLC) (2). Moreover, MET is associated with drug resistance and MET gene amplification is known as one of the mechanisms related with acquired resistance to EGFR tyrosine kinase inhibitor (TKI) therapy (3). MET protein expression was reported in 22-75% of NSCLC and increased MET expression was associated with phospho-MET expression, suggesting that MET overexpression may be related to activation of the MET pathway (4-6). Meanwhile, MET mutation, particularly MET exon 14 skipping mutation, which leads to loss of the c-Cbl-binding site and impaired c-Cbl-mediated ubiquitination and degradation of MET, thus resulting in sustained activation of MET, has been frequently reported in sarcomatoid carcinoma of the lung (the most aggressive entity of

NSCLC) (7-9). Therefore, MET is considered as a potential therapeutic target for NSCLC and several therapies (e.g., HGF antagonists, anti-MET monoclonal antibodies, and MET TKIs) have been developed and under clinical trials (1).

Cancer develops diverse ways to evade anti-tumor immunity. One of the representative mechanisms is hijacking of immune checkpoint pathway, which refers to a variety of inhibitory interactions between T cells and antigen presenting cells or cancer cells. These pathways deliver inhibitory signals to immune cells, thereby contributing to the maintenance of self-tolerance and the regulation of immune responses under physiologic conditions (10). Representatively, the CTLA4/CD80 or CD86 pathway is important for the regulation of T cell at the initial stage of T cell activation, while the PD-1/PD-1 ligands (PD-Ls) pathway mainly involves in the inhibition of effector T cells at the site of inflammation in peripheral tissues (10). However, tumor cells utilize immune checkpoints to evade the immune system. PD-1 is expressed on immune cells, particularly in activated T cells, while PD-Ls, PD-L1 and PD-L2, are mainly expressed on antigen-presenting cells and tumor cells (10). Engagement of PD-1 by PD-Ls suppresses T-cell proliferation and function and induces T-cell apoptosis and anergy. However, interactions between PD-1 and PD-Ls are reversible, and thus

PD-1/PD-L1 blockade restores T-cell function and anti-tumor immune responses (11).

Recently, CTLA4 and PD-1/PD-L1 blocking antibodies have emerged as a novel therapeutic strategy for cancer immunotherapy (11). However, portion of patients with cancer show response to immune checkpoint blockades, demanding on the discovery of predictive biomarkers and novel therapeutic strategy to enhance the efficacy of immune checkpoint blockades. PD-L1 expression in tumor or immune cells detected by immunohistochemistry is associated with the therapeutic efficacy of PD-1/PD-L1 blockades in cancer patients (11), thus being used as a predictive biomarker in clinical practice. PD-L1 expression can be endogenously induced by oncogenic signaling pathways activated in the tumor cells or exogenously (adaptively) by cytokines (e.g., interferon-γ) secreted from reactive immune cells (10). Previous studies demonstrated a positive correlation between MET and PD-L1 expression (12-15). However, the role of MET in the modulation of anti-tumor immune responses and the regulation of immune checkpoint pathways remains unclear. Thus, this study was intended to investigate whether MET is involved in the regulation of immune checkpoint pathways and immune cell function.

MATERIALS AND METHODS

1. Cell lines and regents

Lung adenocarcinoma cell lines including H596 (harboring MET exon 14 skipping mutation), H1993 (harboring MET gene amplification), H23 and H522 (MET wild-type), and a gastric carcinoma cell line, Hs746T (harboring MET exon 14 skipping mutation and gene amplification) were used throughout the study. Hs746T cells were maintained in Dulbecco Modified Eagles Medium (DMEM; Welgene) supplemented with 10% fetal bovine serum (FBS; Welgene) and 1% antibiotics in a humidified incubator with 5% CO_2 at 37°C. H23, H596, H1993 and PBMC cells were maintained in RPMI-1640 medium (Welgene) supplemented with 10% FBS and 1% antibiotics in a humidified incubator with 5% CO_2 at 37°C. PHA665752 (MET inhibitor), Crizotinib (MET inhibitor) were purchased from Sigma Aldrich and Selleckchem, respectively, and dissolved in dimethyl sulfoxide (DMSO). Recombinant human hepatocyte growth factor (rhHGF) was purchased from (ProSpec-Tany TechnoGene Ltd.) and dissolved in distilled water.

2. Small interfering RNA (siRNA) transfections

The specific siRNA targeting human MET were synthesized (Bioneer,

Daejeon, Korea) to achieve the specific downregulation of MET. The sequences of MET siRNAs and scramble (sc) siRNA are described in Table 1. Hs746T and H1993 cells were seeded in 6-well plates and grown to 60–70 % confluence. Cells were then transfected with 100 nM of siRNA using 6 μl of Lipofectamin2000 (Invitrogen) in Opti-MEM media (Qiagen). After 6 h, media was replaced with complete media and the cells were harvested 48 h after transfection.

3. Microarray analysis

Hs746T cells were treated with a MET inhibitor or transfected with MET siRNA and subject to total RNA extraction using TRIzol reagent (Invitrogen). Microarray was performed using Agilent Human 44K (V2) Gene Expression Microarray according to the following protocol by E-BIOGEN (Seoul, Korea).

3.1. Target labeling and hybridization to microarray

For each RNA, the synthesis of target cRNA probes and hybridization were performed using Agilent's LowInput QuickAmp Labeling Kit (Agilent Technologies) according to the manufacturer's instructions. Briefly, each 25 ng total RNA was and T7 promoter primer mix and incubated at 65°C for 10 min. cDNA master mix (5× First strand buffer, 0.1 M DTT, 10 mM dNTP mix, RNase-Out, and MMLV-RT) was prepared and added to the reaction mixer. The samples were incubated

at 40°C for 2 h and then the reverse transcription and dsDNA synthesis was terminated. Transcription of dsDNA was performed by adding the transcription master mix (4× Transcription buffer, 0.1 M DTT, NTP mix, 50% PEG, RNase-Out, Inorganic pyrophosphatase, T7-RNA polymerase, and Cyanine 3-CTP) to the dsDNA reaction samples and incubating at 40°C for 2 h. Amplified and labeled cRNA was purified on RNase mini column. Labeled cRNA target was quantified using ND-1000 spectrophotometer. After checking labeling efficiency, each 1650 ng of cyanine 3-labeled cRNA target was subject to the fragmentation of cRNA by adding 10× blocking agent and 25× fragmentation buffer and incubating at 60°C for 30 min. The fragmented cRNA was resuspended with 2× hybridization buffer and directly pipetted onto assembled Agilent Human 44K (V2) Gene Expression Microarray. The arrays were hybridized at 65°C for 17 h using Agilent Hybridization oven, and then washed as the manufacturer's protocol (Agilent Technologies).

3.2. Data acquisition and analysis

The hybridization images were analyzed by Agilent DNA microarray Scanner and the data quantification was performed using Agilent Feature Extraction software 10.7. The average fluorescence intensity for each spot was calculated and local background was subtracted. All

data normalization and selection of fold-changed genes were performed using GeneSpringGX 7.3.1 (Agilent Technologies). Normalization for Agilent one-color method was performed including data transformation, set measurements less than 5.0 to 5.0 and per chip, and normalize to 50^{th} percentage. The averages of normalized ratios were calculated by dividing the average of control normalized signal intensity by the average of test normalized signal intensity. Functional annotation of genes was performed according to Gene OntologyTM Consortium (http://www.geneontology.org/index.shtml) by GeneSpringGX 7.3.1.

4. Quantitative real-time reverse transcription PCR (qRT-PCR)

Total RNA was isolated from cells using the TRIzol reagent (Invitrogen). To generate cDNA, total RNA (1 μg) was reverse-transcribed using cDNA synthesis kit (Takara). qRT-PCR was performed using a Step One Plus thermal cycler (Applied Biosystems) in triplicate using the SYBR green (Takara) and specific primers (Table 1). Relative mRNA expression was normalized to GAPDH as a control. The relative changes in gene expression were calculated using the following formula, and the data are represented as fold up-/down-regulation: fold change is indicated as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct$ = (Ct of gene of interest, treated-Ct of internal control, treated)-(Ct of gene of interest,

control–Ct of internal control gene, control; Ct, the threshold cycle number).

5. Flow Cytometry

Cells were harvested and washed with FACS buffer (0.5% BSA in PBS). Cells were stained with the PE-conjugated anti-PD-L1 antibody (eBioscience) or with the isotype control (mouse IgG1κ, eBioscience) for 1 h at room temperature in dark. After washing, cells were subject to flow cytometry using a FACS canto (BD Biosciences). Data analysis and graphical output were performed using FlowJo software.

6. Western Blot

Total cellular protein was extracted using RIPA lysis buffer supplemented with phosphatase and protease inhibitor cocktail and EDTA (Sigma-Aldrich). Total protein level was measured using BCA protein assay kits. Equal amount (20-40 ug) of proteins was subjected to SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred to polyvinylidene difluoride membrane (Millipore). The membranes were blocked with 5% nonfat dry milk in Tris buffered saline-Tween20 buffer (25 mM Tris HCl, 125 mM NaCl, 0.1% Tween20) for 1 h and probed with the indicated primary antibodies against c-MET, phospho-MET, PD-L1 (clone E1L3N) (Cell Signaling), PD-L2 (clone 176611) (R&D systems), phospho-Akt, Akt,

phospho-Erk, Erk (Cell Signaling) or β-actin (Santa Cruz) overnight at 4°C and then HRP-conjugated secondary antibodies (Santa Cruz) for 2 h at room temperature. Antibody binding was detected using a chemiluminescence kit (Amersham Pharmacia Biotech).

7. Co-culture of human peripheral blood mononuclear cells (PBMCs) and tumor cells

Human PBMCs were isolated by Ficoll-Paque Plus (GE Healthcare) density gradient separation from heparinized peripheral blood donated by healthy volunteers. After centrifugation, the PBMC layer was collected, seeded onto a culture plate, and incubated at 37°C in a 5% CO_2 incubator. PBMC were activated with phorbol-12-myristate 13-acetate (PMA) (50 ng/ml) and ionomycin (1 ug/ml) for 8-24 h. Expression of surface PD-1 in T cells was determined by flow cytometry using PE-conjugated anti-human PD-1 (CD279) antibody (eBiosciences). Meanwhile, tumor cells were seeded in 6-well plate and cultured with RPMI-1640 media before co-culture with activated PBMC. To test the effect of tumor cells on immune cell function, PBMC and Hs746T cells were co-cultured at the ratio of 2:1 and for additional 16-24 h, and then PMBC and culture soup were harvested for qRT-PCR and ELISA, respectively. Otherwise, Hs746T was pretreated with crizotinib (10nM) or anti-PD-L1 blocking antibody (40

ug/ml) or mouse IgGκ control for 24 h before co-culture with activated PBMC.

8. Enzyme-linked immunosorbent assay (ELISA) for IFN-γ

The cell-free supernatant from co-cultured system was collected by centrifugation at 14,000 rpm. IFNγ level in cell-free supernatant was determined using Human IFNγ ELISA kit (R&D systems, #DY285-05) according to the manufacturer's protocol.

9. Immunohistochemistry (IHC)

Tissue microarrays were constructed from 2-mm diameter cores derived from representative tumor areas of formalin-fixed paraffin-embedded tissue (FFPE) blocks of 789 patients with NSCLC (approved by the Institutional Review Board of SNUH, No. H-1404-100-572). IHC for PD-L1 and c-MET was performed using rabbit anti-PD-L1 (clone E1L3N) and c-MET (clone SP44, Ventana Medical Systems) antibodies and the Ventana Benchmark XT automated staining system. PD-L1 and c-MET expression by IHC was graded as 0, 1, 2, and 3 according to the intensity and proportion of positive cells according to the previous studies (12, 16). MET IHC score was defined by the modified criteria used in the clinical trial for the MET inhibitor as follows: 0, absence of staining or any intensity staining in less than 50% of tumor cells; 1, weak to moderate intensity staining in more than 50%

of tumor cells; 2, moderate to strong intensity staining in more than 50% of tumor cells; 3, strong intensity staining in more than 50% of tumor cells. The PD-L1 immunohistochemistry were evaluated based on the intensity and proportion of membranous and/or cytoplasmic staining in tumor cells and scored as follows: 0, negative; 1, weak or moderate in <10% of tumor cells; 2, moderate in ≥10% of tumor cells; 3, strong in ≥10% of tumor cells.

To determine the copy number of MET, fluorescence in situ hybridization (FISH) was performed using Vysis LSI MET SpectrumRed and Vysis CEP7 (D7Z1) SpectrumGreen probes (Abbott Molecular Inc., Chicago, IL, USA), according to the manufacturer's instructions. The MET gene copy status was classified according to the University of Colorado Cancer Center (UCCC), which defines high polysomy as ≥ 4 copies in ≥ 40% of cells and gene amplification as presence of tight MET gene clusters, a ratio of MET to CEP7 of ≥ 2 or ≥ 15 copies of MET per cell in ≥ 10% of analyzed cells.

To compare MET expression and PD-L1 expression in cases with NSCLC harboring MET exon 14 skipping mutations previously reported (17), areas representing each MET IHC intensity score were selected for evaluation of PD-L1 expression.

10. Cancer Cell Line Encyclopedia (CCLE) and the Cancer

Genome Atlas (TCGA) data analysis

CCLE data analysis was performed to explore the relationship between MET and PD-L1 expression in variable human cancer cell lines (https://portals.broadinstitute.org/ccle). Genomic analysis was also performed for exploring the associations between PD-L1, MET and immune response-related molecules expression. The level 3 data of TCGA was used which were downloaded from the UCSC Cancer Browser (https://genome-cancer.ucsc.edu) on June 3, 2015. TCGA data included clinical information and mRNA expression data obtained by RNAseq (Illumina HiSeq V2 platform). Samples of 14 cancer types (n = 6114) were included in the analysis for association between MET and PD-L1. Data from 1015 patients with NSCLC was analyzed to explore the expression pattern of immune-related molecules according to the expression status of PD-L1 and MET.

11. Statistical analysis

All statistical analyses were performed using SPSS software (version 21; IBM Corp.) and images were created by the GraphPad Prism 5 software. For patient sample data and *in vitro* studies, the expression levels of several continuous variables, including MET and PD-L1, were compared using unpaired Student *t*-tests. For CCLE and TCGA data,

the statistical significance of continuous variables, such as the mRNA levels of *CD274* (PD-L1) and MET, was calculated using Spearman's correlation or Pearson's correlation analyses. The mRNA levels of T-cell effector response-related genes according to *CD274* and MET status were compared using Kruskal-Wallis tests and one-way ANOVA test. Statistical significances in the difference of categorical values were analyzed using Pearson's chi-square tests. Two-sided p values < 0.05 were considered statistically significant.

Table 1. Sequences of small interfering RNAs and primers used for qRT-PCR

Small interfering RNAs (siRNAs)		
MET	siRNA-1	GAGCCAGCCTGAATGATGA
	siRNA-2	GAACAGCGAGCTAAATATA
	siRNA-3	GAACTGGTGTCCCGGATAT
	siRNA-4	GTAAGTGCCCGAAGTGTAA
Scrambled siRNA		GGAGCAACGAGGATTACCT
Primers for qRT-PCR		
MET	Forward	5'-TGATGATGAGGTGGACACA-3'
	Reverse	5'-CTATGGCAAGGAGCAAAGA-3'
PD-L1	Forward	5'-TATGGTGGTGGTGCCGACTACAA-3'
	Reverse	5'-TGGCTCCCAGAATTACCAAG-3'
IFNγ	Forward	5'-CTAATTATTCGGTAACTGACTTGA-3'
	Reverse	5'-ACAGTTCAGCCATCACTTGGA-3'
GAPDH	Forward	5'-CCCTTCATTGACCTACCTCAACTACAT-3'
	Reverse	5'-ACGATACCAAAGTTGTCATGGAT-3'

RESULTS

Microarray analysis of Hs746T cells after MET inhibition and knockdown showed changes in immune-response related genes including co-inhibitory (immune checkpoint) and co-stimulatory molecules

To comprehensively screen the regulation of gene expression by MET, Hs746T cells were treated with MET inhibitor or transfected with MET siRNA and submitted to microarray analysis as described in Materials and Methods. Of the 34127 probes on microarray, 21402 probes produced robust signals in both sets of Hs746T cells treated with MET inhibitor versus DMSO control and transfected with MET siRNA versus scramble siRNA control. Overall, changes of genome expression in MET inhibitor treated and MET knocked down cells were well-correlated each other. 15.4% (n = 5248) of total genes were significantly changes more than 2-fold by MET inhibitor (8.4% up-regulated and 7.0% down-regulated), whereas 4.3% (n = 1456) of total genes were significantly changes more than 2-fold by MET knockdown (1.9% up-regulated and 2.3% down-regulated) (Fig. 1). GO analysis using these genes revealed that expression of genes relating to variable

pathways including angiogenesis, apoptosis, cell cycle, cell migration, proliferation, DNA repair, extracellular matrix, immune response and inflammatory response, were up- or down-regulated by MET suppression (Fig. 1)

According to GO analysis, both MET inhibition by PHA665752, and knockdown with MET siRNA altered mRNA levels of immune-related genes. Moreover, MET inhibition by inhibitor showed more dramatic changes of immune-related gene mRNA level than knockdown with MET siRNA in this study. Of 1343 immune related genes, relative upregulation of mRNA level was found in 136 genes of MET inhibitor treatment group and in 9 genes of knockdown with siRNA group (equal or greater than 2 folds). Relative downregulation of mRNA level was found in 105 genes of MET inhibitor treatment group and in 16 genes of knockdown with siRNA group (equal or greater than 2 folds). Changes of mRNA expression pattern by MET inhibitor treatment and MET knockdown were positively correlated (Fig. 2A). In general, genes of negative regulator of immune response (e.g., CD274 [PD-L1], PDCD1LG2 [PD-L2], PVRL1, CD276 [B7-H3]) were downregulated by MET inhibition or knockdown, and genes known as co-stimulatory molecules (e.g. TNFSF9 [4-1BBL], CD70) were upregulated by MET suppression. Among representative immune-associated superfamily, genes of B7/CD28 superfamily were

downregulated by MET inhibition or knockdown; especially, PD-L1 and PD-L2 were the ones of most considerably downregulated genes. In contrast, genes of TNF/TNFR superfamily and HLA I superfamily showed diverse changes in mRNA levels (Fig. 2B).

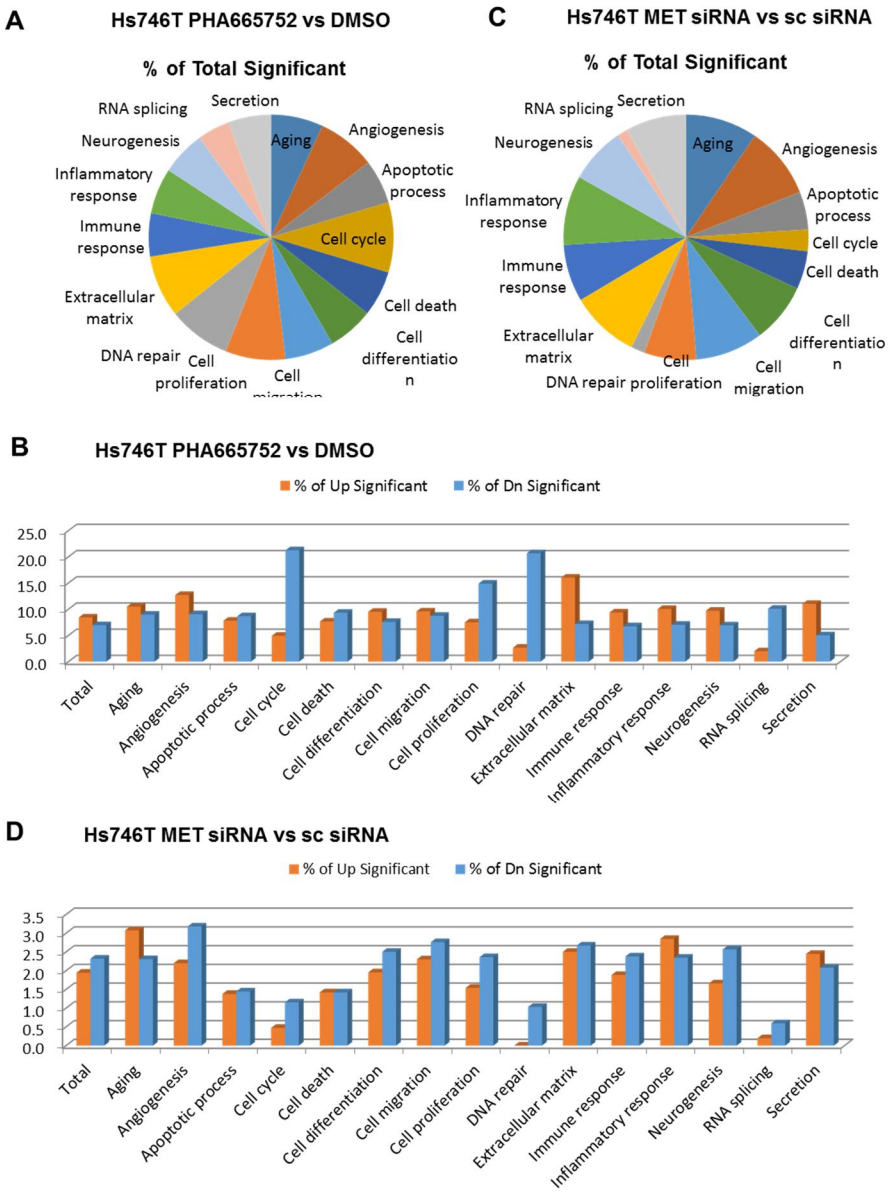


Figure 1. Gene Ontology (GO) analysis for microarray data of Hs746T cells after MET inhibition and knockdown.

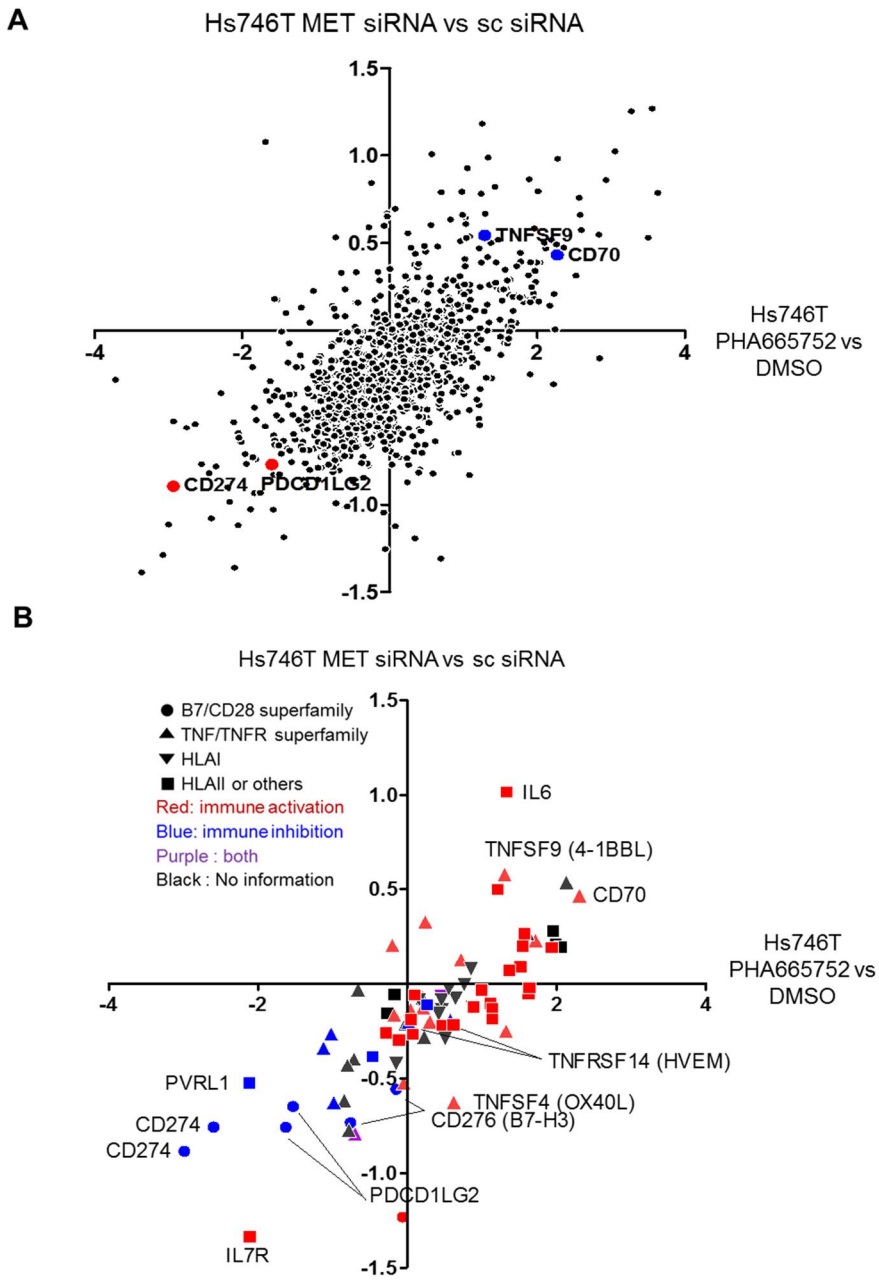


Figure 2. Analysis of co-inhibitory and co-stimulatory molecules and HLA molecules in microarray data of Hs746T cells after MET inhibition and knockdown.

Correlations between MET and PD-1 ligands expression in human cancer cell lines and human cancer tissues (CCLE and TCGA data analysis, IHC study)

According to CCLE database, total 1018 cancer cell lines had their mRNA expression data and all that cancer cell lines were analyzed in this study. In general, CD274 (PD-L1) mRNA level was positively correlated with MET mRNA level in all cancer cell lines. This positive correlation was higher in non-hematolymphoid malignancy cell lines than in all cancer cell lines. ($r = 0.470$, $p < 0.001$ versus $r = 0.430$, $p < 0.001$) (Fig. 3A & C). In contrast, PDCD1LG2 (PD-L2) mRNA level showed only weak correlation with MET mRNA level in all cancer cell lines and also in non-hematolymphoid malignancy cell lines (rho = 0.278, $p < 0.001$; rho = 0.218, $p < 0.001$, respectively) (Fig. 3B & D).

In TCGA data, correlation between MET mRNA and CD274 or PDCD1LG2 mRNA level varied among cancer types. Lung adenocarcinoma and bladder cancer showed relatively strong positive correlation between MET and CD274 ($r = 0.401$, $p < 0.001$ and $r = 0.385$, $p < 0.001$, respectively) and breast cancer and bladder cancer showed relatively strong positive correlation between MET and PDCD1LG2 ($r = 0.383$, $p < 0.001$ and $r = 0.355$, $p < 0.001$, respectively). Stomach cancer showed only weak positive correlation between MET and CD274 levels and no correlation between MET and

PDCD1LG2 levels ($r = 0.137$, $p = 0.005$ and $r = 0.015$, $p = 0.754$, respectively). Consistent with CCLE data, diffuse large B cell lymphoma, one of major hematolymphoid malignancy, showed no definite correlation between MET and CD274 or PDCD1LG2 ($r = 0.12$, $p < 0.415$ and $r = -0.045$, $p = 0.762$), even though only limited number of cases were included in this analysis.

For validation, immunohistochemistry of MET, PD-L1 and PD-L2 was done with 789 human NSCLC. In NSCLC tumor samples, MET IHC score 0, 1, 2, and 3 was 65.4% (516/789), 19.5% (154/789), 11.3% (89/789), and 3.8% (30/789), respectively. PD-L1 score 0, 1, 2 and 3 was 17.6% (139/789), 38.5% (304/789), 34.7% (274/789), and 9.1% (72/789), respectively. PD-L2 score 0, 1, 2, and 3 was 18.2% (145/789), 38.6% (308/789), 33.2% (265/789), and 10.0% (80/789), respectively. A significant positive correlation between MET and PD-L1 protein expression was found in NSCLC tumor samples (Fig. 4A). As MET IHC score increased, PD-L1 score 1 to 3 cases were also increased, especially PD-L1 score 3 cases. In case of PD-L2, there was a tendency that PD-L2 score 1 to 3 cases were increased, as MET IHC score increased, but with a weak correlation compared to PD-L1 (Fig. 4B). In contrast, no statistically significant correlation was observed between MET gene copy status and PD-L1 expression (Fig. 4C), although MET expression was higher in cases with MET gene high polysomy or

amplification (Fig. 4D). Meanwhile, when evaluating PD-L1 expression in nine patients with NSCLC harboring MET exon 14 skipping mutation according to the MET expression status, PD-L1 expression tended to be higher in MET IHC score high area in these patients (Fig. 4 E & F).

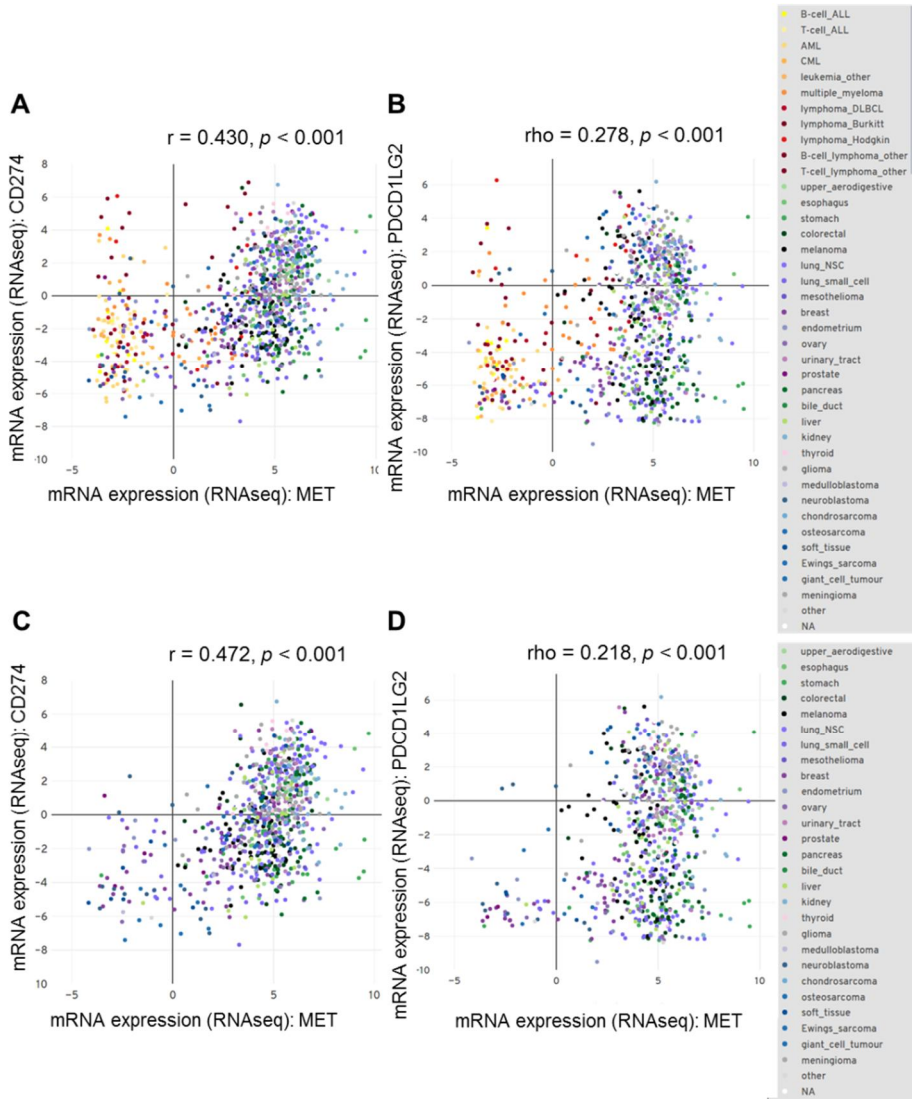


Figure 3. Correlations between MET and PD-1 ligands expression in human cancer cell lines (CCLE data analysis)

Table 2. Correlations between MET and PD-1 ligands expression in human cancer tissues (TCGA data analysis)

Tumor type (n)	MET	PD-L1			PD-L2		
	Mean (median)	Mean(median)	r*	p	Mean(median)	r*	p
Stomach ca (n=415)	11.31 (11.25)	5.35 (5.30)	0.137	0.005	5.84 (6.00)	0.015	0.754
NSCLC (n=1015)	11.40 (11.45)	6.24 (6.07)	0.219†	<0.001	6.50 (6.54)	0.181	<0.001
Lung ADC (n=513)	11.66 (11.84)	6.05 (5.98)	0.401	<0.001	6.46 (6.50)	0.266	<0.001
Lung SqCC (n=502)	11.13 (11.24)	6.43 (6.22)	0.144	0.001	6.53 (6.57)	0.085	0.058
Bladder ca (n=407)	10.56 (10.59)	4.69 (4.32)	0.385	<0.001	4.96 (4.99)	0.355	<0.001
Breast ca (n=1095)	8.31 (8.43)	4.43 (4.38)	0.238	<0.001	5.96 (6.07)	0.383	<0.001
Colon ADC (n=380)	11.77 (11.74)	4.28 (4.14)	-0.098	0.056	4.44 (4.56)	-0.169	0.001
DLBCL (n=48)	7.03 (6.83)	6.30 (6.05)	0.12	0.415	8.25 (8.19)	-0.045	0.762
Eso ca (n=184)	11.53 (11.47)	5.43 (5.35)	0.02	0.792	5.46 (5.41)	-0.046	0.535
GBM (n=157)	7.43 (7.40)	4.88 (4.88)	0.226	0.004	6.63 (6.81)	0.237	0.003
HNSCC (n=519)	11.05 (11.12)	6.36 (6.35)	0.11	0.012	6.65 (6.63)	0.174	<0.001
Kidney RCC (n=534)	12.60 (12.59)	5.35 (5.38)	0.19	<0.001	6.40 (6.45)	0.108	0.013
Mesothelioma (n=87)	11.70 (12.13)	4.76 (4.39)	0.022	0.842	6.70 (6.55)	-0.079	0.468
Sarcoma (n=258)	8.47 (8.23)	4.11 (4.06)	0.124	0.047	7.06 (7.29)	0.122	0.05

Abbreviations: NSCLC, non-small cell lung cancer; ADC, adenocarcinoma; SqCC, squamous cell carcinoma; DLBCL, diffuse large B cell lymphoma; Eso, esopohageal; GBM, glioblastoma; HNSCC, head and neck squamous cell carcinoma; RCC, renal cell carcinoma. *Pearson's r. †Spearman's rho.

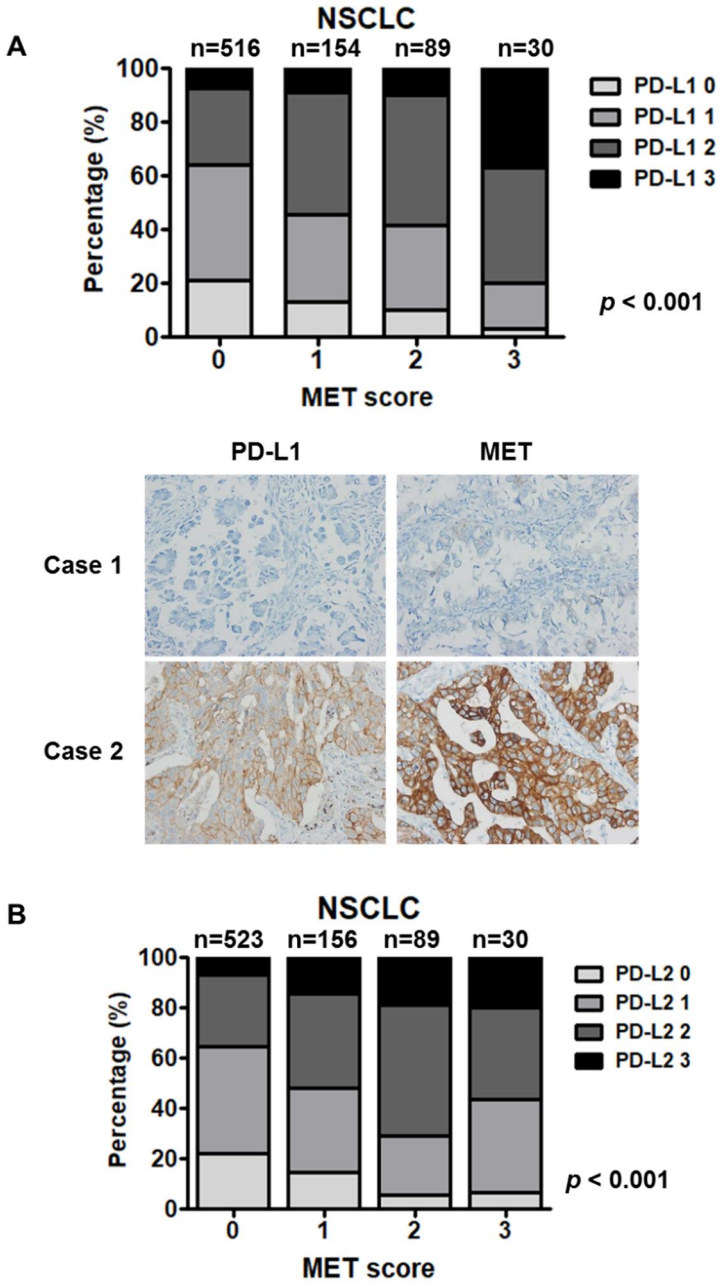


Figure 4. Immunohistochemical analysis for MET and PD-L1 (A) and PD-L2 (B) in human non-small cell lung cancer tissues

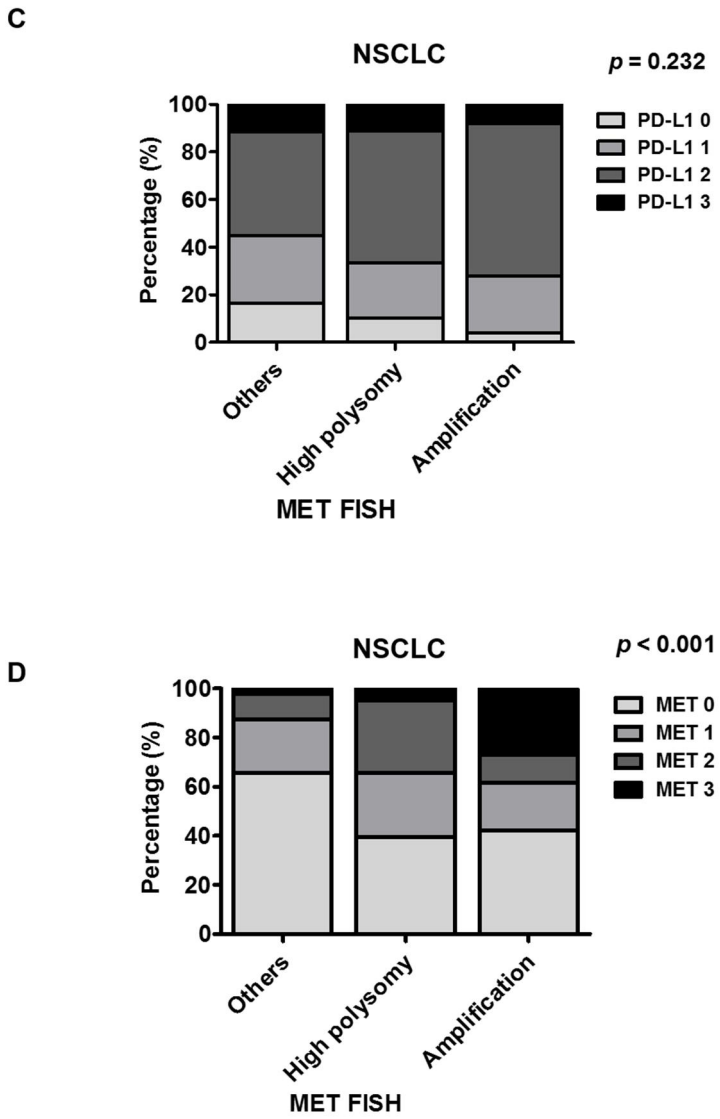


Figure 4 (continued). (C) Relationship of MET gene copy status and PD-L1 expression (D) MET expression.

E PD-L1 expression pattern in MET-mutated NSCLC according to the MET IHC score

MET IHC score	0	1	2	3
Case 1	Negative	Negative	n.a.	n.a.
Case 2	Negative	Negative	Positive	n.a.
Case 3	Negative	Negative	Positive	n.a.
Case 4	n.a.	Negative	Negative	Negative
Case 5	Negative	Negative	n.a.	Positive
Case 6	Negative	Negative	n.a.	Negative
Case 7	Positive	Positive	n.a.	n.a.
Case 8	Positive	Negative	Positive	Positive
Case 9	Negative	n.a.	Positive	n.a.

F

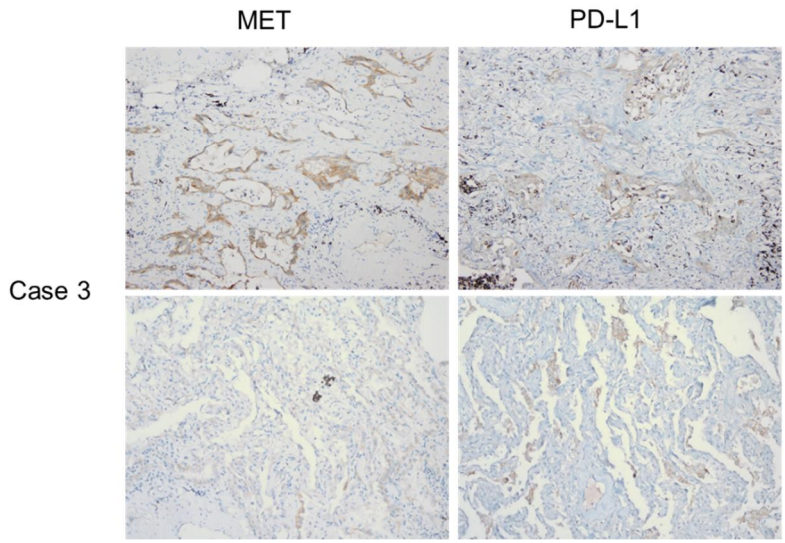


Figure 4 (continued). (E) PD-L1 expression pattern in tumor areas of variable MET IHC intensities in NSCLC cases with MET exon 14 skipping mutation. (F) A representative case (Case 3) shows PD-L1 expression in MET IHC-positive area but not in MET-IHC negative area.

Basal expression levels of PD-L1 and PD-L2 in cancer cell lines with MET mutation and amplification

To determine the relationship between the expression of PD-L1 and MET activation, 4 types of NSCLC cell lines and one gastric cancer cell lines with different MET status were examined. H596 and Hs746T have mutant MET (exon 14 skipping), while H23 and H522 are wild-type MET. Hs746T cell line has also MET amplification and H1993 has MET amplification. MET expression and phosphorylation was higher in Hs746T and H1993 cells. qRT-PCR and western blot showed that PD-L1 expression was higher in H522 (MET wild-type) and Hs746T (MET mutated and amplified) cell lines than that in H23, H522, H1993 cell lines (Fig. 5A-C). These results suggest a positive relationship between PD-L1 expression and MET activation but oncogenic pathways other than MET might control PD-L1 expression in tumor cells as expected.

Based on these basal data, H23, H522 and H596 cells were subject to HGF treatment and Hs746T and H1993 cells were subject to MET inhibition experiments.

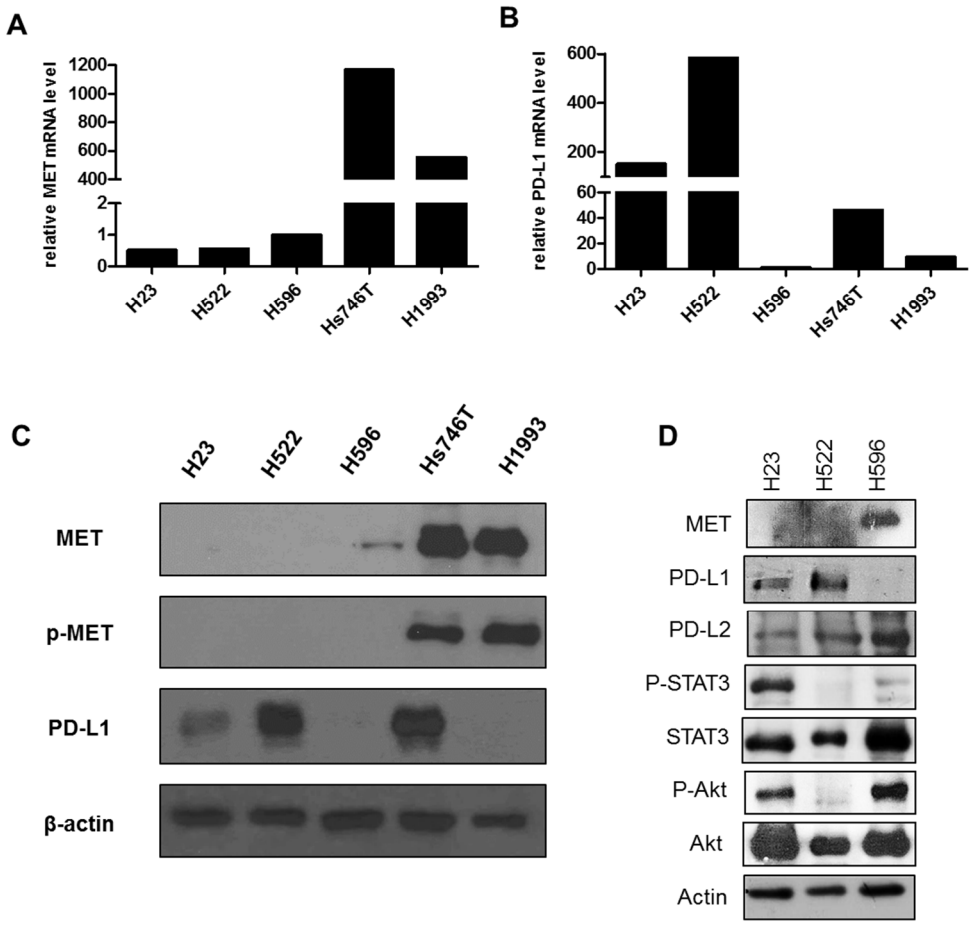


Figure 5. Basal expression levels of MET and PD-L1 in cell lines used in this study. Baseline expression of PD-L1 and MET activity in cell lines. (A, B) mRNA expression of MET and PD-L1 was determined by qRT-PCR and normalized GAPDH. (C, D) The protein expression levels of MET, phospho-MET, PD-L1, PD-L2, and signaling molecules were determined by Western blot. β-actin was used as loading reference.

MET activation by HGF treatment induces PD-L1 expression in human cancer cell lines

To examine whether MET signaling induce PD-L1 expression, rhHGF (50 ng/ml) was used to stimulate H596 cells (harboring MET exon skipping mutation), H23 and H522 cells (MET wild-type). After 2 h, incubation with rhHGF resulted in a significant increase of PD-L1 mRNA expression in H596 and H23 cells (Fig. 6A). Especially, H596 cells showed higher fold changes than H23 cells in mRNA levels. Consistently, PD-L1 protein expression was also significantly increased by rhHGF treatment in H23, H522 and H596 cells (Fig. 6B). This result indicates that MET signaling up-regulates the transcriptional induction of PD-L1. Interestingly, PD-L1 expression in H596 cells, which was low at basal level, was strongly induced after rhHGF treatment (Fig. 6).

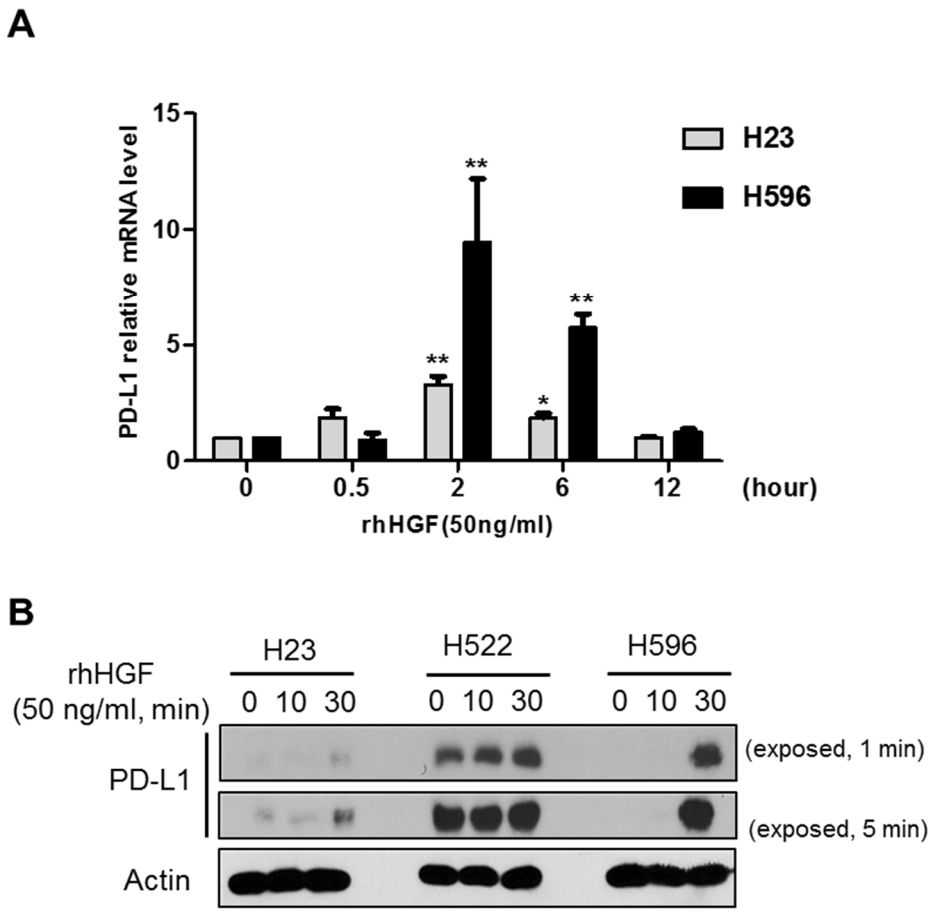
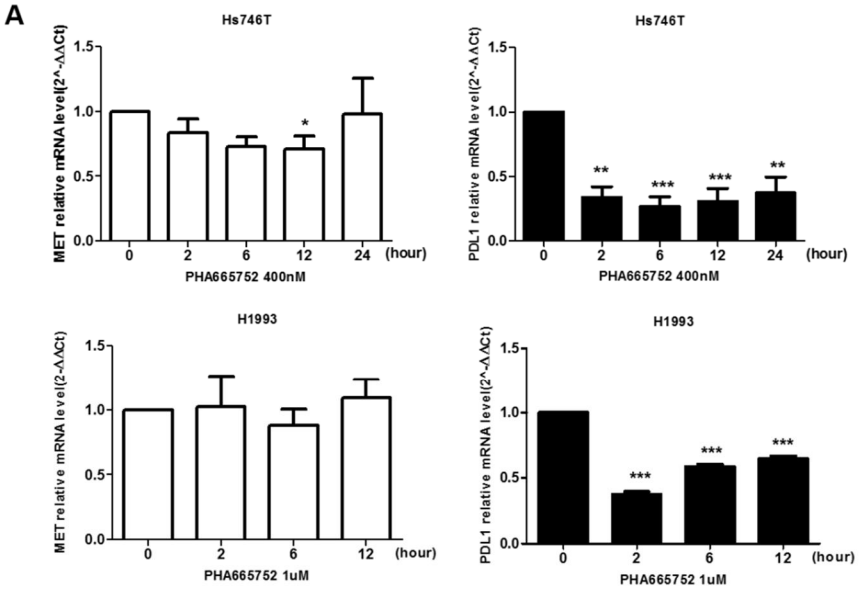


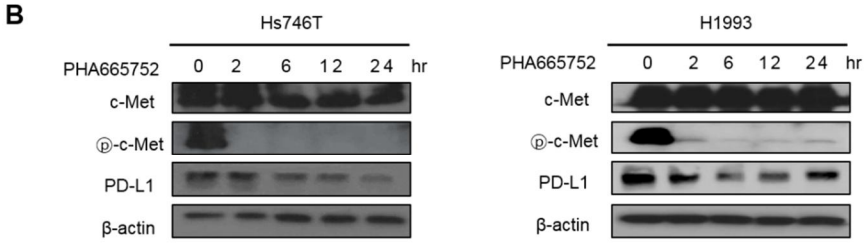
Figure 6. Change of PD-L1 expression in cells treated with HGF. H23, H522, H596 cells were seed at 6-well plate and starved overnight in serum free media. After treated with rhHGF (50 ng/ml) for indicated times, PD-L1 expression level was examined. (A) The mRNA expression of PD-L1 was determined by qRT-PCR and normalized GAPDH. (B) Protein expression of PD-L1 was determined by Western blot. Data represent the means ± SDs of at least three independent experiments or are representative of three independent experiments. All *p* values were calculated using unpaired Student's *t*-tests. *$p < 0.05$, **$p < 0.01$.

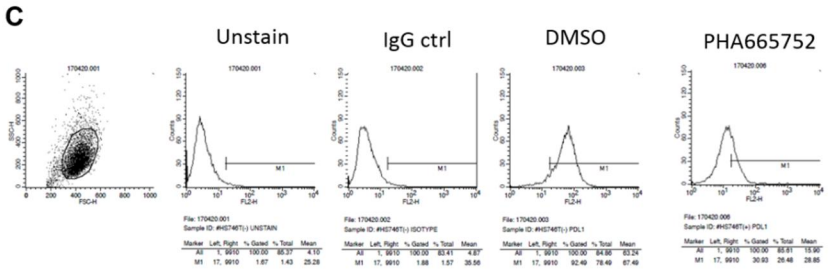
MET inhibition and knockdown reduces PD-L1 expression

To further determine if MET signaling pathway up-regulate PD-L1 expression, Hs746T and H1993 cells were treated with a functional MET inhibitor, PHA665752 and MET/ALK bispecific inhibitor, crizotinib. PHA665752 blocked MET activation and decreased MET phosphorylation. After MET inhibitor treatment, PD-L1 mRNA expression was significantly reduced in both Hs746T and H1993 cells (Fig. 7A). Especially, Hs746T cell which has both MET mutation and amplification showed more sustained effect of MET blockade. Similarly, as represented in Fig. 7B, surface expression of PD-L1 significantly decreased in Hs746T after PHA665752 treatment. Crizotinib treatment also reduced PDL1 expression both protein and mRNA levels in Hs746T cells (Fig. 7D)

To further analyze the effect of MET on PD-L1 expression, four different MET-targeted siRNAs were used to knockdown MET expression. As illustrated in Fig. 8, MET siRNA 1, 2 and 3 efficiently knocked down MET expression and concomitantly reduced PD-L1 expression in both mRNA and protein levels. Taken together, these data indicated that inhibiting MET signaling pathway reduces PD-L1 expression in lung and gastric cancer cell.


A
Hs746T
MET relative mRNA level(2^-ΔΔCt)
1.5
1.0
0.5
0.0
0
2
6
12
24
(hour)
PHA665752 400nM
*
Hs746T
PDL1 relative mRNA level(2^-ΔΔCt)
**
***
***
**
PHA665752 400nM
H1993
MET relative mRNA level(2-ΔΔCt)
PHA665752 1uM
H1993
PDL1 relative mRNA level(2^-ΔΔCt)
***
***
***
PHA665752 1uM


B
Hs746T
PHA665752
0 2 6 12 24 hr
c-Met
ⓟ-c-Met
PD-L1
β-actin
H1993
PHA665752
0 2 6 12 24 hr
c-Met
ⓟ-c-Met
PD-L1
β-actin


C
Unstain
IgG ctrl
DMSO
PHA665752

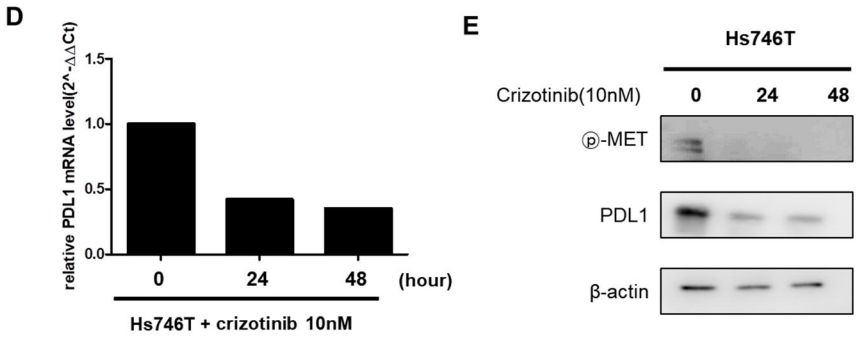


Figure 7. Change of PD-L1 expression in cells treated with MET inhibitor. Hs746T and H1993 cells were treated with each of 400 nM and 1 uM PHA665752 at indicate time. (A) mRNA expression of MET and PD-L1 was determined by qRT-PCR and normalized GAPDH at the indicated time points. (B) Western blot was performed to show protein levels of total MET, phospho-MET and PD-L1. (C) Surface expression of PD-L1 on Hs746T cells was evaluated by flow cytometry as compare with isotype control. Hs746T cells were treated with 400 nM PHA665752 for 6 h. (D, E) Hs746T was treated with crizotinib 10 nM for 24, 48 h. Cells were then subject to qRT-PCR for PD-L1 and Western blot for p-MET and PD-L1. Data represent the means ± SDs of at least three independent experiments or are representative of three independent experiments. All p values were calculated using unpaired Student's *t*-tests. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

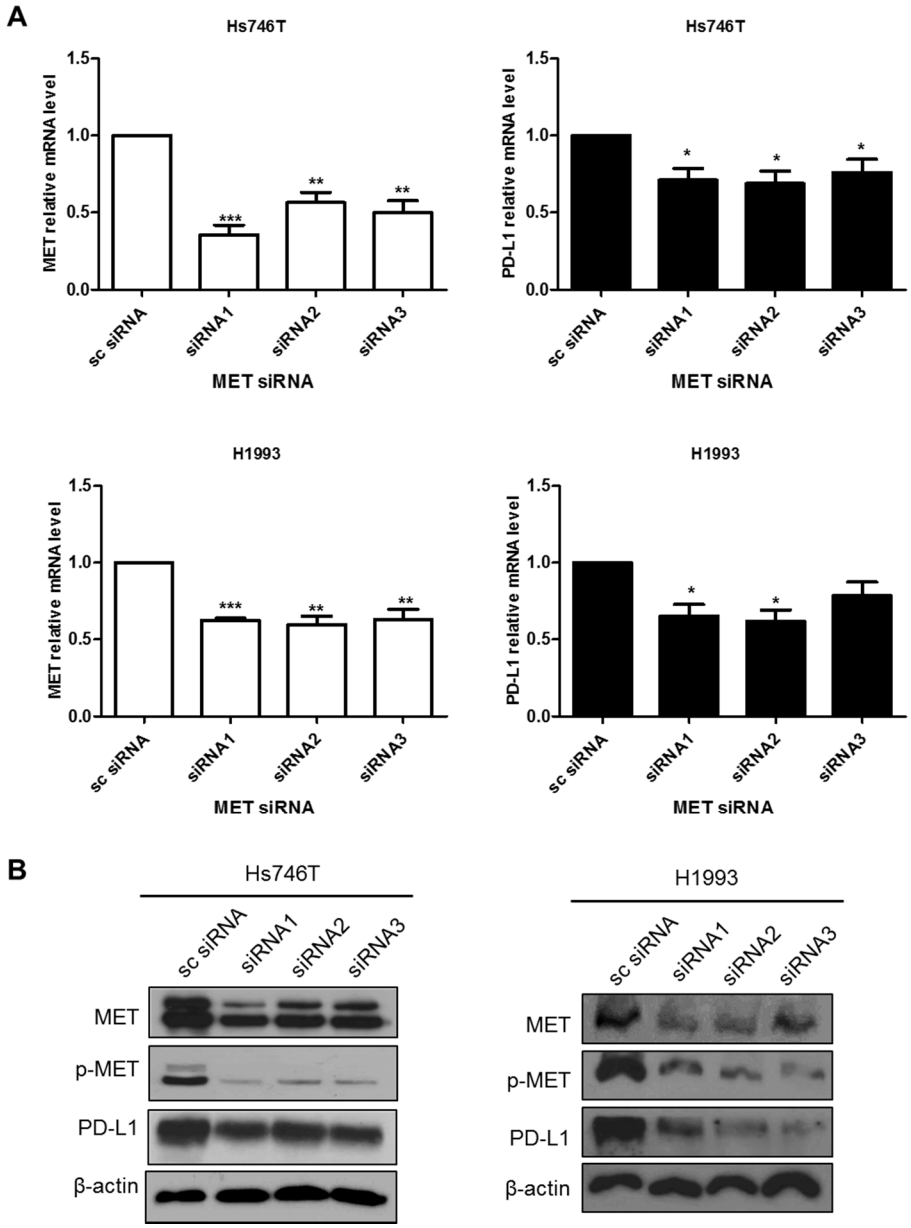
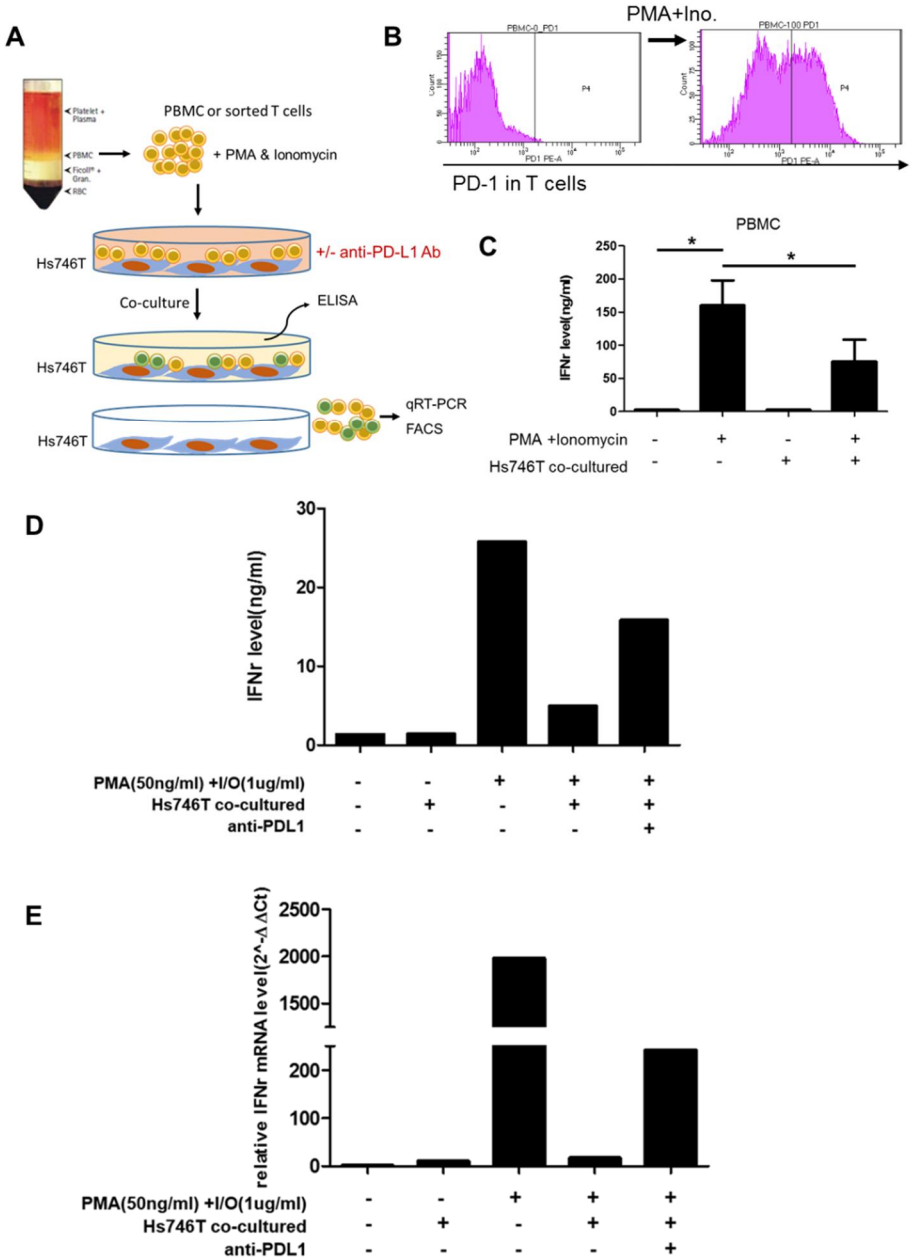


Figure 8. Change of PD-L1 expression in cells transfected with MET siRNAs. Hs746T and H1993 cells were transiently transfected with 100 nM of each MET-targeted siRNA for 48 h. (A) mRNA expression of MET and PD-L1 was determined by qRT-PCR and normalized GAPDH at the indicated time points. (B) Western blot was performed to evaluate protein levels of total MET, p-MET and PD-L1.

Data represent the means ± SDs of at least three independent experiments or are representative of three independent experiments. All p values were calculated using unpaired Student's t-tests. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

PD-L1 expressed in MET-overexpressing cancer cells suppresses the function of PBMC and T cells

The above data raise questions on if MET suppress immune responses by inducing PD-L1 expression via PD-1/PD-L1 pathway. To address this, *in vitro* co-culture system using PBMC and MET-high and PD-L1-high Hs746T cells was developed as schematically shown in Fig. 9A. PBMC from healthy donor were stimulated with PMA and ionomycin, which up-regulated PD-1 expression on T cells and cytokine (IFNγ) secretion (Fig. 9B & C). Co-culture of stimulated PBMC with Hs746T cells resulted in a significant inhibition of IFNγ secretion from PBMC (Fig. 9C). Decreased production of IFNγ from PBMC by co-culture with Hs746T cells was reversed by anti-PD-L1 blocking antibody (Fig. 9D & E) or MET inhibitor (crizotinib) (Fig. 9F & G). These data indicate that Hs746T cells suppress IFNγ production from immune cells in a MET and PD-L1 dependent manner.


A
Platelet + Plasma
PBMC
Ficoll® + Gran.
RBC
PBMC or sorted T cells
+ PMA & Ionomycin
Hs746T
+/- anti-PD-L1 Ab
Co-culture
ELISA
Hs746T
qRT-PCR
FACS
Hs746T
B
PMA+Ino.
PBMC-0 PD1
PBMC-100 PD1
P4
P4
Count
PD1 PE-A
PD-1 in T cells
C
PBMC
IFNr level(ng/ml)
250
200
150
100
50
0
*
*
PMA +Ionomycin - + - +
Hs746T co-cultured - - + +
D
IFNr level(ng/ml)
30
20
10
0
PMA(50ng/ml) +I/O(1ug/ml) - - + + +
Hs746T co-cultured - + - + +
anti-PDL1 - - - - +
E
relative IFNr mRNA level(2^-Δ ΔCt)
2500
2000
1500
200
100
0
PMA(50ng/ml) +I/O(1ug/ml) - - + + +
Hs746T co-cultured - + - + +
anti-PDL1 - - - - +

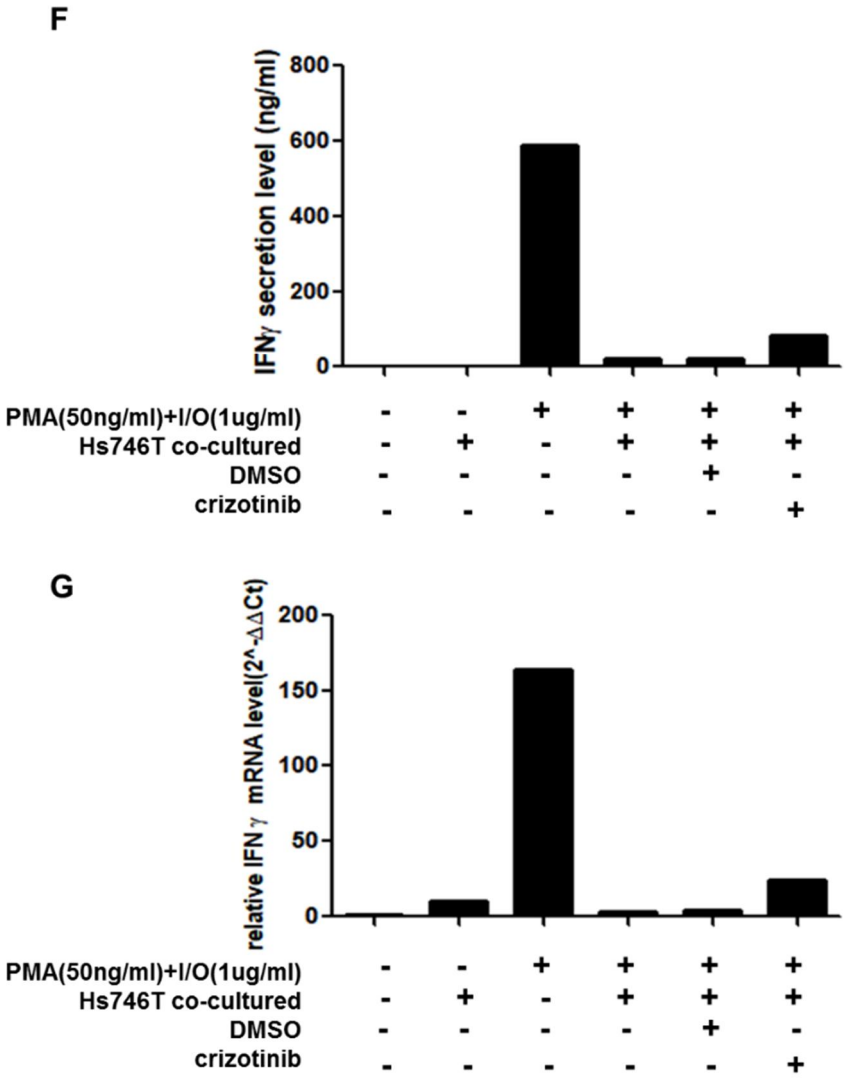
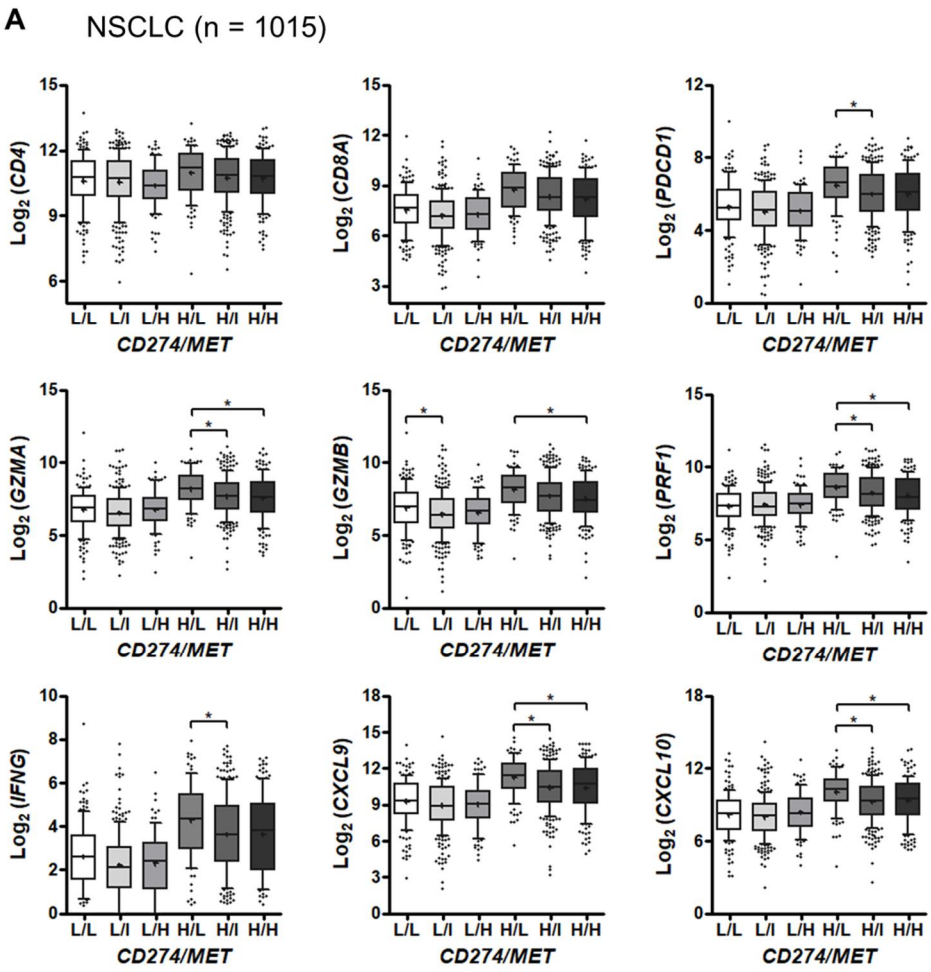


Figure 9. Co-culture of peripheral blood mononuclear cells (PBMC) and PD-L1 expressing Hs746T cells. (A) Schematic procedure of co-culture experiment. (B) Surface expression of PD-1 on PBMCs was evaluated by flow cytometry (T cell gating by APC-conjugated anti-CD3 Ab) (C-G) PBMC were stimulated by PMA (50 ng/ml) and ionomycin (1 ug/ml) for 8 h. Hs746T cells were pre-treated with anti-PD-L1 blocking antibody (40ug/ml) 4 h or with crizotinib (10 nM) 24 h then co-culture with stimulated PBMC for additional 16 h. IFNγ

production was determined by ELISA and qRT-PCR. Data represent the means ± SDs of at least three independent experiments or are representative of three independent experiments. All p values were calculated using unpaired Student's t-tests. *$p < 0.05$.

Landscape of MET, PD-1 ligands and T-cell effector molecules expression in human cancers (TCGA data analysis)

To explore a potential biologic and clinical relevance of MET and PD-L1 expression, TCGA dataset for NSCLC and gastric cancer was analyzed. In NSCLC cases, some of T cell effector molecules, including PD-1 (PCDC1), granzyme A (GZMA), granzyme B (GZMB), perforin (PRF1), IFNγ (IFNG), CXCL9 and CXCL10, showed inverse correlation with MET expression, especially in PD-L1 (CD274) high group rather than in PD-L1 low group. In contrast, no significant correlation was observed between MET/PD-L1 and T-cell effector genes expression in gastric cancer. These data suggest that MET overexpression might be related with poor repertoire for adaptive immune response in PD-L1 expressing human lung cancer.


A
NSCLC (n = 1015)
Log2 (CD4)
Log2 (CD8A)
Log2 (PDCD1)
Log2 (GZMA)
Log2 (GZMB)
Log2 (PRF1)
Log2 (IFNG)
Log2 (CXCL9)
Log2 (CXCL10)
L/L L/I L/H H/L H/I H/H
CD274/MET

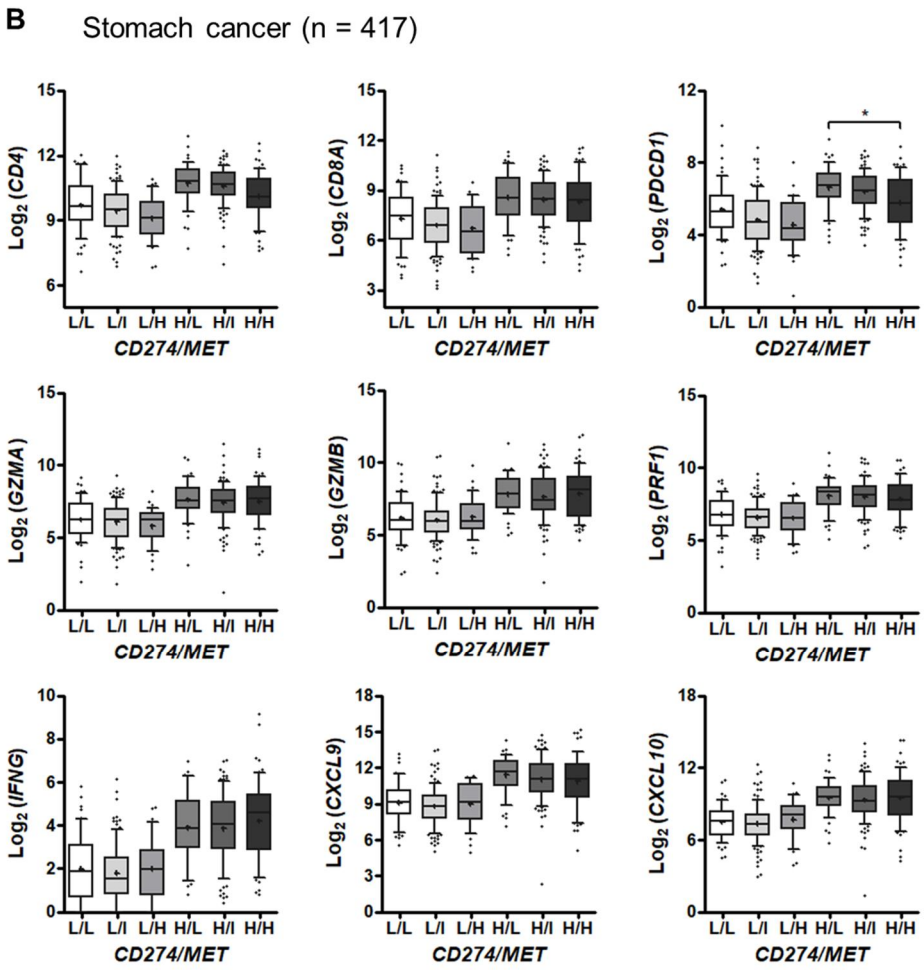


Figure 10. Expression patterns of immune-related genes according to the MET and PD-L1 expressions in human cancer tissues (TCGA data analysis). The expression levels of T cell effector molecules, including *CD4, CD8A, PDCD1, GZMA, GZMB, PRF1, IFNG, CXCL9,* and *CXCL10*, were comparatively analyzed according to the PD-L1 (*CD274)*and *MET* expression status in non-small cell lung cancer cases (n = 1015) and gastric cancer (n = 417) from TCGA dataset. Cases were dichotomized into PD-L1 (*CD274*) low versus high group based on the median value, and trichotomised into *MET* low (less than 25 percentile), intermediate (25-75 percentile), versus high (more than 75 percentile) group. The mRNA levels of those molecules were

compared by Kruskall Wallis test and one-way ANOVA test. The whiskers are drawn down to the 10th percentile and up to the 90th. The middle line of the box is plotted at the median and "+" denotes the mean. Points below and above the whiskers are drawn as individual points. (Abbreviation: N, Negative; P, Positive; L, Low; H, High.). *p < 0.05

DISCUSSION

MET is one of the most commonly altered oncogenes across variable tumor types (1). In addition, MET is a targetable molecule using small molecule inhibitors and monoclonal antibodies under clinical trials (2). MET contributes to tumor progression and aggressiveness by diverse mechanisms including epithelial mesenchymal transition, angiogenesis and drug resistance (18). However, little has been known for the role of MET in immune escape of tumor. To comprehensively investigate the influence of MET on expression of immune-related genes, microarray analysis was performed using MET-amplified and mutated cells line (Hs746T) after functional MET signaling inhibition and MET knockdown by siRNA. Set of genes involved in immune response and inflammatory response were significantly changed in their expressions, and the number of these genes was comparable to those of genes altered by MET inhibition and related to angiogenesis, cell death, differentiation, and migration. Of note, when looking into the membrane bound immune-related molecules, including co-stimulatory and co-inhibitory molecules belonging to B7/CD28 family or TNF/TNFR superfamily and HLA molecules, co-inhibitory molecules including PD-L1, PD-L2, B7-H3 and PVRL1, were decreased, while co-stimulatory molecules, including CD70, 4-1BBL, OX40L (by MET

inhibitor for this molecule) and ICAM1, were increased by MET inhibition/knockdown. These observations suggest that MET might have an important role in immune escape of tumor by up-regulating co-inhibitory molecules and down-regulating co-stimulatory molecules.

Microarray data revealed that PD-L1 is one of the most significantly down-regulated genes by MET suppression. It was previously reported that MET and PD-L1 expression showed a positive correlation in lung cancer, gastric cancer, renal cell carcinoma, and esophageal cancer (12-15, 19-21). Moreover, PD-L1 overexpression was observed in NSCLC with acquired resistance to EGFR TKI relating to MET amplification (22, 23). In addition, high MET expression negatively affected the outcome during EGFR-targeting therapy but was associated with more favorable results with PD-1/PD-L1-directed therapy (5). However, the role of MET for PD-L1 expression in EGFR-TKI-naive cells remains unclear. For unbiased evaluation of the correlations between MET and PD-L1 and PD-L2 expression, CCLE and TCGA data were analyzed. CCLE data, excluding hematolymphoid cells, showed that PD-L1 and PD-L2 (less strength for the latter) expression has a significant positive correlation with MET expression. In TCGA data, PD-L1 and PD-L2 expression showed a significant positive correlation with MET expression in NSCLC (especially adenocarcinoma), stomach cancer, bladder cancer,

breast cancer and glioblastoma, but not in colon cancer. Of note, PD-L1 expression has a stronger relationship with MET expression than PD-L2 in TCGA analysis, which was also observed in immunohistochemical analysis using human NSCLC tissues. Moreover, MET expression level tended to be correlated with PD-L1 expression level in several patients with NSCLC harboring MET exon 14 skipping mutation.

To further validate that MET is involved in PD-L1 upregulation, several cells lines having diverse MET alterations were selected and treated with MET ligand (HGF) or functional inhibitor. HGF treatment in H596 and H23 cells increased PD-L1 mRNA and protein expression in a much higher level in H596 cells (harboring MET exon 14 skipping mutation) than in H23 cells (MET wild-type). This finding suggests that consistent MET activation by HGF in MET-mutated H596 cells, as previously reported (24), might be involved in PD-L1 upregulation. By contrast, MET inhibition using functional inhibitor or MET-specific siRNAs in Hs746T and H1993 cells decreased MET phosphorylation and PD-L1 expression at both mRNA and protein levels. These findings indicate that both MET activation and overexpression might influence on PD-L1 expression in tumor cells. Meanwhile, basal expression level of PD-L1 in cell lines having MET alteration was variable. PD-L1 expression was highest in Hs746T cells, which harbors

both MET mutation and amplification. Although H1993 cells, harboring MET amplification, had a phosphorylated MET level comparable to Hs746T cells in western blot, PD-L1 expression level was much lower than Hs746T cells. Based on these observations, it is suspected that factors other than MET signaling activation might affect on the PD-L1 expression in MET-altered cancer cells. Given that Hs746T cells have an epithelial to mesenchymal transition (EMT) phenotype with low E-cadherin and high vimentin expression and H1993 cells have an epithelial phenotype with high E-cadherin and low vimentin expression (data not shown) and that EMT has been known to increase PD-L1 expression (25, 26), it is possible that EMT would be one of the factors affecting PD-L1 expression in MET-altered tumor.

Finally, if MET-mediated PD-L1 suppresses immune cells function was addressed using co-culture system. PBMC stimulated with PMA and ionomycin showed an increased PD-1 surface expression and IFNγ production, which was compromised by co-culture of MET and PD-L1-high Hs746T cells. Of note, decreased IFNγ production by PBMC co-cultures with Hs746T cells was partly recovered by PD-L1 blocking antibody or MET inhibitor suggesting that MET and PD-L1-high human cancer cells suppressed immune cell function via in a MET and PD-L1-dependent way. However, partial recovery of IFNγ production

from PBMC co-cultured with Hs746T cells by -PD-L1 blocking antibody suggests that mechanisms other than PD-L1 induction might also be important for MET-mediated immunosuppression. Relating to this, it is hypothesized that MET-targeted therapy may be considered as combination therapy with PD-1/PD-L1 blockades for cancer immunotherapy. When MET-mediated PD-L1 expression was reducing by MET inhibitor, tumor cell could lost a target, i.e. PD-L1, for PD-1/PD-L1 blocking antibodies. However, there are another interactions between tumor cells and immune cells mediated by PD-1, as represented by PD-1-CD80 interactions. This study suggests that MET downregulates immune stimulatory molecules like 4-1BBL, CD70 and OX40L. Thus, MET inhibitor has a potential to upregulate these molecules *in vivo* and stimulating anti-tumor immune responses. If tumor are dependent on PD-L1 for immune evasion, PD-L1 downregulation by MET inhibitor and PD-1/PD-L1 blockades could synergistic effect for the control of PD-1/PD-L1-mediated immune evasion. However, the efficacy of combining MET inhibitors and PD-1/PD-L1 blockade need more *in vivo* or clinical studies.

This study has several limitations. First, MET-mediated immune evasion was investigated in a way restricted to PD-L1. Given that expressions of dozens of immune-related genes were changed by MET inhibition in microarray analysis, it would be necessary to study on

other immune-related molecules to comprehensively understand the role of MET in regulation of immune response in tumor. Second, the signaling pathway involved in MET-mediated PD-L1 expression could not be clearly elucidated in this study using human cancer cell lines. It was previously reported that MET upregulates PD-L1 expression in mouse renal cell carcinoma cells in a RAS and heme oxygenase-1-dependent manner and contributes to evasion from immune cell-mediated apoptosis (27). STAT3, AKT, ERK or NF-κB pathways are considered as candidates for MET-mediated PD-L1 regulation and under investigation. Finally, a role of MET or MET-induced PD-L1 in immune escape of tumor was not evaluated using *in vivo* system. The efficacy of combined MET inhibitor and PD-1/PD-L1 blockades should be examined *in vivo* system as mentioned above. In an indirect way, TCGA data was analyzed for T cell effector gene expressions according to MET and PD-L1 status. MET high expression was related to decreased T cell effector genes expression in PD-L1 high NSCLC, suggestive of immunosuppressive role of MET in this tumor. However, this relationship was not observed in gastric cancer, indicating a different role or influence of MET in immune evasion across the patients with different tumor types.

In conclusion, this study demonstrates that MET plays a role for immune evasion of tumor by up-regulating PD-L1 expression and that

MET overexpression/activation is related to immunosuppressive phenotype. MET is suggested as a target not only for targeted therapy but also for cancer immunotherapy, and combined MET and PD-L1 targeting therapy might be considered in patients with cancer, thus demanding further studies.

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국문 초록

연구 목적: MET 활성화는 비소세포폐암을 비롯한 여러 암종에서 흔히 발견되며 암의 성장과 전이를 촉진하는 것으로 알려져 있다. 하지만 MET과 종양세포의 면역회피기작 사이의 관련성은 아직 명확히 밝혀지지 않았다. 특히 암의 면역회피에 중요하게 작용하는 면역단백질 중 하나인 PD-L1은 종양세포에서 내에서 여러 신호전달경로에 의해 발현되는데, 본 연구에서는 MET의 활성화가 종양의 면역회피기작에 미치는 영향을 밝히고자 하였다. 또한 MET의 발현에 따른 PD-L1의 발현조절 기전을 규명함으로써 면역치료제의 새로운 바이오마커 (biomarker), 병용 치료제 등의 가능성을 제시하고자 하였다.

연구 방법: MET 유전자에 돌연변이 혹은 증폭이 일어난 세포주에서 MET 억제제 처리 또는 siRNA를 이용한 MET 발현 저해 시 유전체 발현의 변화를 microarray를 이용하여 분석하였고, 특히 면역반응 관련 유전자 발현의 변화가 있음을 확인하였다. 이를 토대로 MET 유전자의 돌연변이와 증폭이 없는 세포주와 있는 세포주에서 PD-L1, PD-L2 발현을 비교하였다. 또한 HGF를 처리하여 MET 신호전달을 활성화 시켰을 때 PD-L1과 PD-L2의 발현이 유도되는지 확인하였고, 반대로 MET 저해제 혹은 MET siRNA를 처리하여 MET을 억제하였을 때 PD-L1의 발현 변화를 조사하였다. 그리고, MET 돌연변이를 가진 세포주가 발현하는 PD-L1이 PD-1과의 결합을 통해 면역세포의 기능을 억제하는 지 알아보기 위해 혈액에서 분리한 단핵세포와 함께 배양하여 단핵세포의 IFNγ 분비량 및 세

포사멸의 변화를 측정하였다. 또한 Cancer Cell Line Encyclopedia (CCLE), the Cancer Genome Atlas (TCGA) 분석과 폐암 면역조직화학 염색을 시행하였다.

연구 결과: Microarray 분석을 통하여 MET을 억제 시 면역억제분자들의 발현이 감소하고 면역자극분자들의 발현이 증가함을 확인하였다. 암 세포주에서 MET의 활성화가 PD-L1의 발현을 전사 수준과 단백질 발현 수준에서 증가시킴을 확인하였다. 특히 MET에 돌연변이가 있는 세포주에서 HGF에 의해 PD-L1이 크게 유도됨을 알 수 있었다. 또 MET 증폭 및 돌연변이 세포주에서 MET의 신호전달을 억제했을 때 PD-L1의 감소를 확인하였다. 암 세포주에서 MET에 의해 유도된 PD-L1이 혈액 내의 단핵세포의 기능을 억제하여 면역 회피 반응을 일으키는 것을 관찰하였다. MET과 PD-L1 발현이 양의 상관관계가 있음을 CCLE 와 TCGA를 통해 확인하였고, 환자의 폐암 조직에서 검증하였다. 또한 폐암환자에서 MET의 과발현은 PD-L1 양성 종양에서 T 세포 기능 저하와 관련됨을 TCGA 분석을 통해 확인하였다.

결론: 이번 연구를 통해 종양세포에서 MET의 활성화와 과발현이 PD-L1 발현을 유도하고 이를 통해 면역 회피 반응을 증가시킨다는 것을 증명하였다. 이를 토대로 면역세포의 PD-1/PD-L1 결합을 억제하는 면역치료제와 MET 저해제를 병용하였을 때 치료효과의 상승이 있을 것으로 예상 할 수 있다.

주요어: MET, 면역체크포인트, 암 면역치료, programmed cell death ligand 1 (PD-L1), PD-1/PD-L1 경로

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