Abstract. Non-small cell lung cancer (NSCLC) is one of the most severe malignant tumor types worldwide. Recent studies have reported an important role of PD-L1 in mediating immune evasion in the tumor microenvironment. In addition, increasing research has indicated that the expression of PD-L1 is related to the epithelial-mesenchymal transition (EMT) process, but the related mechanisms remain to be explored. In the present study, we explored the molecular mechanisms underlying the regulation of PD-L1 expression during the EMT process in NSCLC cells treated with transforming growth factor-β1 (TGF-β1) and fibroblast growth factor 2 (FGF2). The phenotypic alteration associated with EMT was evaluated by western blotting and confirmed by a wound-healing assay. The results revealed that EMT markedly promoted the expression of PD-L1 in the A549 cell line, while having no obvious influence on the H1650 and H1975 cells. Furthermore, the AKT pathway inhibitor LY294002, the ERK pathway inhibitor PD98059 and the TAK1 pathway inhibitor 5Z-7 inhibited the expression of PD-L1 in A549 and H1650 cells, but not in H1975 cells, during the EMT process. Moreover, our study indicated that the AKT, ERK and TAK1 pathways regulated the expression of PD-L1 by mediating transportation of the transcription factor Stat3 and the p65 subunit of NF-κB from the cytoplasm to the nucleus, with such findings determined by western blotting and flow cytometric analyses. Furthermore, the expression of PD-L1 was significantly increased following treatment with gefitinib in a tumor xenograft model. In summary, our results support the role of ERK, AKT and TAK1 in mediating the expression of PD-L1 during the EMT process, and indicate a promising strategy of PD-L1-targeted therapy for the clinical treatment of NSCLC.

Introduction

Lung cancer is one of the most common and fatal malignant tumor types worldwide (1). Non-small cell lung cancer (NSCLC) accounts for 80-85% of all lung cancer cases (2). Since 2003, three generations of TKI drugs have received FDA approval for the clinical treatment of NSCLC (3). However, the increasing rate of drug resistance in patients treated with molecular targeted therapy is becoming a severe challenge for treatment. Moreover, even patients who exhibit sensitivity to targeted drugs at the start of treatment can become resistant within a few months (4,5). The molecular mechanisms of drug resistance, regarding both mutational and non-mutational pathways, have only recently begun to be fully explored (6); however, at present, accumulating evidence indicates that epithelial-mesenchymal transition (EMT) plays an important role in the resistance to targeted therapy in a non-mutational manner (7).

EMT is a process by which epithelial cells are converted into mesenchymal cells. This involves a phenotypic switch of cellular characteristics, whereby cells lose their adhesive properties and cell-cell contacts while acquiring migratory properties, which can promote tumor progression. The process of EMT is mediated by a change in the expression of cell surface proteins and the induction of cytoskeletal rearrangement, among other phenotypic alterations (8,9). Diverse signaling molecules can trigger EMT, including hepatocyte growth factor (HGF), epidermal growth factor (EGF), fibroblast growth factor (FGF) (10,11). In particular, transforming growth factor-β1 (TGF-β1) is considered to be a key regulator in the EMT pathway (12). TGF-β1 belongs to a family of related polypeptide factors with shared structural motifs, which serve vital roles in processes such as cell growth, differentiation and oxidative stress (13). TGF-β-mediated EMT has
been identified in many different tumor types, including lung cancer (14). TGF-β promotes EMT-related gene transcription in a Smad3-dependent manner through the β-integrin signal transduction pathway or the mitogen-activated protein kinase (MAPK) signaling pathway, in order to influence gene expression and ultimately transform epithelial cells from an epithelial phenotype to a stromal cell phenotype, thus promoting the occurrence of EMT (15-17).

Programmed death-1 (PD-1) is so named due to its association with apoptosis, and is mainly expressed in CD4+ and CD8+ T lymphocytes, B lymphocytes, NK (natural killer) cells and regulatory T cells (Tregs) (18,19). The two ligands of PD-1 are PD-L1 (also known as CD274 or B7-H1) and PD-L2 (also known as CD273 or B7-DC) (19). Cytokines, such as IL-4 and IL-10, activate the PD-1/PD-L1 pathway, which affects T cells with high expression of PD-1 molecules on their surface or tumor cells with high surface-expression of PD-L1 (20,21). As a result, T cell function is inhibited and the immune system is suppressed in its recognition and attack of tumor cells. Recent studies have indicated that the EMT in tumor cells is associated with immune escape, which results in insensitivity to targeted drugs (22,23).

In this study, we first speculated on the effect of the EMT process induced by TGF-β1 and FGF2 on the expression of PD-L1 in the NSCLC cell lines A549 (wild-type), H1650 (E746_A750 deletion) and H1975 (harboring the mutation of T790 in EGFR). We then investigated the molecular mechanisms underlying the regulation of PD-L1 expression during the EMT process, by treating the three NSCLC cell lines with the AKT pathway inhibitor Ly294002, the ERK pathway inhibitor PD98059 and the TAK1 pathway inhibitor 5Z-7. Furthermore, we assessed the expression of PD-L1 in the NSCLC cells following treatment with EGFR-TKI (gefitinib) (24,25). Collectively, these tests aimed to provide a theoretical basis for the development of new methods to reverse drug resistance in NSCLC.

Materials and methods

Reagents. Antibodies against E-cadherin (cat. no. 14472), N-cadherin (cat. no. 14215), vimentin (cat. no. 5741), mTOR (cat. no. 2983), phosho-mTOR (Ser 2448) (cat. no. 2983), Akt (cat. no. 4685), phosho-Akt (Ser 473) (cat. no. 4060), p44/42 MAPK (Erk1/2) (cat. no. 4695), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (cat. no. 4370), TAK1 (cat. no. 4505), p65 (cat. no. 8242), GADPH (cat. no. 5174) and human Basic Fibroblast Growth Factor (hFGF basic/FGF2) (cat. no. 8910) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). PD-L1 (cat. no. 205921) was purchased from Abcam (Cambridge, MA, USA). Gefitinib (cat. no. S1105) and PD98059 (cat. no. S1177) were purchased from Selleck Chemicals (Houston, TX, USA) and stock solution was prepared in dimethyl sulfoxide (DMSO) at 10 mM. 5Z-7-oxozeaenol (5Z-7) was obtained from Sigma-Aldrich (Rehovot, Israel) and dissolved in DMSO at 10 mM. 5Z-7-oxozeaenol (5Z-7) was obtained from Sigma-Aldrich. LY294002 (cat. no. S1105) and PD98059 (cat. no. S1177) were purchased from Selleck Chemicals (Houston, TX, USA) and stock solution was prepared in dimethyl sulfoxide (DMSO) at 10 mM. 5Z-7-oxozeaenol (5Z-7) was obtained from Sigma-Aldrich (Rehovot, Israel) and dissolved in DMSO at 10 mM. 5Z-7-oxozeaenol (5Z-7) was obtained from Sigma-Aldrich (Rehovot, Israel) and dissolved in DMSO at 10 mM.

Cell lines. Non-small cell lung cancer cell lines A549, NCI-H1975 and NCI-H1650 were purchased from the China Infrastructure of Cell Line Resources (Beijing, China). The cells were grown in McCoy’s medium (Gibco; Invitrogen, Carlsbad, Calif) or RPMI-1640 modified medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco; Invitrogen, Carlsbad, Calif), 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were maintained in humidified air containing 5% CO2 at 37°C in a monolayer culture.

Wound healing assay. Cells (5x10^5) in 6-well plates were allowed to grow until reaching 80% confluency. After starvation overnight, wounds were scratched on the cell surface using a 100 μl micropipette tip. The cells were treated with 10 ng/ml TGF-β1 plus 10 ng/ml FGF2 or 10 ng/ml TGF-β1 plus 10 ng/ml FGF2 for 48 h. The changes of the wound area were captured by Nikon camera.

Immunofluorescence staining. Cells 5x10^5 were seeded on 6-well plates coated with coverslips in McCoy’s medium or RPMI-1640 medium. Following starvation overnight in serum-free medium, the cells were treated with 10 ng/ml TGF-β1 plus 10 ng/ml FGF2 for 48 h. The cells were fixed for 20 min in paraformaldehyde and permeabilized in 0.2% Triton X-100 for 5 min. Then, F-actin was stained with Rhodamine phalloidin and the nuclei with DAPI. The images were captured using a Leica SP5 spectral confocal microscope.

Western blot analysis. The cells in 6-well plates were washed with cold phosphate-buffered saline (PBS) twice and lysed with 80 μl RIPA solution containing 1 mM PMSF in each well. Lysates were incubated on ice and vortexed every 5 min, in total 30 min. Then the supernatant was collected after centrifugation (15,000 x g for 5 min at 4°C). The concentration of the total protein was assessed using the BCA Protein Assay Kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). Total protein (35 μg) was subjected to 10-12% SDS-PAGE and blotted on PVDF membranes (EMD Millipore, Bedford, MA, USA). Then the membranes were incubated with primary antibodies mentioned above (1:1,000 dilution) overnight at 4°C. The results were detected using the Odyssey detection system (LI-COR Biosciences, Lincoln, NE, USA).

Flow cytometric analysis. Cells were plated in 6-well plates (1-5x10^5) and treated with 0.05% trypsin. Then, the cells were harvested by centrifugation (800 x g, 5 min). Flow cytometric cells were stained with the PE-conjugated anti-human PD-L1 clone M1H1. The cells were assessed using Accuri™ C6 (BD Biosciences, Franklin Lakes, NJ, USA). The data was analyzed using FlowJo software (TreeStar, Inc., Ashland, OR, USA) and the PD-L1 levels were determined by calculating the Median Fluorescence Intensity (MFI).

Nuclear translocation analysis. Cells were harvested with trypsin-EDTA, and then centrifuged at 500 x g for 5 min. The
cells were then transferred to a 1.5 ml microcentrifuge tube and pelleted by centrifugation at 500 x g for 2-3 min. A pipette was used to carefully remove and discard the supernatant, leaving the cell pellet as dry as possible. Ice-cold CER I was added to the cell pellet. The tube was then vortexed vigorously on the highest setting for 15 sec to fully suspend the cell pellet and subsequently the tube was incubated on ice for 10 min. Ice-cold CER II was added to the tube and then the tube was vortexed for 5 sec on the highest setting. Incubation followed on ice for 1 min and then the tube was centrifuged for 5 min at a maximum speed (~16,000 x g). The supernatant was then immediately transferred to a clean pre-chilled tube and vortexed on the highest setting in ice-cold NER. Subsequently, the sample was placed on ice and vortexing continued for 15 sec every 10 min, for a total of 40 min. The tube was then centrifuged at maximum speed (~16,000 x g) in a microcentrifuge for 10 min. Finally, the supernatant (nuclear extract) fraction was immediately transferred to a clean pre-chilled tube and the extracts were stored at -80˚C until use.

Animals and tumor xenograft experiments. As mentioned in our previous study (24), total 12 five- to six-week-old female BALB/c nude mice were raised under specific pathogen-free conditions. Approximately 2x10⁶ H1975 cells were injected subcutaneously into the right flank region of nude mice. The tumor volume was assessed every three days. Mice bearing tumors ~50 mm³ in volume were randomized into six mice per group (n=6). The two group were administrated every day for 21 days by oral gavage as followed: (a) saline; (b) gefitinib. At harvest, the mice were sacrificed and the tumor tissues were fixed in formalin for immunohistochemistry.

Immunohistochemistry. A series of 3-µm sections were obtained from each paraffin block. After heat immobilization, deparaffinization and rehydration, the sections were treated with 10 mM sodium citrate buffer at pH 6.0 in boiling temperature for antigen retrieval. Then the tissue sections were incubated with the primary antibodies PD-L1 (1:100) overnight at 4˚C and incubated with the secondary antibody biotin (cat. no. 926-32211; (LI-COR Biosciences) for 20 min at room temperature. Targeted proteins PD-L1 were visualized using peroxidase substrate diaminobenzidine (DAB). Staining intensities were estimated in five random fields per section by three independent observers individually using microscope equipped with camera.

Statistics. Data were computed using Student’s t-test (two-tailed) or one-way ANOVA analysis. All values were presented as the mean ± SD. P<0.05 was considered to indicate a statistically significant difference.

Results

FGF2 plus TGF-β1 induced EMT in NSCLC cell lines. In this study, we explored the effects of FGF2 plus TGF-β1 on promoting the EMT process in three NSCLC cell lines: A549
Let al: PD-L1 REGULATION IN NSCLC DURING EMT PROCESS

Materials and Methods

We used three human lung cancer cell lines with different EGFR mutations: A549 (wild-type EGFR), H1650 (E746_A750 deletion) and H1975 (T790M). We stimulated the cells with TGF-β1, FGF2 or FGF2 plus TGF-β1 for 48 h in serum-free medium. The EMT phenotype was confirmed in the FGF2 plus TGF-β1 group when the expression of E-cadherin (an epithelial marker) was decreased and the expression of vimentin and N-cadherin (mesenchymal markers) were markedly increased compared with the other groups (Fig. 1A-C). Subsequently, cell migration capacity was assessed by a wound healing assay. We observed that the migration ability of cells treated with FGF2 plus TGF-β1 was enhanced in comparison with the other groups at both 24 and 48 h (Fig. 1D-F). These results indicated that treatment with FGF2 plus TGF-β1 downregulated E-cadherin, upregulated vimentin and N-cadherin and increased migration ability in all three NSCLC cell lines.

In addition to affecting cell migratory ability and EMT-related protein expression, morphological changes further confirmed the process of EMT. An increase in cell size mediated by FGF2 plus TGF-β1 was one of the predominant characterizations of EMT. As shown in Fig. 1G and H, the A549 cells treated with FGF2 plus TGF-β1 for 48 h were markedly enlarged compared with the control cells. Collectively,
these results indicated that FGF2 plus TGF-β1 treatment is an effective way of promoting the induction of a physiological EMT phenotype.

**EMT promotes the expression of PD-L1 in the wild-type EGFR NSCLC cell line.** The expression of PD-L1 in A549 cells was markedly increased during the EMT process induced by FGF2 plus TGF-β1 treatment. As shown in Fig. 2A, the PD-L1-positive staining on the surface of A549 cells was significantly increased after treatment with FGF2 plus TGF-β1 as determined by flow cytometric analysis (P=0.0029). Moreover, this result was further confirmed by RT-PCR analysis. The mRNA level of PD-L1 during the EMT process was higher than that in the control cells (P=0.009) (Fig. 2B). These results indicated that the EMT process markedly promoted the expression of PD-L1 in A549 cells.

**Related signaling pathways during the EMT process in wild-type EGFR A549 cells.** To further explore the molecular mechanisms of EMT-induced PD-L1 expression, we assessed several EMT-related signaling pathways. As shown in Fig. 3A, the cells were treated with the AKT inhibitor LY294002, the ERK inhibitor PD98059 and the TAK1 inhibitor 5Z-7 after induction of EMT. Following treatment, we observed that the expression of phosphorylated AKT, phosphorylated mTOR, phosphorylated ERK was inhibited compared to FGF2 plus TGF-β1 treatment. Furthermore, the expression of PD-L1 was decreased following treatment with the inhibitors (Fig. 3B). According to previous research, phosphorylated proteins mediate downstream transcription factors in their signaling pathways. Our study revealed that the inhibitors of AKT, ERK and TAK1 reduced the expression of Stat3 in the nucleus to a similar extent. Additionally, treatment with AKT inhibitor (LY294002) and TAK1 inhibitor (5Z-7), but not ERK inhibitor (PD98059), elicited decreased nuclear expression of p65 (Fig. 3C). The results confirmed our hypothesis that inhibition of AKT, ERK and TAK1 phosphorylation could inhibit the import of Stat3 transcription factor into the nucleus, and thus inhibit the expression. Furthermore, our results confirmed that inhibition of AKT and TAK1 phosphorylation could further inhibit the nuclear import of the p65 subunit of NF-κB, thus indicating its synergistic role in influencing the expression of PD-L1.

**Related signaling pathways regulate PD-L1 expression in H1650 cells while having no influence on H1975 cells during the EMT process.** In this study, we further explored the regulatory mechanism underlying PD-L1 expression in the H1650 cell line. As shown in Fig. 4A, the expression of PD-L1 during the EMT process underwent no significant change in the H1650 cells. And the expression of relative proteins was decreased after the treatment of inhibitors (Fig. 4B). In addition, the inhibitors of AKT (LY294002), ERK (PD98059) and TAK1 (5Z-7) decreased the expression of PD-L1 (Fig. 4C).

In addition, we explored the influence of EMT on the T790M EGFR mutant cell line H1975. As shown in Fig. 4D, the expression of PD-L1 had no influence on the expression of PD-L1 (P=0.3739). Even following the inhibition of
relative proteins (Fig. 4E), the expression of PD-L1 had no changes (Fig. 4F). These results revealed that the signaling pathways of AKT, ERK and TAK1 served an important role in promoting the expression of PD-L1 during the EMT process in H1650 cells, but not in H1975 cells.

Gefitinib promotes the expression of PD-L1 in H1975 xenograft nude mice. In the present study, we also assessed the expression of PD-L1 in the tumor tissue of an H1975 xenograft nude mouse model. As shown in Fig. 5, the expression of PD-L1 in the gefitinib treatment group was markedly higher compared with that in the control group. This result revealed that gefitinib promoted the expression of PD-L1 in the H1975 xenograft nude mice.

Discussion

Currently, researchers are becoming increasingly aware that the occurrence and development of tumors are processes involving mutual antagonism between the tumor and host cells. The interaction between the tumor and host serves an important role in tumor progression and metastasis. Furthermore, the host immune system and the immune microenvironment are considered to be crucial determinants of tumor progression and metastasis. Under normal conditions, the immune system of the host body should be able to recognize and kill the malignant cells to remove or control the growth of tumor tissue. However, the immune system of cancer patients is typically inhibited. One of the most obvious characteristics of this inhibition is the clustering of immune suppressor cells around the tumor mass in its microenvironment. PD-L1 is one of the main pathways involved in tumor immune evasion. PD-L1 mRNA is widely expressed in normal human tissues and organs, while the PD-L1 protein is rarely expressed in normal human tissues, being limited to embryonic tonsil tissue, macrophage-like cells, lung cells and liver cells at a low level of expression (25). This phenomenon reveals that the expression of PD-L1 receives post-transcriptional regulation under normal physiological conditions. However, PD-L1 is abundantly expressed on the cell membranes of various tumor cells. Moreover, PD-L1 is expressed in tumor lesions, but not in metastatic cells in most tumors (25). These findings indicate that PD-L1 is selectively expressed in tumor tissues, and that its expression is closely related to the tumor microenvironment.

Lung cancer is one of the most common and fatal malignant tumor types worldwide. Non-small cell lung cancer (NSCLC) accounts for 80-85% of all lung cancer cases (26). Since 2003, three generations of TKI drugs have received FDA approval for the clinical treatment of NSCLC (27). However, the increasing rate of drug resistance in patients treated with chemotherapy increasingly becomes a serious challenge; even patients who are sensitive to chemotherapeutic drugs at the start of treatment develop resistance within a few months (28). The molecular mechanisms of drug resistance, including both mutational and non-mutational, have only recently begun to be explored (29-32). At present, accumulating evidence has indicated that the epithelial-mesenchymal transition (EMT) plays an important role in chemo-resistance in a non-mutational way. In the present study, we selected three NSCLC cell lines, A549 (wild-type EGFR), NCI-H1975 (L858R and T790M mutation; EGFR-TKI-resistant) and NCI-H1650 (E746_A750 deletion mutation; EGFR-TKI-sensitive), and explored the relationship between the expression of PD-L1 and the EMT process.

Numerous primary studies have demonstrated that EMT is strongly associated with cancer metastasis (33-34). Cadherin
switching is a main characteristic alteration in several types of cancer, including NSCLC. The inactivation of the epithelial marker E-cadherin is the pivotal event of EMT in tumor progression (9). Furthermore, activation of certain mesenchymal markers, such as vimentin and N-cadherin, may play a positive role in the process of metastasis since the normal cell-cell and cell-matrix contacts are disrupted (35). The aforementioned changes promote cell migration and contribute to loss of cell adhesion. A recent study suggested a relationship between PD-L1 expression and the EMT process in promoting the occurrence of immune evasion and metastasis progression (22). In this study, we created an effective way of promoting the induction of a physiological-like EMT phenotype in A549 cells. Additionally, our data elucidated the key role of EMT in triggering the expression of PD-L1 in the A549 cell line. We further explored the mechanisms of EMT in promoting the expression of PD-L1. The results indicated that the AKT, ERK and TAK1 pathways play synergistic roles in regulating the expression of PD-L1 during the EMT process, by mediating nuclear import of the transcription factor Stat3 and the p65 subunit of NF-κB in A549 cells. These regulatory mechanisms may be applied to the EGFR-TKI sensitive cell line H1650, but not the EGFR-TKI resistant cell line H1975. Furthermore, gefitinib, an oral EGFR-TKI, increased the expression of PD-L1 in an H1975 xenograft tumor. Collectively, the aforementioned results revealed that EMT could mediate PD-L1 expression via multiple pathways, and thereby may serve an important role in immune evasion in the tumor microenvironment. However, our research is in the primary stage and further study is warranted to confirm the present conclusions.

In the clinical treatment of NSCLC, certain patients exhibit primary drug resistance to EGFR-TKI without having gene mutations. Considering the results of previous studies together with our findings leads us to make the assumption that EMT plays an important role in promoting the expression of PD-L1 in NSCLC cases expressing either wild-type EGFR or activated EGFR mutant by mediating the AKT-mTOR, ERK and TAK1-p65 pathways, but not in cases with EGFR-TKI resistance. Therefore, EMT may play a crucial role in primary drug resistance to EGFR-TKI in patients without mutation by promoting immune evasion, while having little effect on NSCLC with EGFR-TKI-resistant gene mutation (T790M). Although the process of tumor metastasis is regulated by various key factors, such as the circulatory system (26), the phosphorylation of Smad2 and Smad3 (37), and diverse growth factors in the tumor microenvironment (38), EMT may be one of the key cross-linking components mediating all of the necessary elements during the process of metastasis. Therefore, inhibition of EMT induction could be applied as a novel therapeutic method for the treatment of NSCLC harboring wild-type EGFR or EGFR-TKI-sensitizing mutation.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors’ contributions

LF and ZTJ conceived and designed the study. LF, YY and XZ performed the experiments. LL and WJD reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experimental protocols were approved by the Institutional Review Board of the Department of Laboratory Animal Science of Peking university Health Science Center (Beijing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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