Nagilactone E increases PD-L1 expression through activation of c-Jun in lung cancer cells

CHEN Yu-Chi¹, HUANG Mu-Yang¹, ZHANG Le-Le¹,², FENG Zhe-Ling¹, JIANG Xiao-Ming¹, YUAN Luo-Wei¹, HUANG Run-Yue³, LIU Bo³, YU Hua¹, WANG Yi-Tao¹, CHEN Xiu-Ping¹, LIN Li-Gen¹, LU Jin-Jian¹*

¹State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Macao 999078, China; ²School of Medicine, Chengdu University, Chengdu 610106, China; ³The Second Affiliated Hospital of Guangzhou University of Chinese Medicine (Guangdong Provincial Hospital of Chinese Medicine), Guangzhou 510120, China

Available online 20 Jul., 2020

[ABSTRACT] Nagilactone E (NLE), a natural product with anticancer activities, is isolated from Podocarpus nagi. In this study, we reported that NLE increased programmed death ligand 1 (PD-L1) expressions at both protein and mRNA levels in human lung cancer cells, and enhanced its localization on the cell membrane. Mechanistically, NLE increased the phosphorylation and expression of c-Jun, and promoted the localization of c-Jun in the nucleus, while silencing of c-Jun by small interfering RNA (siRNA) reduced NLE-induced PD-L1. Further study showed that NLE activated the c-Jun N-terminal kinases (JNK), the upstream of c-Jun, and its inhibitor SP600125 reversed the NLE-increased PD-L1. Moreover, NLE-induced PD-L1 increased the binding intensity of PD-1 on the cell surface. In summary, NLE upregulates the expression of PD-L1 in lung cancer cells through the activation of JNK-c-Jun axis, which has the potential to combine with the PD-1/PD-L1 antibody therapies in lung cancer.

[KEY WORDS] Programmed death ligand 1; Nagilactone E; Lung cancer; c-Jun; JNK


Introduction

Lung cancer is the most commonly diagnosed cancer and the leading cause of cancer death, which occupied 18.4% of the total cancer deaths in the world [1]. Immune checkpoint inhibitors, particularly inhibitors of programmed cell death protein 1 (PD-1) and its ligand programmed death ligand 1 (PD-L1), have revolutionized the treatment of lung cancer in recent years. PD-L1 also known as CD274 or B7-H1, which is expressed in several cancer cells and immune cells, binds to PD-1 expressed on the surface of T cells to suppress the immune activity of T cells, leading to immune escape of tumor cells in the tumor microenvironment [2,3]. Anti-PD-1 antibodies (nivolumab and pembrolizumab) and anti-PD-L1 antibodies (atezolizumab and durvalumab) have been approved by food and drug administration (FDA) for the treatment of lung cancer. However, most lung cancer patients still cannot benefit from the anti-PD-1/PD-L1 therapies [4-9]. It has been reported that anticancer compounds such as PARP inhibitors, CDK4/6 inhibitors, HDAC3 inhibitors and neddylation inhibitors, could upregulate PD-L1 expression and enhance the effects of PD-1/PD-L1 antibodies [10-13], suggesting that combination with anti-tumor drugs that could upregulate PD-L1 expression is a potential strategy to enhance the efficacy of PD-1/PD-L1 antibodies [12]. Natural products provide abundant resource library for the anticancer drug development, and also exhibit promising potential to improve the therapeutic efficacy through combination strategy [14,15]. In this case, we considered whether any natural product could alter the PD-L1 expression and modify the efficacy of PD-1/PD-L1 blocking therapies.

Podocarpus nagi (Thunb.) Pilg is originated and widely distributed in East Asia, and is one of the oldest gymno-
The seeds of *P. nagi* are up to 30% oil content, which is used as the raw material for the production of edible oils and industrial oils [17]. *P. nagi* seed oil has effects of improving inflammation and lipid metabolism, which is associated with its presence of rare non-methylene interrupted fatty acids [18, 19]. The seed oil from *P. nagi* contains many diterpenoids, some of which exhibit anti-bacterial, anti-inflammatory and anti-atherosclerotic effects [20-22]. Previous studies in our group have found that nine novel diterpenoids and nor-diterpenoids obtained from *P. nagi* have cytotoxicity and autophagy induction effect on cancer cells [23]. Nagilactone E (NLE), a dinorditerpenoid isolated from *P. nagi*, induced G2 phase cell cycle arrest by downregulating cyclin B1, and inhibited TGF-β1-induced epithelial-mesenchymal transition, migration and invasion in non-small cell lung cancer cells [24, 25]. Here, NLE was identified to increase the protein and mRNA expressions of PD-L1 in lung cancer cells due to the activation of JNK - c-Jun axis, which has the potential to combine with the PD-1/PD-L1 antibody therapies.

**Materials and Methods**

**Reagents**

Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), penicillin-streptomycin, phosphate-buffered saline (PBS) and trypsin-EDTA were purchased from Gibco (Carlsbad, CA, USA). Triton-X100, paraformaldehyde (PFA) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Bovine serum albumin (BSA) was purchased from Beyotime (Shanghai, China). Hoechst 33342 was obtained from Molecular Probes (Grand Island, NE, USA). SP600125 was obtained from Selleck Chemicals (Houston, TX, USA). NLE was obtained from Dr. LIN Li-Gen’s lab from University of Macau and the purity of NLE was determined as about 99.99% [23].

**Cell culture**

Human lung cancer NCI-H460 and NCI-H1975 cells were purchased from KeyGen Biotech (Jiangsu, China) and Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China), respectively. Human lung cancer A549 cells were purchased from American Type Culture Collection (Rockville, MD, USA). The cells were cultured in the RPMI 1640 medium that supplemented with 10% FBS, 100 U·mL⁻¹ penicillin and 100 μg·mL⁻¹ streptomycin, and were maintained in the 5% CO₂ incubator at 37 °C.

**Western blot**

The cells were lysed by using the SDS buffer to extract proteins. The proteins were separated by the SDS-PAGE, and transferred to the PVDF membranes. Then the membranes were blocked with 5% non-fat dried milk and were incubated with the primary antibodies. After that, the membranes were washed and incubated with HRP-conjugated secondary antibodies. Finally, the protein bands were visualized by ECL detection reagent (GE healthcare, Buckinghamshire, UK). The antibodies used were as follows: PD-L1 (#13684), p-c-Jun (S63) (#2361), p-c-Jun (S73) (#3270), c-Jun (#9165), p-JNK (T183/Y185) (#4671), JNK (#9252), Histone H3 (#4499), GAPDH (#5174), HRP-linked anti-rabbit IgG (#7074) and HRP-linked anti-mouse IgG (#7076). All antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA).

**Quantitative real-time PCR**

The total RNA was extracted by Trizol (Invitrogen, Carlsbad, CA, USA). The CDNA was synthesized according to the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) protocol. Quantitative real-time PCR (qRT-PCR) was using SYBR Green reagent (Roche, Basel, Switzerland), and was performed in Viia 7 Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). The primers used were as follows: PD-L1: 5’-CAAT-GTGAACCGCACACTGAGAGA-3’ (forward) and 5’-GGA-TAAGATGGGTCCAGAGA-3’ (reverse); GAPDH: 5’-GCCACACCAACTCCTCCACCTTT-3’ (forward) and 5’-TGCCTGTAAGCAAATTCGGTGTCATA-3’ (reverse).

**Flow cytometry**

The cells were trypsinized and re-suspended in 100 μL of flow cytometry incubation buffer (PBS with 0.5% BSA), then incubated with the fluorescent-labeled antibodies for 45 min at room temperature. The cells were washed with PBS and analyzed in BD LSRFortessa cytometry (BD Biosciences, Franklin Lakes, NJ, USA). The data were analyzed by FlowJo 7.6 (FlowJo LLC, Ashland, OR, USA). The antibodies used were as follows: PE-labeled mouse anti-human PD-L1 (#557924, BD Bioscience, Franklin Lakes, NJ, USA) and PE-labeled mouse IgG1 (#555749, BD Bioscience, Franklin Lakes, NJ, USA).

**Immunofluorescence**

The cells were seeded in the confocal dish, and were fixed in 4% PFA for 30 min after drug treatment, then were incubated with the fluorescent-labeled antibodies for 45 min at room temperature. The cells were washed with PBS and analyzed in BD LSRFortessa cytometry (BD Biosciences, Franklin Lakes, NJ, USA). The antibodies used were as follows: PE-labeled mouse anti-human PD-L1 (#557924, BD Bioscience, Franklin Lakes, NJ, USA) and PE-labeled mouse IgG1 (#555749, BD Bioscience, Franklin Lakes, NJ, USA).

**Separation of nuclear and cytoplasmic proteins**

The cells were lysed by using cytoplasmic lysis buffer (50 mmol·L⁻¹ Tris-HCl, 0.5% Triton-X100, 137.5 mmol·L⁻¹ NaCl, 10% glycerol, 5 mmol·L⁻¹ EDTA, 1% phosphatase inhibitor, 1% PMSF) for 15 min on ice. The cytoplasmic proteins were collected from the supernatants after centrifuging at 500 × g for 5 min at 4 °C. Then the precipitations were washed by cytoplasmic lysis buffer through the centrifuge at the same condition, and were lysed by using the SDS buffer.
to extract the nuclear proteins. The proteins were analyzed by western blot.

**siRNA interference**

Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) was used to transfect the c-Jun and negative control (NC) siRNA to the cells in the serum-free RPMI 1640 medium. After 6 h transfection, the medium was replaced by 10% FBS RPMI 1640 medium, and followed culture 18 h before the treatment of NLE. The target sequences used were as follows: c-Jun: 5'-GGAGACGAGGUGCAAC-GCUUTT-3' (sense) and 5'-AAGCCUGGCAACCCGU-UCCTT-3' (antisense); NC: 5'-UUCUCCGAACGUUGACGUG-CACGUTT-3' (sense) and 5'-ACGUGACACGUUCCG-GAGAAATT-3' (antisense).

**PD-L1 binding assay**

The cells were trypsinized and re-suspended in 100 μL of flow cytometry incubation buffer (PBS with 0.5% BSA), then incubated with the recombinant protein of PE-labeled human PD-1 (#71243-2, BPS Bioscience, San Diego, CA, USA) for 1 h at room temperature. The cells were washed with PBS and analyzed in BD LSRII Fortessa cytometry (BD Biosciences, Franklin Lakes, NJ, USA). The binding of PD-1 on the cell surface was analyzed by FlowJo 7.6 (FlowJo LLC, Ashland, OR, USA).

**Statistical analysis**

The data were presented as means ± SD (standard deviation). The significance was analyzed by GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA) with the Student t test or one-way ANOVA. P value < 0.05 was considered as statistically significant.

**Results**

**NLE promoted PD-L1 expression in human lung cancer cells**

In order to discover the potential PD-L1 modulator in lung cancer cells, we screened the effects of several natural product-derived compounds on PD-L1 expression. 0.5 to 2 μmol·L⁻¹ of NLE (Fig. 1A) obviously increased PD-L1 protein after 24 h treatment in lung cancer NCI-H460 cells (Fig. 1B). The mRNA levels of PD-L1 were detected by qRT-PCR in NCI-H460 cells, which treated with NLE from 0.25 to 2 μmol·L⁻¹ for 3 h (Fig. 1C) or treated with 1 μmol·L⁻¹ of NLE from 1 to 24 h (Fig. 1D). NLE induced dramatic upregulation of PD-L1 mRNA level at the concentration higher than 0.5 μmol·L⁻¹. NLE also promoted the maximum increase of PD-L1 mRNA at 3 h treatment. In addition, NLE increased the PD-L1 protein level in human lung cancer NCI-H1975 and A549 cells (Fig. 1E). Taken together, these results indicate that NLE upregulates PD-L1 in both mRNA and protein level in human lung cancer cells.

**NLE increased cell-surface localization of PD-L1 in lung cancer cells**

PD-L1 expressed on the cell surface is its mature form, which binds to PD-1 on the surface of T cells and inhibits the T cell immune function [28]. Here, we firstly used flow cytometry to determine whether NLE could increase cell-surface PD-L1. As expected, NLE significantly increased the cell-surface PD-L1 compared to the control group after 24 h NLE (1 μmol·L⁻¹) treatment in NCI-H460 cells (Fig. 2A). In addition, the immunofluorescence staining result also showed that NLE upregulated the cell-surface PD-L1 (Fig. 2B).

**NLE activated c-Jun in lung cancer cells**

Because NLE both increased the protein and mRNA expressions of PD-L1 in lung cancer cells, we consider that NLE regulates PD-L1 in the transcriptional levels. The transcription factors such as NF-κB, STAT1, IRF-1 and c-Jun can translocate into the nucleus and bind to the specific sites of PD-L1 gene promoter or enhancer to induce its transcription [28]. After 1, 3, 6, 9, 12 and 24 h NLE (1 μmol·L⁻¹) treatment in NCI-H460 cells, p-c-Jun (S63), p-c-Jun (S73) and c-Jun were increased after 1 h (Fig. 3A). However, NLE could not activate NF-κB, STAT1 and IRF-1 in NCI-H460 cells (data not shown). Phosphorylations of c-Jun at Ser-63 and Ser-73 activate its transcriptional activity, also led to its own transcription. The results suggest that c-Jun is the most likely transcription factor that upregulates PD-L1 induced by NLE.

**Knockdown of c-Jun attenuated the upregulation of PD-L1 induced by NLE**

To investigate whether the upregulation of PD-L1 induced by NLE is through the activation of c-Jun in human lung cancer, NCI-H460 cells were transiently transfected with c-Jun specific siRNA for 24 h, then treated with NLE (1 μmol·L⁻¹) for another 24 h in western blot assay, or treated with NLE (1 μmol·L⁻¹) for 3 h in qRT-PCR assay. The results showed that knockdown of c-Jun attenuated the upregulation of PD-L1 protein (Fig. 4A) and mRNA (Fig. 4B) that induced by NLE in human lung cancer cells. Furthermore, the flow cytometry results also showed that knockdown of c-Jun decreased the cell-surface expressions of PD-L1 that upregulated by 24 h NLE (1 μmol·L⁻¹) treatment in NCI-H460 cells (Fig. 4C).

**JNK was the upstream of c-Jun activated by NLE to induce PD-L1 expression**

c-Jun N-terminal kinases (JNKs) can bind and phosphorylate c-Jun on Ser-63 and Ser-73 within its transcriptional activation domain [28]. Because NLE obviously activated c-Jun at 1 h in NCI-H460 cells. Therefore, the p-JNK and JNK expressions were detected by western blot after 5, 15, 30, and...
Fig. 1  NLE increased the expressions of PD-L1 in human lung cancer cells. (A) Chemical structure of nagilactone E (NLE). (B) The protein levels of PD-L1 were detected by western blot after NLE (0.25, 0.5, 1 and 2 μmol·L−1) treatment for 24 h in NCI-H460 cells. (C) The mRNA levels of PD-L1 were detected by qRT-PCR after NLE (0.25, 0.5, 1 and 2 μmol·L−1) treatment for 3 h in NCI-H460 cells. (D) The mRNA levels of PD-L1 were detected by qRT-PCR after NLE (1 μmol·L−1) treatment for 1, 3, 6, 9, 12 and 24 h in NCI-H460 cells. (E) The protein levels of PD-L1 were detected by western blot after NLE (1 μmol·L−1) treatment for 24 h in NCI-H1975 cells and A549 cells. *P < 0.05, **P < 0.01 and ***P < 0.001 vs control

60 min NLE (1 μmol·L−1) treatment in NCI-H460 cells (Fig. 5A). The activation of JNK was observed at early time points (after 15 min). Inhibiting the function of JNK with the JNK inhibitor SP600125 (20 μmol·L−1) attenuated the upregulation of PD-L1 and c-Jun protein (Fig. 5B) and PD-L1 mRNA (Fig. 5C) that induced by NLE (1 μmol·L−1) in NCI-H460 cells. These results suggest that JNK is the upstream of c-Jun which increases PD-L1 expression by NLE treatment.

**NLE promoted the interaction of PE labeled PD-1 and human lung cancer cells**

The expressed PD-L1 in cancer cells needs to bind the PD-1, then to perform the function of immune suppression. In order to determine whether the NLE-increased PD-L1 has the physiological function, the NLE-treated cells were incubated with PE-labeled human PD-1 (Fig. 6A) to investigate the interaction between PD-1 and PD-L1. As shown in Fig. 6B, NLE significantly upregulated the binding of PD-1 to NCI-H460 cells, which indicated the NLE-induced PD-L1 has the physiological function.

**Discussion**

In the present study, we identified that the NLE increases the expressions of PD-L1 in lung cancer cells at protein and mRNA levels, and enhances its localization on the cell membrane. In our previous study, NLE was found to arrest the cell cycle by downregulating cyclin B1, and inhib-
Several studies have demonstrated some small molecule compounds, which have the function to inhibit the cell growth and increase the expression of PD-L1 in tumor cells, can improve the PD-1/PD-L1 antibody therapies [10-13]. Accordingly, the PD-L1 upregulation ability and anti-tumor capacity of NLE showed its potential to enhance the anti-PD-1/PD-L1 therapies. However, compared to the dramatic upregulation of PD-1/PD-L1 binding that induced by IFN-γ (data not shown), which is a cytokine that promotes the PD-L1 expression in the tumor microenvironment [29], the effect of NLE...
was weaker. But as reported, pevonedistat (NEDD8 inhibitor) promoted the similar fold change of PD-1/PD-L1 binding as NLE, and synergized the effect of anti-PD-L1 antibody [13].

Besides, although we did verify that the NLE-induced PD-L1 has the physiological function by the cell-surface binding assay in this study, more experiments such as the T-cell killing assay by using the peripheral blood mononuclear cell (PBMC) as well as animal experiments can be applied to further confirm the function of changed PD-L1.

Mechanistically, NLE induces the phosphorylation and the nuclear localization of c-Jun through the activation of JNK, and then promotes the transcription of PD-L1 in NCI-H460 cells (Fig. 6C). The transcriptional regulation of PD-L1 is complicated, and many transcription factors such as NF-κB, STAT1, IRF-1 and c-Jun are involved in this process [29]. Among them, the phosphorylation and total amount of c-Jun protein were raised obviously just after 1 h treatment of NLE.

There is a little different between the results of immunofluorescence and western blot that test the nuclear and cytoplasmic proteins in Figs. 3B & 3C. The cytoplasmic c-Jun was up-regulated after NLE treatment in western blot results, but the change cannot be clearly observed in immunofluorescence experiments. It should be because the fluorescence of nuclear c-Jun is extremely bright in immunofluorescence assay, which may cover up the changing of fluorescence in the cytoplasm. Anyway, the obvious upregulation of nuclear c-Jun was observed in both experiments. The activation of c-Jun mediates the upregulation of PD-L1 in many cancer types that contain Hodgkin lymphomas, melanoma, renal cancer and non-small cell lung cancer, which could directly bind to the AP-1 site in the PD-L1 gene and promote the transcription of PD-L1 [30-33].

We also observed JNK, the upstream of c-Jun [28], was rapidly phosphorylated after 15 min treatment of NLE. Corresponding to this, the JNK inhibition or c-Jun

---

Fig. 4  Knockdown of c-Jun attenuated the upregulation of PD-L1 that induced by NLE in NCI-H460 cells. Cells were transiently transfected with c-Jun siRNA for 24 h, and treated with NLE (1 μmol·L⁻¹) for 24 h to detect the protein expressions by western blot (A), or treated with NLE (1 μmol·L⁻¹) for 3 h to detect the PD-L1 mRNA levels by qRT-PCR (B), or treated with NLE (1 μmol·L⁻¹) for 24 h to detect the cell-surface expressions of PD-L1 by flow cytometry (C). *P < 0.05, **P < 0.01 and ***P < 0.001
**Fig. 5** JNK was the upstream of c-Jun activated by NLE to induce PD-L1 expression in NCI-H460 cells. (A) p-JNK and JNK expressions were detected by western blot after NLE (1 μmol·L\(^{-1}\)) treatment for 5, 15, 30, and 60 min. Cells were pre-treated with the JNK inhibitor SP600125 (20 μmol·L\(^{-1}\)) for 1 h, and cultured with NLE (1 μmol·L\(^{-1}\)) for 24 h to detect the protein expressions by western blot (B), or cultured with NLE (1 μmol·L\(^{-1}\)) for 3 h to detect the PD-L1 mRNA levels by qRT-PCR (C). *P < 0.05, **P < 0.01 and ***P < 0.001

**Fig. 6** NLE promoted the interaction of PE labeled PD-1 and NCI-H460 cells. (A) The schematic of cell-surface PD-1 binding assay. (B) Cell-surface PD-1 binding was detected by flow cytometry after NLE (1 μmol·L\(^{-1}\)) treatment for 24 h. (C) The mechanism of NLE upregulates PD-L1 expression. NLE increases protein and mRNA expressions of PD-L1 through the JNK - c-Jun axis in lung cancer cells. *P < 0.05, **P < 0.01 and ***P < 0.001
silencing could significantly reduce the NLE inducing PD-L1 in protein and mRNA levels.

In our recent study, NLE was identified as a protein synthesis inhibitor [19]. As reported, several protein synthesis inhibitors such as deoxynivalenol, anisomycin and ricine have the ability to activate JNK by the ribotoxic stress response [19]. The ribotoxic stress response may also mediate the NLE to activate JNK and then to increase PD-L1. The mechanism that NLE can inhibit protein synthesis but upregulate PD-L1 protein remains unclear. Interestingly, besides the JNK/c-Jun, using AZD6244 to inhibit the activity of ERK, another member of the MAPK family, also could inhibit NLE-induced-PD-L1 in NCI-H460 cells. However, ERK was not activated at less than 9 h of NLE treatment, and AZD6244 could not inhibit NLE-induced c-Jun (data not shown). Considering the c-Jun was activated at 1 h and the PD-L1 mRNA was increased at 3 h of NLE treatment, it seems that ERK is not the upstream of c-Jun-induced PD-L1 in NLE treatment. NCI-H460 cells are KRAS-mutated lung cancer cell lines with ERK activation [20], and ERK may be involved in the upregulation of PD-L1 through other members of the AP-1 family that could interact with c-Jun in NCI-H460 cells. These results revealed the complexity of PD-L1 regulation.

In summary, this study demonstrated that NLE upregulates the expression of PD-L1 in lung cancer cells by the activation of JNK/c-Jun axis, which shows the potential to combine with the PD-1/PD-L1 antibody therapies.

References

[28] Zhang X, Zhao G, Zhang Y, et al. Activation of JNK signaling in osteoblasts is inversely correlated with collagen synthesis in


Cite this article as: CHEN Yu-Chi, HUANG Mu-Yang, ZHANG Le-Le, FENG Zhe-Ling, JIANG Xiao-Ming, YUAN Luo-Wei, HUANG Run-Yue, LIU Bo, YU Hua, WANG Yi-Tao, CHEN Xiu-Ping, LIN Li-Gen, LU Jin-Jian. Nagilactone E increases PD-L1 expression through activation of c-Jun in lung cancer cells [J]. Chin J Nat Med, 2020, 18(7): 517-525.