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EGFR-TKI down-regulates PD-L1 in EGFR mutant NSCLC through inhibiting NF-κB

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ABSTRACT

Non-small-cell lung cancer (NSCLC) is a severe disease threatening human health. Targeted therapy of epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) has obtained potent efficacy in the treatment of NSCLC patients. However, the effects of EGFR-TKIs on tumor immune microenvironment are unclear. In this study, we show that NSCLCs with EGFR mutation express higher programmed cell death ligand 1 (PD-L1) than NSCLCs with wild type EGFR. The EGFR activation is also associated with high expression of PD-L1. The EGFR-TKI gefitinib can reduce PD-L1 expression, *via* inhibiting NF-κB, in EGFR mutant NSCLC *in vitro* and *in vivo*. These findings elucidate a novel anti-tumor mechanism of EGFR-TKI and provide the possibility of combined strategy of targeted therapy and immunotherapy for EGFR mutant NSCLC patients.

1. Introduction

Lung cancer is the leading cause of cancer-related deaths around the world [1]. Non-small-cell lung cancer (NSCLC) is the most common histological type, which accounts above 80% of all lung cancer [2]. In spite of a rapid development of traditional operation, platinum-based chemotherapy and radiotherapy, the advanced and metastatic NSCLC patients still have a poor prognosis [3]. Therefore, novel and more effective strategies are urgently required. Currently, the targeted therapy of epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs), gefitinib and erlotinib, has become the first-line therapy in EGFR mutant NSCLC patients [4]. In the tumor microenvironment, the immune inhibitory cells (Treg, MDSC, TAM) and cytokines (TGF-β, IL-10) can lead to immune tolerance and escapes of tumor cells [5]. Immunotherapies targeting immune "checkpoints" such as programmed cell death 1 (PD-1), programmed cell death ligand 1 (PD-L1) and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), have been reported to obviously prolong the median progression-free survival of patients suffering tumors [6,7]. However, the effects of EGFR-TKIs on tumor immune microenvironment are unclear.

Recently, it is reported that NSCLC cell lines harboring EGFR mutations express a higher level of PD-L1 than those with other mutations [8], but the potential mechanism remains unexplored. Therefore, we hypothesized that EGFR-TKIs could reduce the expression of PD-L1 and ameliorate the immune response in EGFR mutant NSCLC. In this study, we found that EGFR-TKIs reduced the expression of PD-L1 in both EGFR-TKIs sensitive and acquired-resistant NSCLC *in vitro* and *in vivo*, which is dependent on NF-κB signaling pathway. These findings provide a novel anti-tumor mechanism of EGFR-TKIs and clues for the individual strategies for the combination of EGFR-TKIs and immunotherapy.

2. Materials and methods

2.1. Cell lines and regents

The human NSCLC cell lines PC-9, HCC827, NCI-H1650, NCI-H1299, NCI-H460 and SPC-1A were purchased from the American TypeCulture Collection (Mannassas, VA, USA) and cultured in RPMI-1640 (Hyclone, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin. These cell lines were last tested and authenticated by short tandem repeat profiling in September, 2014. Gefitinib (Iressa,ZD1839) was purchased from AstraZeneca and dissolved in dimethyl sulfoxide (DMSO). EGFR-

Abbreviations: CTLA-4, cytotoxic T lymphocyte-associated antigen-4; DMSO, dimethyl sulfoxide; EGFR, epidermal growth factor receptor; LNA, locked nucleic acid; NSCLC, Non-small-cell lung cancer; PCR, polymerase chain reaction; PD-1, programmed cell death 1; PD-L1, programmed cell death ligand 1; PNA, peptide nucleic acid; PR, EGFR-TKIs resistant PC-9 cells; TKIs, tyrosine kinase inhibitors; VEGF, vascular endothelial growth factor.

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TKIs resistant PC-9 cells (PR) cells were generated by treatment with 1 μM gefitinib for three months, and cultured with medium containing 1 nM gefitinib. The $IC_{50}$to gefitinib was assessed by Cell Counting Kit-8 (Boster, Wuhan, China). PR cells did not harbor T790 mutation as identified by EGFR mutation detection.

2.2. EGFR mutation detection

EGFR mutations were identified by the peptide nucleic acid (PNA)–locked nucleicacid (LNA) polymerase chain reaction (PCR) clamp method according to the manufacturer's directions (Roche, NJ, USA).

2.3. In vivo xenograft experiment

Severe combined immune-deficient mice (female, 4–6-week old) were purchased from the Chinese Academy of Medical Sciences (Beijing, China). Mice were raised in laminar flow cabinets under specific pathogen-free conditions. For xenograft experiments, 3×10^5 PC-9 cells were resuspended in 200 μl PBS and injected into the right armpit of nude mice. The tumor growth was determined weekly. When the tumor volume reached about 500 mm^3, the engrafted mice were divided into two groups: vehicle and gefitinib group. The mice in gefitinib group were given gefitinib (20 mg/kg, i.g.) resuspended in 100 μlNS with 1% methylcellulose and 0.2% Tween-80, while mice of vehicle group were given 100 μl NS with 1% methylcellulose and 0.2% Tween-80. When the lowest tumor volume in the gefitinib group reduced to ~200 mm^3 (43 days post tumor implantation), all the mice were sacrificed and the tumor tissues were harvested for experiments. Mouse care and use was performed in accordance with local ethical guidelines.

2.4. Small interfering RNA (siRNA) transfection

NF-κB p65 gene expression was inhibited by siRNA obtained commercially from Genepharma (Shanghai, China). We transfected p65 siRNA and negative control siRNA into PC-9 and PR cell lines respectively at a concentration of 60 nM according to the manufacturer's protocol. Then the medium was replaced at 6 h after transfection and cells were further culturing for 48 h. Gene silencing was confirmed by western blotting.

2.5. Supplementary materials and methods

Additional materials and methods, including flow cytometry, real-time PCR, immunofluorescence, and western blotting are shown in the supplementary information.

2.6. Statistical analysis

All quantitative data were expressed as the mean ± s.d. Statistical analysis was performed by the independent samples t-testor one-way ANOVA. The difference was considered statistically significant when $P < 0.05$. All statistical analyses were carried out with SPSS18.0 software.

3. Results

3.1. The expression of PD-L1 in NSCLC is regulated by EGFR activation

To determine the relationship between the expression of PD-L1 and EGFR activation, we examined 6 types of NSCLC cell lines with different EGFR status. PC-9, HCC827 and H1650 harbored mutant EGFR on exon 19 (Supplementary Fig. 1), while H460, H1299 and SPC-1A harboring wild-type EGFR. EGFR mutations on exon 19 in NSCLC have been shown to involve in constitutive tyrosine kinase phosphorylation and unattenuated signaling [9]. Flow cytometric analysis revealed that the expression of PD-L1 was much higher in EGFR mutant cell lines (PC-9, HCC827 and H1650) than that in EGFR wild-type cell lines (H460, H1299 and SPC-A1) (Fig. 1A and B). These results suggest a positive association between PD-L1 expression and EGFR activation.

In order to further verify whether EGFR activation could induce PD-L1 expression, we utilized recombinant human EGF (100 ng/ml), a well-known EGFR ligand, to stimulate H460 cells, one of NSCLC cell lines harboring wild-type EGFR. After 24 h, incubation with EGF resulted in a significant increase of PD-L1 mRNA and protein in H460 cells (Fig. 1C and D). These results indicate that the expression of PD-L1 was positively regulated by EGFR activation.

3.2. EGFR-TKIs reduce the expression of PD-L1 in EGFR-TKI sensitive and acquired-resistant NSCLCs

Small molecular TKIs of EGFR, gefitinib and erlotinib, have been approved and shown efficacy for the first-line therapy of EGFR mutation positive NSCLCs [10]. Moreover, the main anti-tumor mechanisms of EGFR-TKIs have been proved to inhibit angiogenesis and induce apoptosis [11,12]. However, the effect of EGFR-TKIs on immune microenvironment of tumor has not been reported. According to our above findings, we hypothesized that TKIs of EGFR might reduce the expression of PD-L1 in EGFR mutant NSCLCs. We first stimulated PC-9 cells with different concentrations of gefitinib (0, 2.5, 5, 10, and 100 nM). After incubation for 48 h, we found that the expression of PD-L1 was down-regulated in PC-9 cells in a dose-dependent manner (Fig. 2A and B). We next treated PC-9 cells with 100 nM gefitinib for different times (0, 6, 12, 24, and 48 h). Similarly, we also observed a gradual PD-L1 decrease in PC-9 cells (Fig. 2C and D). Immuofluorescence staining further confirmed the reduction of PD-L1 by gefitinib (Fig. 2E). Likewise, the expression of PD-L1 of HCC827 cells, another EGFR mutant NSCLCs, was down-regulated by gefitinib in dose- and time-dependent manners (Supplementary Fig. 2A). Of interest, EGF could partially reverse the reduction of PD-L1 induced by gefitnib (Supplementary Fig. 2B). These data indicate that gefitinib could reduce the expression of PD-L1 in EGFR-TKI sensitive NSCLCs.

In spite of exciting efficacy of EGFR-TKIs treatment, it ultimately caused acquired drug-resistance, and the medium response durations ranged 8 to 13 months [13]. It's known that gefitinib treatment couldn't affect growth or apoptosis of EGFR-TKI resistant NSCLCs. However, whether gefitinib affect the expression of PD-L1 in gefitinib acquired-resistant NSCLC remains unclear.

We established the gefitinib acquired-resistant PC-9 cells (PR), in which IC50 to gefitinib reached ~20 μM (ten times higher than that to gefitinib-sensitive PC-9) (Supplementary Table 1). We found that the expression of PD-L1 in PR cells was much higher than that in PC-9 cells (Fig. 2F), which implied the alternative signaling might regulate PD-L1. Next, we treated PR with different concentrations of gefitinib (0, 2.5, 5, 10, and 100 nM) for 48 h or treated with 100 nM gefitinib for different times (0, 12, 24, and 48 h). Interestingly, consistent with the effect of gefitinib on PC-9, the expression of PD-L1 in PR was also reduced in dose- and time-dependent manners (Fig. 2G, H and Supplementary Fig. 2C, D). These results suggest that gefitinib could reduce the expression of PD-L1 in gefitinib acquired-resistant NSCLCs.

3.3. Gefitinib reduces the expression of PD-L1 in PC-9 cells in vivo

We next questioned whether gefitinib could reduce PD-L1in NSCLCs *in vivo*. We subcutaneously injected PC-9 cells (3×10^5)

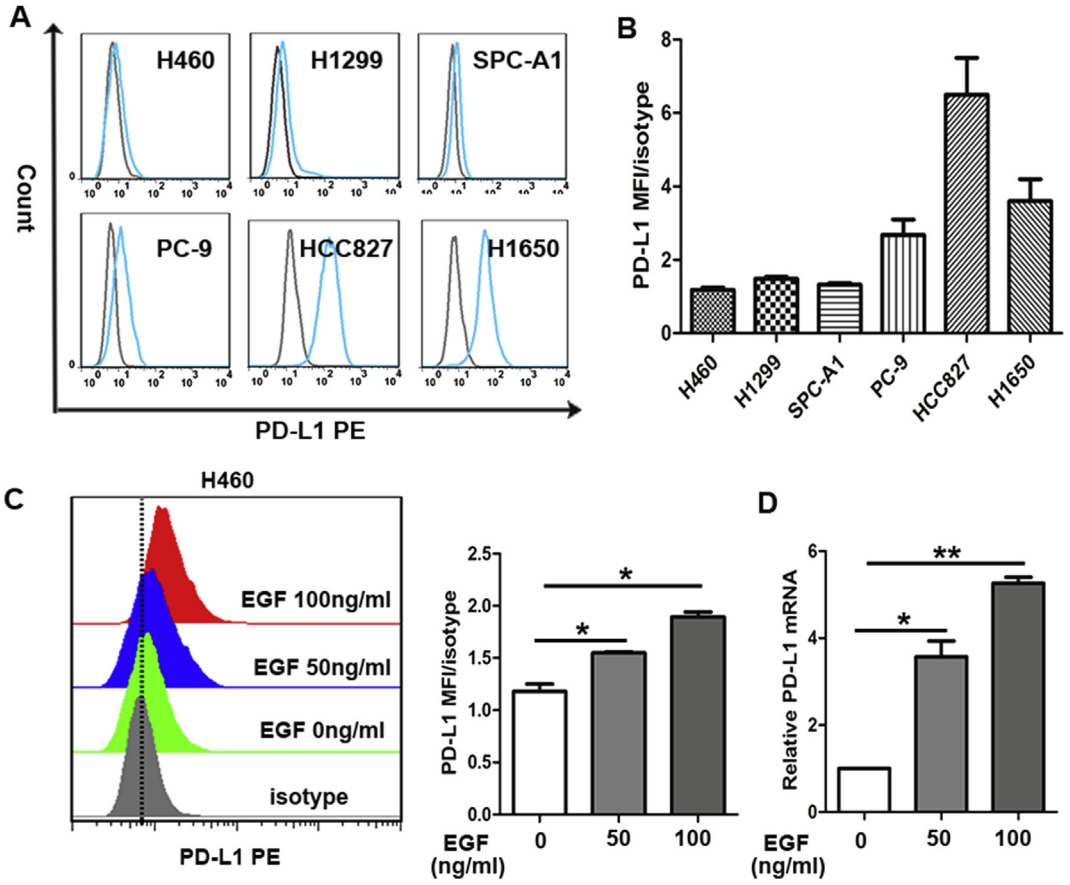

Fig. 1. EGFR activation is associated with PD-L1 expression in NSCLC. (A) The representative data of PD-L1 determined by flow cytometry in EGFR wild type NSCLC (H460, H1299 and SPC-A1) and EGFR mutant NSCLC (PC-9, HCC827, and H1650). (B) The statistical analysis of PD-L1 flow cytometry. (C) After H460 cells were treated with human recombinant EGF (0, 50, and 100 ng/ml) for 48 h, PD-L1 was examined by flow cytometry. (D) The mRNA expression of PD-L1 in H460 treated with EGF was examined by Real-time PCR and normalized toβ-actin. All experiments were performed threetimes and data were expressed as mean ± s.d. *P < 0.05, **P < 0.01.

into 6-8 week-old female nude mice. As the volume of tumor reached about 1000 mm^3, the mice were given gefitinib (20 mg/kg, i.g.) every other day until the volume of tumor decreased to 200 mm^3 (Fig. 3A). The volume of tumors from the gefitinib group was much smaller than that from the vehicle group (Fig. 3B and C). Consistently, the weight of tumors in the gefitinib group was also significantly lower than that in the vehicle group ($p < 0.05$, Fig. 3D).

Then the expression of PD-L1 in tumors from grafted mice was assessed by flow cytometry and immunofluorescence. Consistent with the results *in vitro*, we found that the expression of PD-L1 in gefitinib group was much lower than that in the vehicle group (Fig. 3E-G). These findings indicate that gefitinib could reduce the expression of PD-L1 in PC-9 cells *in vivo*.

3.4. The reduced expression of PD-L1 by gefitinib is dependent on NF-κB signaling pathway

NF-κB signaling pathway plays a key role in inducing PD-L1 expression in monocytes [14]. NF-κB is also one of the most important downstream pathways of EGFR activation in regulating proliferation and chemotherapy resistance of tumor cells [15]. Thus we hypothesized that NF-κB signaling pathway could be involved in regulation of PD-L1 by EGFR activation. To test our hypothesis, we examined the NF-κB expression in EGFR wild-type and mutant NSCLC cell lines. We found that the nuclear NF-κB expression was higher in EGFR mutant cell lines than that in EGFR wild-type cell lines (Fig. 4A). Moreover, the expression of PD-L1 was also down-regulated by PTDC, a chemical NF-κB inhibitor, in a dose-dependent manner in PC-9 cells (Fig. 4B). These data indicate that NF-κB might be required for the elevated PD-L1 expression in EGFR mutated NSCLCs.

To further confirm the role of NF-κB in transcriptional stimulation of PD-L1, we silenced endogenous p65 expression by a specific siRNA. PC-9 and PR cell lines were transfected with p65 siRNA or negative control siRNA. Western blotting assay showed that p65 siRNA could efficiently inhibit p65 expression but not β-actin after 48 h of siRNA transfection compared to the effect of negative control siRNA (Fig. 4C). In addition, in comparison to negative control group, a significant attenuation of PD-L1 expression was observed both in PC-9 and PR cell lines 48 h after treatment with p65 siRNA (Fig. 4D). The results were consistent with the effect of PDTC. Collectively, these data indicate that NF-κB is required for the elevated PD-L1 expression in EGFR mutated NSCLCs.

To determine whether the reduced expression of PD-L1 by gefitinib was dependent on NF-κB, we examined the NF-κB activity in PC-9 and H1299 cells treated with different doses of gefitinib. As shown in Fig. 4E, the NF-κB activity was significantly inhibited by gefitinib in PC-9 cells. However, gefitinib could not inhibit the NF-κB activity in H1299 (Supplementary Fig. 3). Consistently, the results of immunofluorescence revealed that gefitinib significantly

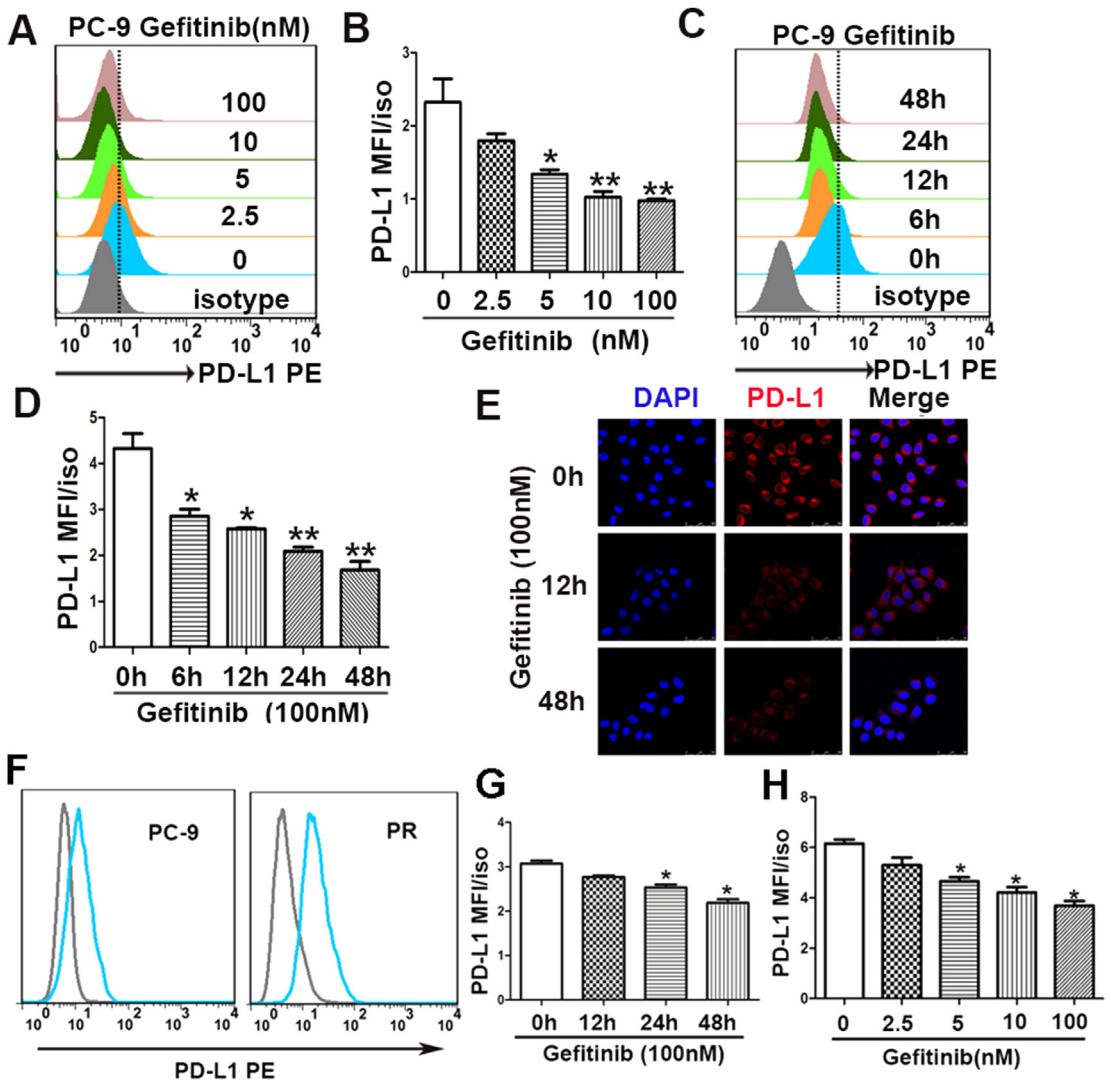

Fig. 2. EGFR-TKI reduces the expression of PD-L1 in EGFR sensitive and acquired-resistant NSCLC. (A) After PC-9 cells were treated with different doses of gefitinib (0, 2.5, 5, 10, 100 nM) for 48 h, PD-L1 was examined by flow cytometry. (B) The statistical analysis of PD-L1 expression in (A). Experiments were performed threetimes and data were expressed as mean ± s.d. Compared to control group (the white column), *P < 0.05, **P < 0.01. (C) After PC-9 cells were treated with 100 nM gefitinib for different time (0, 6, 12, 24, 48 h), PD-L1 was examined by flow cytometry. (D) The statistical analysis of PD-L1 expression in (C). Experiments were performed threetimes and data were expressed as mean ± s.d. Compared to control group (the white column), *P < 0.05, **P < 0.01. (E) Immuofluorescence staining of PD-L1 in PC-9 cells treated with gefitinib. (F) Flow cytometric analysis of PD-L1 expression in PC-9 and PR. (G) PD-L1 expression in PR cells was examined by flow cytometry after treated with 100 nM gefitinib for different time (0, 12, 24, 48 h). The experiments were performed threetimes and data were expressed as mean ± s.d. Compared to control group (the white column), *P < 0.05. (H) PD-L1 expression in PR cells was examined by flow cytometry after treated with different dose of gefitinib (0, 2.5, 5, 10, 100 nM) for 48 h. The experiments were performed threetimes and data were expressed as mean ± s.d. Compared to control group (the white column), *P < 0.05, **P < 0.01.

attenuated NF-κB activity in tumors compared with that treated with vehicle (Fig. 4F). Taken together, gefitinib reduced PD-L1 expression dependent of NF-κB signaling pathway.

4. Discussion

EGFR-TKIs were initially found to promote tumor regression through inhibiting proliferation and inducing apoptosis of tumor cells. They can also decrease vascular endothelial growth factor (VEGF) expression and tumor angiogenesis [11]. However, whether EGFR-TKIs affect the tumor immune microenvironment has not been reported.

PD-L1 is a co-inhibitory molecule expressed generally on APCs (macrophages, DCs), activated T cells, B cells and tumor cells [16]. By binding to its receptor PD-1, which is mainly expressed in activated T cells, PD-L1 induces the apoptosis, anergy, unresponsiveness, and exhaustion of T cells [17,18]. Overexpression of PD-L1 has been reported to correlate with poor prognosis in a number of human cancers, including breast cancer, kidney cancer, ovarian cancer and NSCLCs [19].

In the present study, we found that NSCLC cell lines harboring EGFR mutations showed a higher level of PD-L1 than those with wild type EGFR. Moreover, administration of EGF caused a significant increase of PD-L1 in EGFR wild type NSCLCs. These findings indicated that constitute EGFR activation is associated with expression of PD-L1, which is consistent with few recent studies. A positive correlation between EGFR mutations and PD-L1 overexpression has been reported in surgically resected NSCLC [20], whereas other oncogenic mutations, such as K-ras and ALK, were not associated with PD-L1 elevation [21], suggesting that EGFR

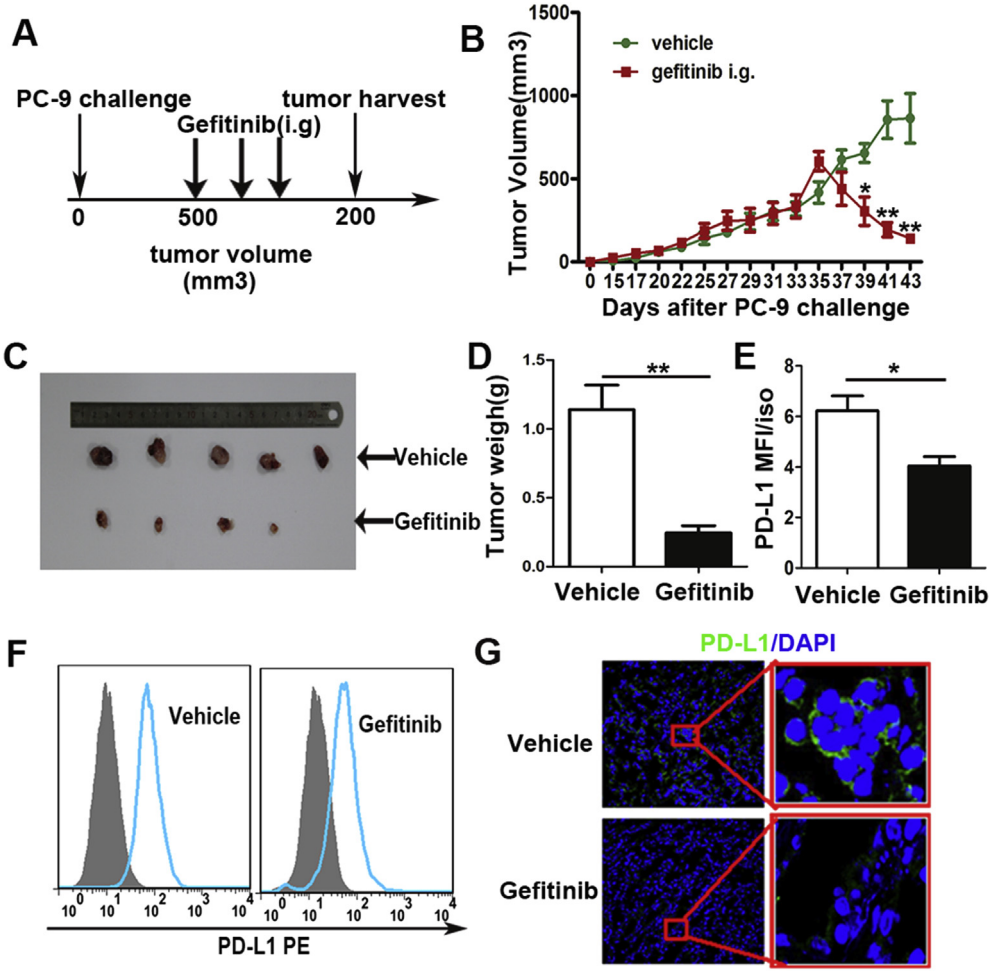

Fig. 3. Geftinib reduces the tumor growth and PD-L1 expression of tumor cells in PC-9-xenografted mice model. (A) The schedule of experiment *in vivo*. 7×10^5 PC-9 cells were injected into the right armpit of nude mice. When the tumor volume reached about 500 mm^3, the engrafted mice were given gefitinib (20 mg/kg, i.g.) or vehicle (100 μl NS). When the tumor volume in the gefitinib group reduced to ~200 mm^3 (43 days post tumor implantation), all the mice were sacrificed and the tumor tissues were harvested for experiments. (B) The curve of xenografts tumor volumes (n = 5 in each group). The results were expressed as mean ± s.d. of n = 5 mice each group. (C) Images of xenografted tumor at harvest day. (D) Tumor weight of xenografted tumor at harvest day. The results were expressed as mean ± s.d. of n = 5 in vehicle group, n = 4 in gefitinib group. Compared to vehicle group, $^*p < 0.05$, $^{**}p < 0.01$. (E) Flow cytometricanalysis of PD-L1 expression in tumor cells. The results were expressed as mean ± s.d. of n = 5 in vehicle group, n = 4 in gefitinib group. Compared to vehicle group, $^*p < 0.05$, $^{**}p < 0.01$. (F) PD-L1 expression of tumor cells in vehicle and gefitnib group. (G) Immuofluorescence staining of PD-L1 of tumor cells in vehicle and gefitnib group.

activation, rather than K-ras or ALK, regulates the PD-L1 expression in NSCLCs.

NF-κB was found to bind the promoter of PD-L1 and promote its expression in monocytes [14]. We next speculated EGFR might induce PD-L1 expression through NF-κB signaling pathway. Consistent with our speculations, nuclear NF-κB expression in EGFR mutant NSCLC was higher than that in wild type NSCLC. In addition, inhibition of NF-κB with PDTC and siRNA reduced the expression of PD-L1. Moreover, we found that gefitinib reduced PD-L1 expression in gefitinib-sensitive NSCLC *in vitro* and *in vivo* through inhibiting NF-κB pathway. These findings illustrated a novel anti-tumor mechanism of EGFR-TKIs treatment in EGFR mutant NSCLC.

Given the NSCLC patients treated with EGFR-TKIs ultimately acquired drug-resistance owing to T790M mutation and c-met amplification [22,23], and EGFR-TKIs could not inhibit cell proliferation or induce apoptosis of tumor cells, whether they also regulate PD-L1 in the acquired resistant NSCLCs is not clear. We found EGFR-TKIs acquired resistant NSCLC expressed higher PD-L1 than sensitive NSCLC, which further suggests that the increased PD-L1 may correlate with the EGFR-TKIs acquired resistance. Interestingly, gefitinib could partly reduce the PD-L1 expression in gefitinib acquired resistant NSCLC. This could be explained by inhibition of NF-κB activity by gefitinib.

Recently, immunotherapy has been the mainstream of cancer treatment. The development of antibodies of “immune checkpoint”, such as CTLA-4, PD-1 and PD-L1, results in an increase in median survival in patients with melanoma, renal cancer and NSCLC. Therefore, we speculate that in EGFR-TKIs sensitive NSCLC patients, the targeted therapy may have a synergistic effect with CTLA-4 antibody, but may not with PD-1/PD-L1 antibody treatment, whose efficacy is dependent on high expression PD-L1 on tumor cells. Likewise, wild type EGFR NSCLC patient with low expression of PD-L1 could not response to PD-1/PD-L1 antibody treatment. However, in EGFR-TKIs acquired-resistant NSCLC patients, EGFR-TKIs combination with PD-L1/PD-1 may bring a better effect. These speculations, which are needed to be further proved, would provide novel combined strategies for NSCLC patients harboring various EGFR mutations.

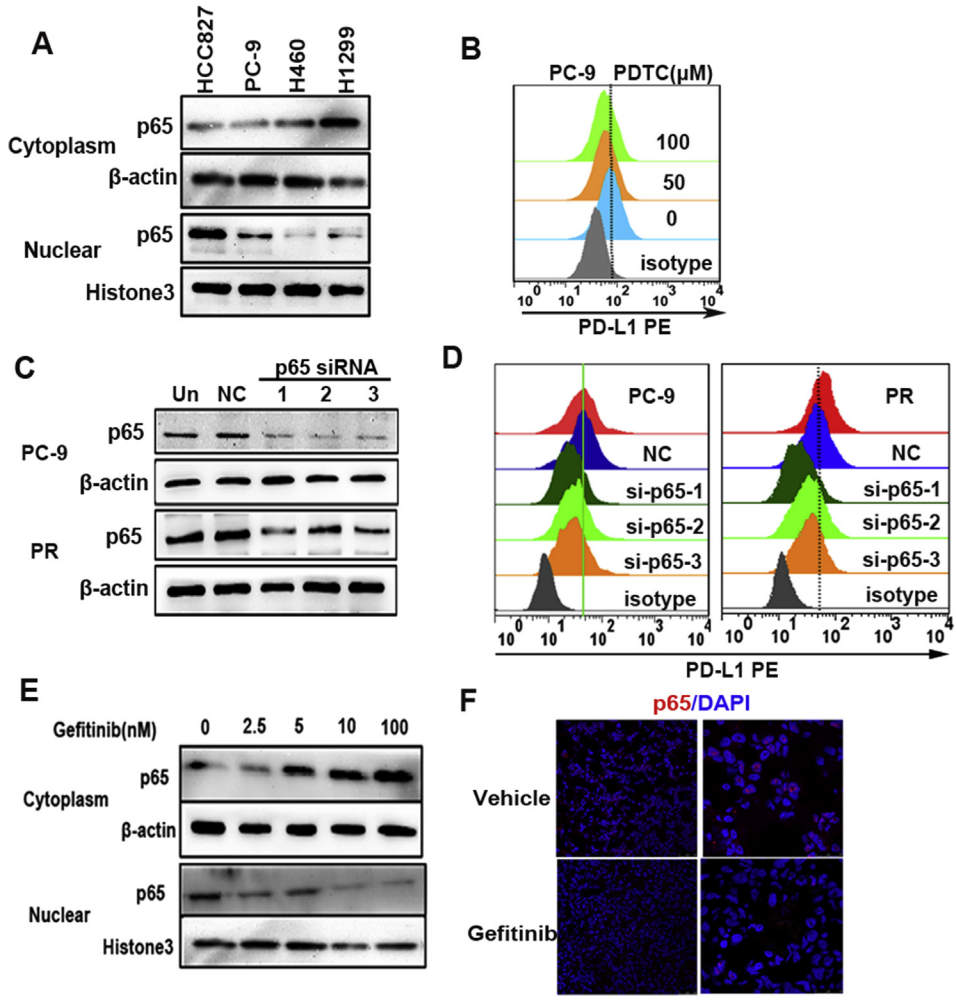

Fig. 4. NF-κB pathway is involved in reduction of PD-L1 by EGFR-TKI. (A) The cytoplasmic and nuclear expressionof NF-κB in EGFR mutant and wild type NSCLC. (B) Flow cytometric analysis of PD-L1 in PC-9 treated with different dose of PDTC (0, 50, 100 μM) for 48 h. (C) Representative western blotting assay evaluating p65 expression of PC-9 and PR untreated (Un) or 48 h after negative control (NC) or p65 siRNA transfection. (D) Flow cytometric analysis of PD-L1 in PC-9 and PR untreated (Un) or transfected with negative control (NC) or p65 siRNA 48 h later. (E) The NF-κB activity in PC-9 cells treated with different dose of gefitinib (0, 2.5, 5, 10, 100 nM) for 48 h. (F) Immuofluorescence staining of NF-κB activity in PC-9 xenografted tumor in vehicle and gefitinib group.

In summary, our results showed that EGFR activation could induce PD-L1 expression and EGFR-TKIs could inhibit the induction of PD-L1 on EGFR mutant NSCLC *in vitro* and *in vivo*, which were, at least partially, dependent on NF-κB pathway. These results not only illustrated a novel anti-tumor mechanism of EGFR-TKIs, but also provided potential thinking to the combined therapeutic effect of EGFR-TKIs and immunotherapy.

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Conflicts of interest

All authors declare that there are no conflicts of interest in this study.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.030.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.030.

References

[1] A. Jemal, R. Siegel, J. Xu, E. Ward, Cancer statistics, 2010, CA Cancer J. Clin. 60 (2010) 277–300.
[2] K. Florian, H. Wolfgang, S. Andreas, P. Andreas, S. Thomas, G. Richard, A. Jutta, N.S. Meinhard, S. William, T. Alexandar, J. Herbert, K. Karin, Z. August, F. Josef, O. Wilhelm, F. Michael, Longitudinal analysis of 2293 NSCLC patients: a comprehensive study from the TYROL registry, Lung Cancer 87 (2015) 193–200.
[3] M.D. Fisher, A. D'Orazio, Phase II and III trials: comparison of four chemotherapy regimens in advanced non small-cell lung cancer (ECOG 1594), Clin. Lung Cancer 2 (2000) 21–22.
[4] L.V. Sequist, R.G. Martins, D. Spigel, S.M. Grunberg, A. Spira, P.A. Janne, V.A. Joshi, D. McCollum, T.L. Evans, A. Muzikansky, G.L. Kuhlmann, M. Han, J.S. Goldberg, J. Settleman, A.J. Iafrate, J.A. Engelman, D.A. Haber, B.E. Johnson, T.J. Lynch, First-line gefitinib in patients with advanced non-small-cell lung

cancer harboring somatic EGFR mutations, J. Clin. Oncol. 26 (2008) 2442–2449.
[5] T.F. Gajewski, Y. Meng, H. Harlin, Immune suppression in the tumor microenvironment, J. Immunother. 29 (2006) 233–240.
[6] S.L. Topalian, F.S. Hodi, J.R. Brahmer, S.N. Gettinger, D.C. Smith, D.F. McDermott, J.D. Powderly, R.D. Carvajal, J.A. Sosman, M.B. Atkins, P.D. Leming, D.R. Spigel, S.J. Antonia, L. Horn, C.G. Drake, D.M. Pardoll, L. Chen, W.H. Sharfman, R.A. Anders, J.M. Taube, T.L. McMiller, H. Xu, A.J. Korman, M. Jure-Kunkel, S. Agrawal, D. McDonald, G.D. Kollia, A. Gupta, J.M. Wigginton, M. Sznol, Safety, activity, and immune correlates of anti-PD-1 antibody in cancer, N. Engl. J. Med. 366 (2012) 2443–2454.
[7] E.J. Lipson, C.G. Drake, Ipilimumab: an anti-CTLA-4 antibody for metastatic melanoma, Clin. Cancer Res. 17 (2011) 6958–6962.
[8] E.A. Akbay, S. Koyama, J. Carretero, A. Altabef, J.H. Tchaicha, C.L. Christensen, O.R. Mikse, A.D. Cherniack, E.M. Beauchamp, T.J. Pugh, M.D. Wilkerson, P.E. Fecci, M. Butaney, J.B. Reibel, M. Soucheray, T.J. Cohoon, P.A. Janne, M. Meyerson, D.N. Hayes, G.I. Shapiro, T. Shimamura, L.M. Sholl, S.J. Rodig, G.J. Freeman, P.S. Hammerman, G. Dranoff, K.K. Wong, Activation of the PD-1 pathway contributes to immune escape in EGFR-driven lung tumors, Cancer Discov. 3 (2013) 1355–1363.
[9] H.S. Huang, M. Nagane, C.K. Klingbeil, H. Lin, R. Nishikawa, X.D. Ji, C.M. Huang, G.N. Gill, H.S. Wiley, W.K. Cavenee, The enhanced tumorigenic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling, J. Biological Chem. 272 (1997) 2927–2935.
[10] R. Rosell, E. Carcereny, R. Gervais, A. Vergnenegre, B. Massuti, E. Felip, R. Palmero, R. Garcia-Gomez, C. Pallares, J.M. Sanchez, R. Porta, M. Cobo, P. Garrido, F. Longo, T. Moran, A. Insa, F. De Marinis, R. Corre, I. Bover, A. Illiano, E. Dansin, J. de Castro, M. Milella, N. Reguart, G. Altavilla, U. Jimenez, M. Provencio, M.A. Moreno, J. Terrasa, J. Munoz-Langa, J. Valdivia, D. Isla, M. Domine, O. Molinier, J. Mazieres, N. Baize, R. Garcia-Campelo, G. Robinet, D. Rodriguez-Abreu, G. Lopez-Vivanco, V. Gebbia, L. Ferrera-Delgado, P. Bombaron, R. Bernabe, A. Bearz, A. Artal, E. Cortesi, C. Rolfo, M. Sanchez-Ronco, A. Drozdowskyj, C. Queralt, I. de Aguirre, J.L. Ramirez, J.J. Sanchez, M.A. Molina, M. Taron, L. Paz-Ares, G.F. Pneumocancerologie, A.I.O. Toracica, Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial, Lancet Oncol. 13 (2012) 239–246.
[11] N. Pore, Z. Jiang, A. Gupta, G. Cerniglia, G.D. Kao, A. Maity, EGFR tyrosine kinase inhibitors decrease VEGF expression by both hypoxia-inducible factor (HIF)-1-independent and HIF-1-dependent mechanisms, Cancer Res. 66 (2006) 3197–3204.
[12] S. Tracy, T. Mukohara, M. Hansen, M. Meyerson, B.E. Johnson, P.A. Janne, Gefitinib induces apoptosis in the EGFRL858R non-small-cell lung cancer cell line H3255, Cancer Res. 64 (2004) 7241–7244.
[13] K. Hotta, K. Kiura, H. Ueoka, M. Tabata, K. Fujiwara, T. Kozuki, T. Okada, A. Hisamoto, M. Tanimoto, Effect of gefitinib ('Iressa', ZD1839) on brain metastases in patients with advanced non-small-cell lung cancer, Lung Cancer 46 (2004) 255–261.
[14] G. Huang, Q. Wen, Y. Zhao, Q. Gao, Y. Bai, NF-kappaB plays a key role in inducing CD274 expression in human monocytes after lipopolysaccharide treatment, PLoS One 8 (2013) e61602.
[15] K. Tanaka, I. Babic, D. Nathanson, D. Akhavan, D. Guo, B. Gini, J. Dang, S. Zhu, H. Yang, J. De Jesus, A.N. Amzajerdi, Y. Zhang, C.C. Dibble, H. Dan, A. Rinkenbaugh, W.H. Yong, H.V. Vinters, J.F. Gera, W.K. Cavenee, T.F. Cloughesy, B.D. Manning, A.S. Baldwin, P.S. Mischel, Oncogenic EGFR signaling activates an mTORC2-NF-kappaB pathway that promotes chemotherapy resistance, Cancer Discov. 1 (2011) 524–538.
[16] S.J. Lee, B.C. Jang, S.W. Lee, Y.I. Yang, S.I. Suh, Y.M. Park, S. Oh, J.G. Shin, S. Yao, L. Chen, I.H. Choi, Interferon regulatory factor-1 is prerequisite to the constitutive expression and IFN-gamma-induced upregulation of B7-H1 (CD274), FEBS Lett. 580 (2006) 755–762.
[17] H. Dong, S.E. Strome, D.R. Salomao, H. Tamura, F. Hirano, D.B. Flies, P.C. Roche, J. Lu, G. Zhu, K. Tamada, V.A. Lennon, E. Celis, L. Chen, Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion, Nat. Med. 8 (2002) 793–800.
[18] D.L. Barber, E.J. Wherry, D. Masopust, B. Zhu, J.P. Allison, A.H. Sharpe, G.J. Freeman, R. Ahmed, Restoring function in exhausted CD8 T cells during chronic viral infection, Nature 439 (2006) 682–687.
[19] K.A. Schalper, V. Velcheti, D.E. Carvajal-Hausdorf, V. Anagnostou, K. Syrigos, S. Gettinger, L. Chen, R. Herbst, D.L. Rimm, Tumor infiltrating lymphocytes are associated with epithelial expression of PD-L1 protein, PD-L1 mRNA and better outcome in non-small cell lung cancer, Mod. Pathol. 27 (2014) 493a–494a.
[20] K. Azuma, K. Ota, A. Kawahara, S. Hattori, E. Iwama, T. Harada, K. Matsumoto, K. Takayama, S. Takamori, M. Kage, T. Hoshino, Y. Nakanishi, I. Okamoto, Association of PD-L1 overexpression with activating EGFR mutations in surgically resected nonsmall-cell lung cancer, Ann. Oncol. 25 (2014) 1935–1940.
[21] A. D'Incecco, M. Andreozzi, V. Ludovini, E. Rossi, A. Capodanno, L. Landi, C. Tibaldi, G. Minuti, J. Salvini, E. Coppi, A. Chella, G. Fontanini, M.E. Filice, L. Tornillo, R.M. Incensati, S. Sani, L. Crino, L. Terracciano, F. Cappuzzo, PD-1 and PD-L1 expression in molecularly selected non-small-cell lung cancer patients, Br. J. Cancer 112 (2015) 95–102.
[22] S.X. Jiang, K. Yamashita, M. Yamamoto, C.J. Piao, A. Umezawa, M. Saegusa, T. Yoshida, M. Katagiri, N. Masuda, K. Hayakawa, I. Okayasu, EGFR genetic heterogeneity of nonsmall cell lung cancers contributing to acquired gefitinib resistance, Int. J. Cancer 123 (2008) 2480–2486.
[23] M. Garofalo, G. Romano, G. Di Leva, G. Nuovo, Y.J. Jeon, A. Ngankeu, J. Sun, F. Lovat, H. Alder, G. Condorelli, J.A. Engelman, M. Ono, J.K. Rho, L. Cascione, S. Volinia, K.P. Nephew, C.M. Croce, EGFR and MET receptor tyrosine kinase-altered microRNA expression induces tumorigenesis and gefitinib resistance in lung cancers, Nat. Med. 18 (2012) 74–82.