

# Gefitinib enhances the anti-tumor immune response against EGFR-mutated NSCLC by upregulating B7H5 expression and activating T cells via CD28H

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**Abstract.** Gefitinib is a sensitive and effective drug to treat non-small-cell lung cancer (NSCLC) carrying the somatic activating mutations of the tyrosine kinase domain of epidermal growth factor receptor (EGFR). In the present study, a new mechanism of action of gefitinib in EGFR-mutated NSCLC cells was discovered using *in vitro* co-culture of NSCLC cells with peripheral blood mononuclear cells (PBMCs). Gefitinib significantly enhanced the cytotoxicity of PBMCs against NSCLC cells expressing mutated EGFR but not in cells expressing wild-type EGFR. Furthermore, it was observed that B7H5 expression was significantly lower in EGFR-mutant cells than in wild-type cells, while inhibition of EGFR by gefitinib

or reduction in EGFR using a small interfering RNA (siRNA) both increased the expression of B7H5 in EGFR-mutated NSCLC cells. In addition, when B7H5 expression was reduced by siRNA, the toxic effect of gefitinib was reduced in the co-culture of PBMCs and EGFR-mutant NSCLC cells. In addition, the siRNA-mediated decrease in expression of the B7H5 receptor CD28H in PBMCs also reduced the toxicity of gefitinib on EGFR-mutated NSCLC. Based on these results, it may be proposed that the B7H5/CD28H axis is involved in NSCLC-mediated immunosuppression when EGFR is over-activated. Gefitinib actively inhibits mutated EGFR, which induces B7H5 expression on the cell surface of NSCLC cells, thereby activating CD28H signaling in immune cells, followed by enhanced cytotoxicity against NSCLC. The present study not only provided new insight into the immune evasion mechanism mediated by EGFR mutations but also identified new targets for immune therapy.

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## Introduction

Lung cancer is one of the most common types of malignant tumor (1). More than one million individuals die of lung cancer every year worldwide, accounting for 26-28% of the total number of cancer-associated deaths, and the morbidity and mortality rates have risen to first place among all tumors (2,3). Approximately 85% of lung cancer cases are non-small cell lung cancer (NSCLC). The lack of effective diagnosis at the early stages of lung cancer means that most patients are already in the advanced stage at the time of diagnosis. In addition, the poor sensitivity of advanced lung cancer to chemotherapy drugs inevitably leads to poor prognosis, with a five-year survival rate of only ~15.6% worldwide (4).

Gefitinib, considered the first-line drug for the standard treatment of epidermal growth factor receptor (EGFR)-mutated advanced NSCLC, inhibits the EGFR tyrosine kinase activity (5). Mutations in EGFR are observed in ~40 and 20% of patients with NSCLC in Asian and non-Asian populations, respectively (6,7). EGFR is mainly located on the plasma membrane of cells and belongs to the receptor tyrosine kinase family. Once bound by EGF ligands, EGFR kinase is activated, which induces the dimerization of the intracellular domains and then activates cell signal transduction (8). EGFR is abnormally activated and overexpressed in numerous different tumor types, including NSCLC, thyroid cancer and colorectal cancer; therefore, EGFR has become an important effector target for drug therapy for cancers (7). The abnormal expression of any protein downstream of EGFR-mediated signaling transduction pathways promotes the erroneous activation of proliferation signals in the cell nucleus, thus inducing unusual hyperplasia of cells. Mutations in genes such as EGFR grant tumor cells the ability to proliferate indefinitely; however, tumor growth must also have an environment suitable for its growth. Tumor cells interact with the body's own cells, including immune cells, vascular endothelial cells and fibroblasts. These interactions constitute the tumor microenvironment (TME), which is involved in tumor angiogenesis and other processes. More importantly, the TME may help tumor cells evade the body's normal immune cells. During tumorigenesis, not only do the body's immune cells select mutant cells, but tumor cells may also regulate normal immune cells. It has been reported that the EGFR-mutated cancer cells are able to form an immunosuppressive TME that reduces immune response of T cells (9).

The B7 family is the most important member of T cell costimulatory/inhibitory molecules and involved in B7/CD28 signaling. B7-1 (CD80) and B7-2 (CD86) were reported to stimulate T-cell activation by binding to CD28 (10). Currently known members of the B7 family include B7-H1 [programmed death ligand 1 (PD-L1)], B7-H2 (ICOSL/B7h/B7RP-1), B7-H3, B7-DC (PD-L2), B7-H4 (B7x/B7S1), B7-H5 and six other members. These molecules all belong to the immunoglobulin superfamily and have 20-39% identity (homology) in their protein sequences (11). They are expressed at different stages of different immune cells and have different roles. B7 molecules participate in various processes, such as the activation of T cells. Studies have indicated that in mouse models, transfecting tumor cells with B7-1 or B7-2 may enhance T cell immunity, thereby inhibiting tumor growth (12). Studies suggested that the loss of B7-H5 may contribute to immune evasion in various cancers, such as pancreatic cancer and NSCLC (13,14). It has been reported that B7-H5 is constitutively expressed in macrophages and may be induced on dendritic cells, and it was additionally demonstrated that the B7-H5/CD28H [also known as transmembrane and immunoglobulin domain containing 2] interaction stimulated human T-cell growth and cytokine production (15). Previous studies by our group have demonstrated that overexpression of B7H5/CD28H is associated with unfavorable survival in human gastric cancer and pancreatic ductal adenocarcinoma (16,17). Therefore, in the present study, B7H5 was selected to further verify its effect in NSCLC cells.

In the present study, it was investigated whether gefitinib is able to enhance the anti-tumor immune response against EGFR-mutated NSCLC by upregulating B7H5 expression

and activating T cells via CD28H. It was also investigated whether in EGFR-mutant NSCLC cells, over-activated EGFR downstream signaling pathways may cause the decrease in the expression of B7-H5 on the tumor cell surface and inhibit the activation of B7-H5/CD28H costimulatory signals, thereby evading the attack of effector T cells.

## Materials and methods

**Cell culture.** NSCLC cell lines (NCI-H1299, NCI-H358, HCC827 and PC-9) were purchased from the American Type Culture Collection. The identity of the NCI-H358 cells was confirmed using short tandem repeat profiling. Among the cell lines used, NCI-H358 and NCI-H1299 harbor wild-type EGFR, while HCC827 and PC-9 cells harbor EGFR mutations, both with 19 exon deletion and E746-A750 deletion (18,19). PC-9 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO; Thermo Fisher Scientific, Inc.), while NCI-H1299, HCC827 and NCI-H358 cells were cultured in RPMI-1640 medium (GIBCO; Thermo Fisher Scientific, Inc.). Both media contained 10% fetal bovine serum (FBS; GIBCO; Thermo Fisher Scientific, Inc.) and all cells were cultured in an incubator with 5% CO<sub>2</sub> at 37°C.

**Isolation of peripheral blood mononuclear cells (PBMCs).** A blood sample taken using BD anticoagulant tubes (BD Biosciences) was diluted with an equal volume of PBS, which was used to dilute freshly collected peripheral blood of a healthy subject (female; age, 35 years; Tongde Hospital of Zhejiang Province, Hangzhou, China; November 2021). The blood was taken from the vein in the antecubital region of the hand and arm. The diluted blood was slowly added into a centrifuge tube containing the lymphocyte separation solution (FICOLL) at a ratio of 1:0.5 (diluted blood/FICOLL solution). The samples were centrifuged at 400 x g at 20°C for 30 min. The middle white film was then transferred into a new 50-ml centrifuge tube and mixed with 3-4 volumes of sterile PBS. The mixture was then centrifuged at 300 x g for 10 min at 20°C and the supernatant was discarded. The cell pellet was resuspended in RPMI-1640 medium with 10% FBS and then cultured at 37°C.

**Small interfering RNA (siRNA) transfection.** EGFR siRNA, Negative siRNA (negative control), CD28H siRNA, EGFR pcDNA3.1-Flag-C and EGFR mutant plasmid (EGFR; mutation site:L747-E749, A750P) were purchased from Guangzhou Ribobio Co., Ltd. The NCI-H358, NCI-H1299, HCC827, PC-9 cells and PBMCs were seeded in a 6-well plate at 1x10<sup>5</sup> cells/well and supplemented with 2 ml of the corresponding medium. When the cells had reached 60-70% confluence, they were transfected with 50 nM siRNA using the Lipofectamine<sup>®</sup> 2000 transfection reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Cells were collected 48-72 h later for the subsequent experiments. The siRNA sequences were as follows: EGFR siRNA 001 forward, 5'-GUAUUUAUGUGGUGACAGATT-3' and reverse, 5'-UCUGUCACCACAUAUUUACTT-3'; EGFR siRNA 002 forward, 5'-CCUUAGCAGUCUUAUCUAATT-3' and reverse, 5'-UUAGAUAGACUGCUAAGGTT-3'; EGFR siRNA 003 forward, 5'-GGAACUGGAUUCUGAAATT-3'

and reverse, 5'-UUUCAGAAUAUCCAGUUCCTT-3'; B7H5 siRNA 001 forward, 5'-GUGCCUGCAUCGUAGGAAUTT-3' and reverse, 5'-AUUCCUACGAUGCAGGCACTT-3'; B7H5 siRNA 002 forward, 5'-GGCACGAUGUGACCUUCUATT-3' and reverse, 5'-UAGAAGGUCACAUCGUGCCTT-3'; B7H5 siRNA 002 forward, 5'-CACGCCGUAUUCCUGUAUTT-3' and reverse, 5'-AUACAGGGAAUACGGCGUGTT-3'; CD28H siRNA 001 forward, 5'-CCGUAGAGAUUCCUGAGUUTT-3' and reverse, 5'-AACUCAGGAAUCUCUACGGTT-3'; CD28H siRNA 002 forward, 5'-CUCCGUGUUAAGUGGACAATT-3' and reverse, 5'-UUGUCCACUUAACACGGAGTT-3'; CD28H siRNA 002 forward, 5'-UCUACAGCAACGUCCUAUATT-3' and reverse, 5'-UAUAGGACGUUGCUGUAGATT-3'; negative control siRNA forward, 5'-UUCUCCGAACGUGUCACG U-3' and reverse, 5'-ACGUGACACGUUCGGAGAA-3'.

**Toxicity test in NSCLC/PBMCs co-culture.** Calcein acetoxy-methyl ester (1 mg/ml; cat. no. PJ693; Dojindo Laboratories, Inc.) was diluted in RPMI-1640 medium. The toxicity assay was performed in a 96-well culture plate. A gradient of diluted effector cells (E) and labeled target cells (T) was added at 100  $\mu$ l/well at E/T ratios of 50/1, 25/1, 12.5/1, 6.25/1, 3.13/1 and 1.56/1, with three replicate wells for each ratio. At the same time, three replicate wells for the spontaneous release group and three replicate wells for the complete release group were set up. The E/T cells were incubated at 37°C in a 5% CO<sub>2</sub> incubator for 4 h and then centrifuged at 300 x g for 5 min at room temperature. The supernatant was transferred into a new well. A multi-functional enzyme label instrument was used to measure the fluorescence (excitation filter, 485 nm; emission filter, 530 nm). The percent-specific lysis was calculated as follows: Percent-specific lysis=[(test lysis-spontaneous lysis)/(maximum lysis-spontaneous lysis)] x100.

**Cell viability.** NSCLC cells were seeded at 3,000 cells/well in 96-well plates. After the cells were completely attached to the wells, the cells were treated with corresponding medium plus 1% FBS for 24 h. Cells were then transfected with B7H5 siRNA or negative controls for 6 h. The supernatant was discarded and cells were incubated in fresh medium for 24 h. The cells were then incubated with 100  $\mu$ l Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Inc.) solution per well for 2-3 h. Finally, the absorbance at 460 nm was measured using an MRX II microplate reader (Dy nex).

**Reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was extracted from NSCLC cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and then reverse-transcribed to cDNA using a Reverse Transcription Kit (cat. no. RR047A; Takara Bio, Inc.). The cDNA was then used as the template for the qPCR step of the RT-qPCR protocol using a SYBR Premix Ex Taq kit (Takara Bio, Inc.) on an Applied Biosystems Real-time PCR System (Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec and annealing at 60°C for 30 sec.  $\beta$ -actin was used as a reference control. The results were analyzed using the 2<sup>- $\Delta\Delta$ C<sub>q</sub></sup> method (20). The primers used were as follows: EGFR forward, 5'-CAGATCGCAAAGGGCATGAA-3' and reverse, 5'-TTGCCTCCTTCTGCATGGTA-3'; B7H5 forward,

5'-AGGGGGCATCTCTGTACTACT-3' and reverse, 5'-GGC TGGACTATGCTCTCCAC-3';  $\beta$ -actin forward, 5'-GACTTA GTTGC GTTACACCCTT-3' and reverse, 5'-ACCTTCACC GTTCCAGTTTT-3'.

**Western blot analysis.** NSCLC cells were harvested and lysed with radioimmunoprecipitation assay lysis buffer containing protease inhibitors (cat. no. P0013C; Beyotime Institute of Biotechnology). The concentration of the proteins was quantified using a bicinchoninic acid protein assay (Sigma-Aldrich; Merck KGaA). Subsequently, 40  $\mu$ g of protein was separated using 10% SDS-PAGE and then electrotransferred onto polyvinylidene fluoride membranes (EMD Millipore). After blocking with 5% nonfat dry milk (cat. no. 6342932; BD Biosciences) in Tris-buffered saline-Tween-20 buffer for 2 h at 37°C, the membranes were incubated with the indicated primary antibodies, anti-EGFR and anti-B7H5 (cat. nos. 4267S and 54979T, respectively; 1:1,000 dilution; Cell Signaling Technology, Inc.) or anti-CD28H [1:1,000 dilution; a kind gift from Professor Yuwen Zhu (Department of Surgery, University of Colorado Anschutz Medical Campus, Aurora, USA) (13,15)] at 4°C overnight. The samples were then washed and incubated with the corresponding secondary antibodies, anti-rabbit IgG HRP-linked antibody (cat. no. 7074S; 1:2,000 dilution; Cell Signaling Technology, Inc.) and anti-mouse IgG HRP-linked antibody (cat. no. 7076S; 1:2,000 dilution; Cell Signaling Technology, Inc.). GAPDH was used as an internal control (cat. no. 2118S; 1:2,000 dilution; Cell Signaling Technology, Inc.). The immunoreactive proteins were visualized using an ECL kit (Bioworld Technology, Inc.). The density of the bands was quantified using Image Lab 5.0 (Bio-Rad Laboratories).

**Immunofluorescence analysis.** NSCLC cells subjected to different treatments were washed with ice-cold PBS and then fixed using 4% paraformaldehyde for 15 min at room temperature, incubated with 5% bovine serum albumin at room temperature for 30 min, and then with primary antibodies, anti-EGFR and anti-B7H5 (cat. nos. 4267S and 54979T; respectively; 1:200 dilution; Cell Signaling Technology, Inc.) at 4°C overnight. The next day, the cells were washed with PBS, incubated with corresponding secondary antibodies goat anti-rabbit IgG (H+L) AF555 (cat. no. A32732; 1:2,000 dilution; Thermo Fisher Scientific, Inc.) at room temperature for 2 h, incubated with DAPI (Sigma-Aldrich; Merck KGaA) nuclear stain at room temperature for 2 min and washed using PBS twice. Finally, the cells were viewed and imaged under an inverted fluorescence microscope (Olympus Corporation).

**Flow cytometric analysis.** In brief, PBMCs in each group were collected into a 15-ml centrifuge tube at 300 x g and 4°C for 10 min. The cell density was adjusted to 2x10<sup>6</sup>/ml after discarding the supernatant. The cells were washed with PBS containing 2% bovine serum albumin (BSA; Biofrox) 3 times, and then resuspended in 0.1 ml of ice-cold washing buffer and incubated with 1  $\mu$ g biotin-CD28H [a kind gift from Professor Yuwen Zhu (Department of Surgery, University of Colorado Anschutz Medical Campus, Aurora, USA) (13,15)] for 45 min at 4°C. The cells were washed three times with 3 ml ice-cold washing buffer and then incubated with 1  $\mu$ g PE-Biotin (cat. no. 409004; BioLegend, Inc.) for 30 min at 4°C.

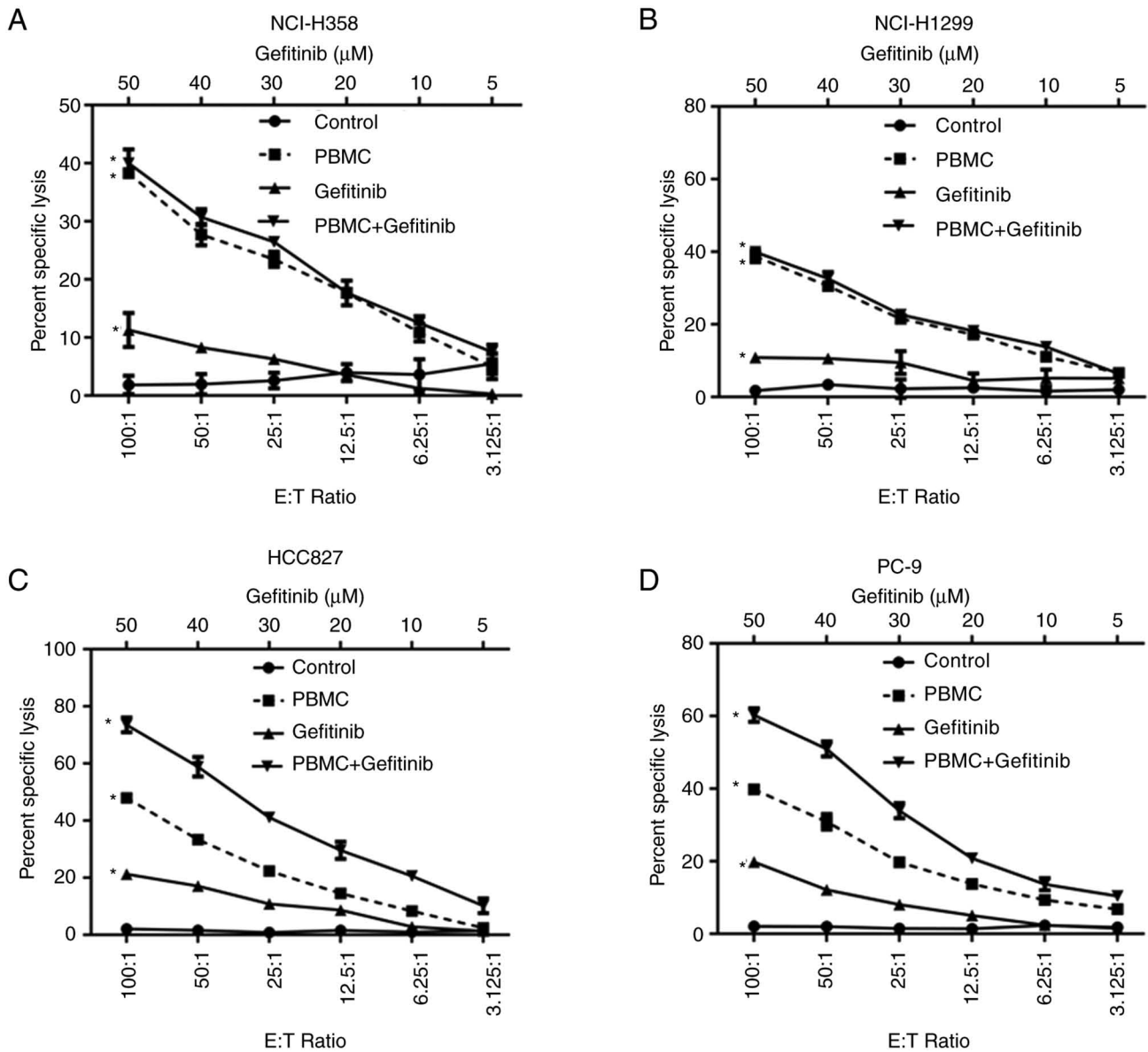


Figure 1. Gefitinib enhances the cytotoxicity of PBMCs in EGFR-mutated NSCLC cells, but not in NSCLC cells with wild-type EGFR. Cell lysis measured by Calcein staining in four NSCLC lines: (A) NCI-H358, (B) NCI-H1299, (C) HCC827 and (D) PC-9. NSCLC cells were cultured with PBMCs at different E/T ratios. \* $P < 0.05$  vs. control. E/T, effector/target; PBMC, peripheral blood mononuclear cell; NSCLC, non-small cell lung cancer.

At the end of the incubation, the cells were washed three times with 3 ml ice-cold washing buffer and resuspended in 0.2 ml PBS for examination on a FACSCalibur flow cytometer (BD Biosciences).

**Tissue samples.** A total of 55 pairs of NSCLC tumor tissues, including 29 EGFR wild-type 26 EGFR Mut and their matched paraneoplastic tissues, were obtained from the First Affiliated Hospital of Huzhou University (Huzhou, China; January 2022) and the detailed information of the patients is provided in Table SI (25 females and 30 males 30; age >65 years, n=29; age <65 years, n=26; age range, 45-85 years; median age, 65.43 years). The present study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Huzhou University (Huzhou, China). All participants provided written informed consent prior to using the tissues for scientific research.

**Immunohistochemical analysis.** The tissue chip (project no. HLugA180Su02; Shanghai Outdo Biotech Co., Ltd.) was baked in an oven at 63°C for 1 h, followed by dewaxing in an automatic dyeing machine (ST5015; Leica Microsystems). Antigens were retrieved by incubation with 0.1 M citric acid at high pressure (50 kPa) at 110°C for 5 min. The chip was blocked with 5% BSA at room temperature for 20 min and rinsed with PBS three times. The primary antibody B7H5 (cat. no. 54979T; 1:300 dilution; Cell Signaling Technology, Inc.) was added dropwise and the array was incubated at room temperature overnight at 4°C. Next, the slide was rinsed with PBS three times, the secondary antibody, anti-rabbit IgG HRP-linked antibody (cat. no. 7074S; 1:2,000 dilution; Cell Signaling Technology, Inc.), was added dropwise, and the slide was incubated at room temperature for 30 min. Finally, 3,3'-diaminobenzidine solution was added dropwise and the color development intensity was observed. Thereafter, the slide was rinsed with tap water

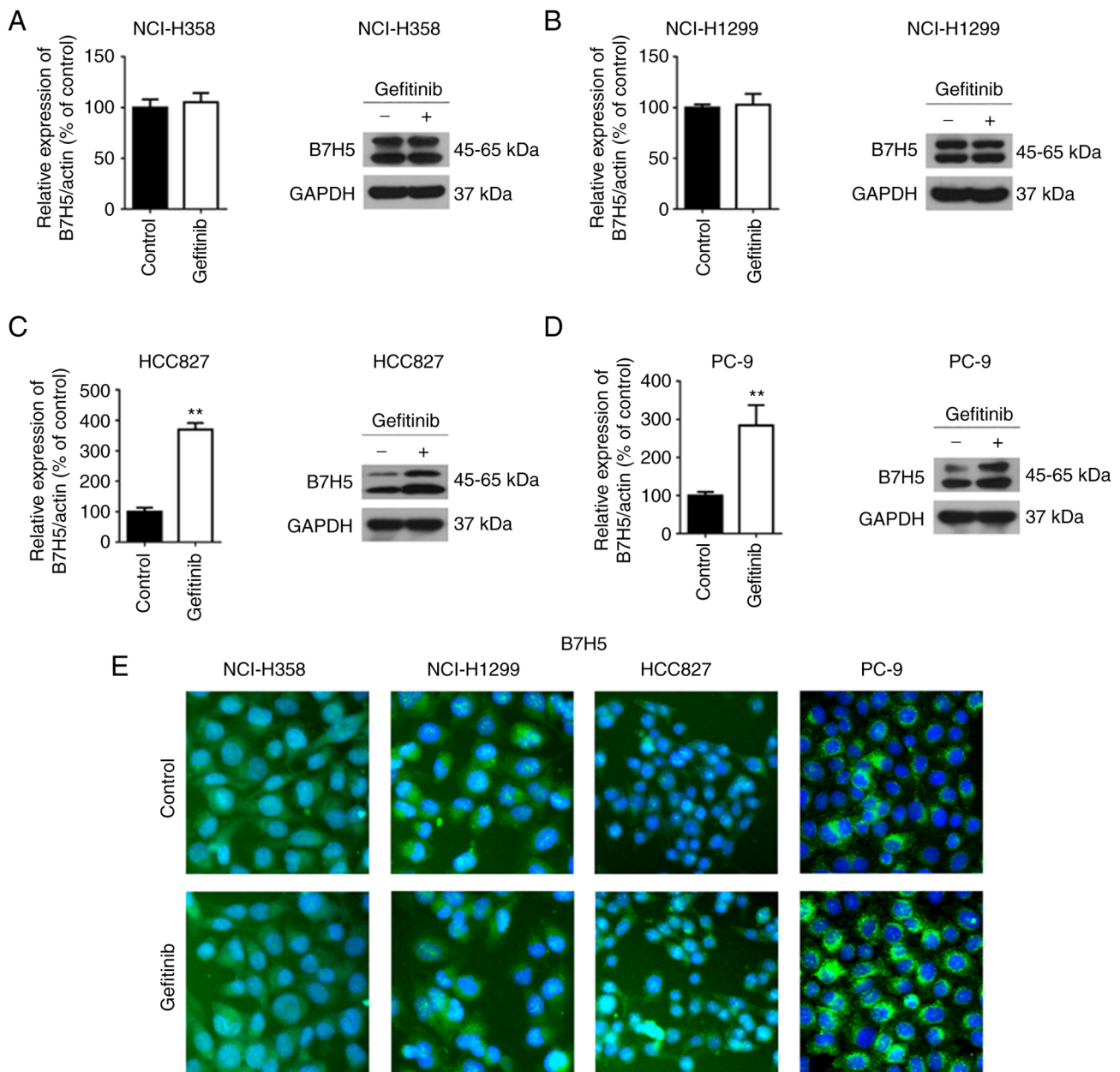


Figure 2. Expression of B7H5 following treatment with Gefitinib in NSCLC cells. (A-D) Reverse transcription-quantitative PCR and western blot analysis of B7H5 expression after treatment with gefitinib in NSCLC cells (A) NCI-H358, (B) NCI-H1299, (C) HCC827 and (D) PC-9. (E) Immunofluorescence determined B7H5 expression (green colour) following treatment with or without gefitinib in NSCLC cells (magnification, x200). \*\*P<0.01 vs. control. NSCLC, non-small cell lung cancer.

for 5 min and Hasteoloy Hematoxylin (Sigma-Aldrich; Merck KGaA) was added dropwise onto the slide, followed by incubation for 1 min. The sample was then submerged in 0.25% hydrochloric acid alcohol for no less than 2 sec, rinsed with tap water for >2 min, dried at room temperature and mounted for a light microscopic (Olympus Corporation) observation.

**Tissue microarray analysis.** The tissue microarray included 87 pairs of tumor tissues and their matched paracancerous tissues, which was analyzed by Shanghai Outdo Biotech Co., Ltd. and the experimental procedures were approved by the Shanghai Outdo Biotech Company ethics committee (YB-M-05-02), which authorized the collection of tissue samples from patients (39 females and 48 males; age >65 years, n=36; age <65 years,

n=51; age range, 44-84 years; median age, 63.07 years); the detailed information is listed in Table SII. An immunohistochemistry protocol was used to analyze the expression of B7H5 and subsequently examine its association with survival.

**Statistical analysis.** Values are expressed as the mean  $\pm$  standard deviation and data were statistically analyzed using GraphPad Prism 5 (GraphPad Software, Inc.). Unpaired Student's t-tests or one-way ANOVA followed by Tukey's post-hoc test were used to analyze differences between two groups or among multiple groups, respectively. Kaplan-Meier survival analysis was used to compare survival, while the log-rank test was performed to determine the significance of differences between groups. All experiments were performed

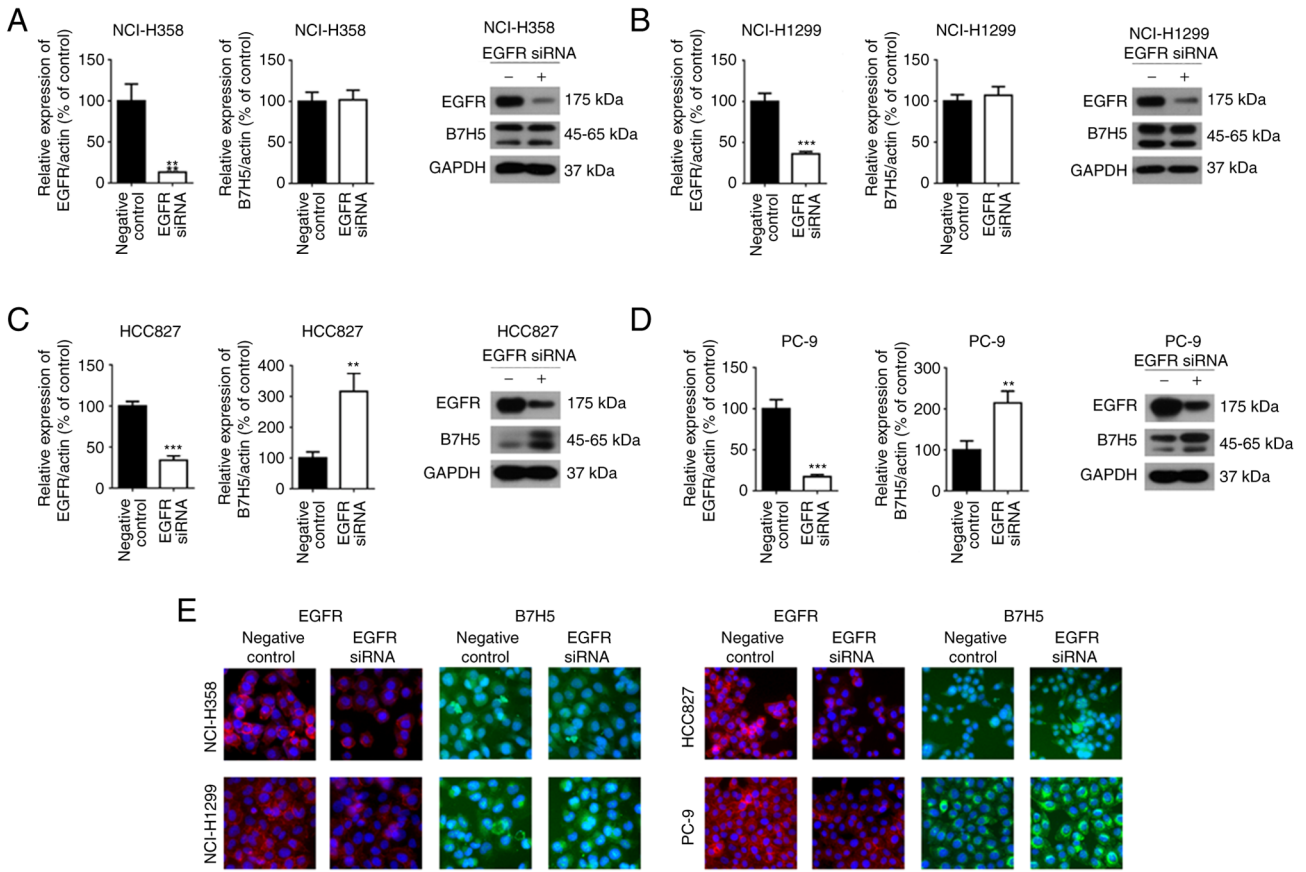


Figure 3. Changes in B7H5 expression in mutant and wild-type NSCLC cell lines after EGFR siRNA interference. (A-D) Reverse transcription-quantitative PCR and western blot analysis of B7H5 and EGFR expression following transfection with an EGFR siRNA in NSCLC cells. (A) NCI-H358, (B) NCI-H1299, (C) HCC827 and (D) PC9. (E) Immunofluorescence analysis B7H5 (green colour) and EGFR (red colour) expression following transfection with EGFR siRNA in NSCLC cells (magnification, x200). \*\*P<0.01, \*\*\*P<0.001 vs. negative control. siRNA, small interfering RNA; NSCLC, non-small cell lung cancer.

as three independent replicates. P<0.05 was considered to indicate a statistically significant difference.

**Results**

*Gefitinib enhances the cytolytic capacity of PBMCs toward EGFR-mutated NSCLC cells.* To assess the anti-tumor cytotoxicity of gefitinib and PBMCs toward NSCLC cells, an *in vitro* model was established by co-culturing PBMCs with four different NSCLC cell lines (NSC-H358, NCI-H1299, HCC827 and PC-9), respectively. The NSCLC cells were stained with Calcein and then mixed with PBMCs at different E/T ratios (100:1, 50:1, 25:1, 12.5:1 and 6.25:1). The cytolytic capacity of PBMCs increased with an increasing E/T ratio in all four cell lines. A 100:1 E/T ratio resulted in 40.01, 39.98, 47.93 and 39.87% lysis of NCI-H358, NCI-H1299, HCC827 and PC9 cells, respectively (Fig. 1A-D). When NSCLC cells were treated with 50 mM gefitinib alone, 11.31 and 10.87% of HNC-H358 and NCI-H1299 cells expressing wild-type EGFR were lysed, respectively, and 21.27 and 19.8% of HCC827 and PC-9 cells expressing mutant EGFR were lysed, respectively (Fig. 1A-D). Furthermore, the combination of gefitinib and PBMCs significantly increased cytolysis from 47.93 and 39.87% to 73.51 and 60.28% in HCC827 and PC-9 cells, respectively, compared with PBMCs alone, but had no significant effects in HNC-H358 and NCI-H1299 cells [combination index: PC9,

0.77; HCC827, 0.588; NCI-H1299, 1.167; and NCI-H358, 1.316; Fig. 1A-D]. The results indicated that gefitinib confers better cytotoxicity in NSCLC cells that carry EGFR mutations, particularly in the presence of PBMCs.

*Gefitinib increases the expression of B7H5 in EGFR-mutated NSCLC cells.* To gain insight into the mechanisms by which gefitinib produces enhanced toxicity against EGFR-mutated NSCLC cells, the expression of B7H5 was examined using RT-qPCR, western blot analysis and immunofluorescence. The results indicated that the mRNA and protein levels of B7H5 were significantly upregulated in EGFR-mutated NSCLC cells (HCC827 and PC-9), but unchanged in wild-type EGFR NSCLC cell lines (NCI-H358 and NCI-H1299) after gefitinib treatment (Fig. 2A-D). Increased expression of B7H5 on the cell plasma membrane was detected in both HCC827 and PC-9 cells, but not in NCI-H358 and NCI-H1299 (Fig. 2E). Next, the effects of EGFR siRNA on the expression of B7H5 in NSCLC cells were examined. It was observed that the expression of B7H5 in EGFR-mutant NSCLC cell lines (HCC827 and PC-9) was upregulated after EGFR siRNA interference, but it was not affected in wild-type EGFR NSCLC cell lines (NCI-H358 and NCI-H1299) (Fig. 3A-E). Regarding the application of siRNA, three siRNAs were mixed together to perform the experiments. Furthermore, the expression of B7H5 was measured in EGFR wild-type

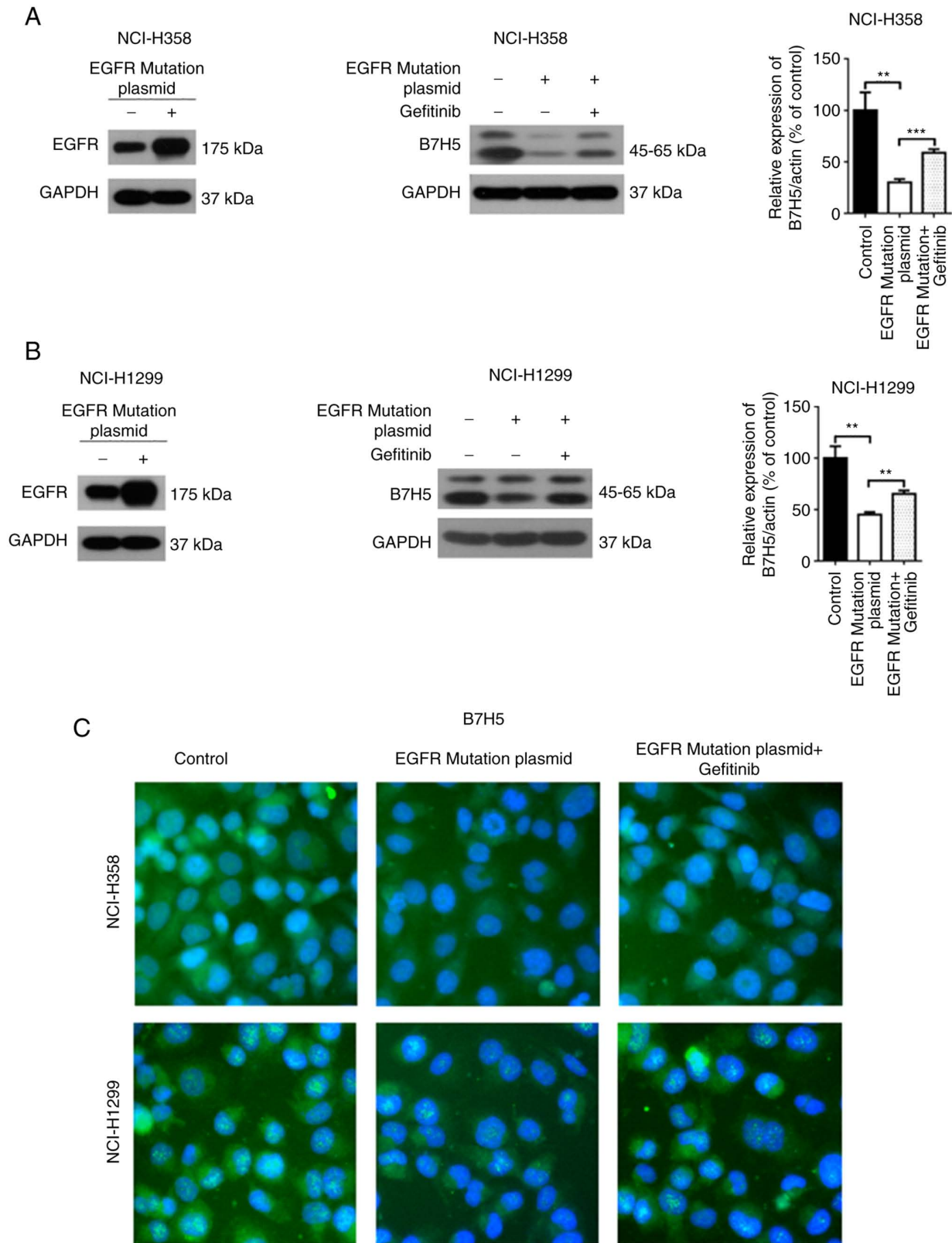


Figure 4. Gefitinib treatment increases B7H5 expression in wild-type NSCLC cell lines expressing EGFR mutants. The levels of EGFR and B7H5 as determined by western blot analysis in (A) NCI-H1299 and (B) NCI-H358 cells. (C) Immunofluorescence analysis of B7H5 expression (green colour) after transfection with the EGFR mutation plasmid or the EGFR mutation plasmid combined with gefitinib in wild-type EGFR NSCLC cells (magnification, x200). \*\*P<0.01, \*\*\*P<0.001. NSCLC, non-small cell lung cancer.

NSCLC cell lines transfected with EGFR mutant plasmid (L747-E749, A750P) and it was indicated that the B7H5 mRNA levels were reduced after expression of the EGFR

mutants (Fig. 4A-C). Gefitinib treatment increased the expression of B7H5 in these cells expressing EGFR mutants (Fig. 4A-C).

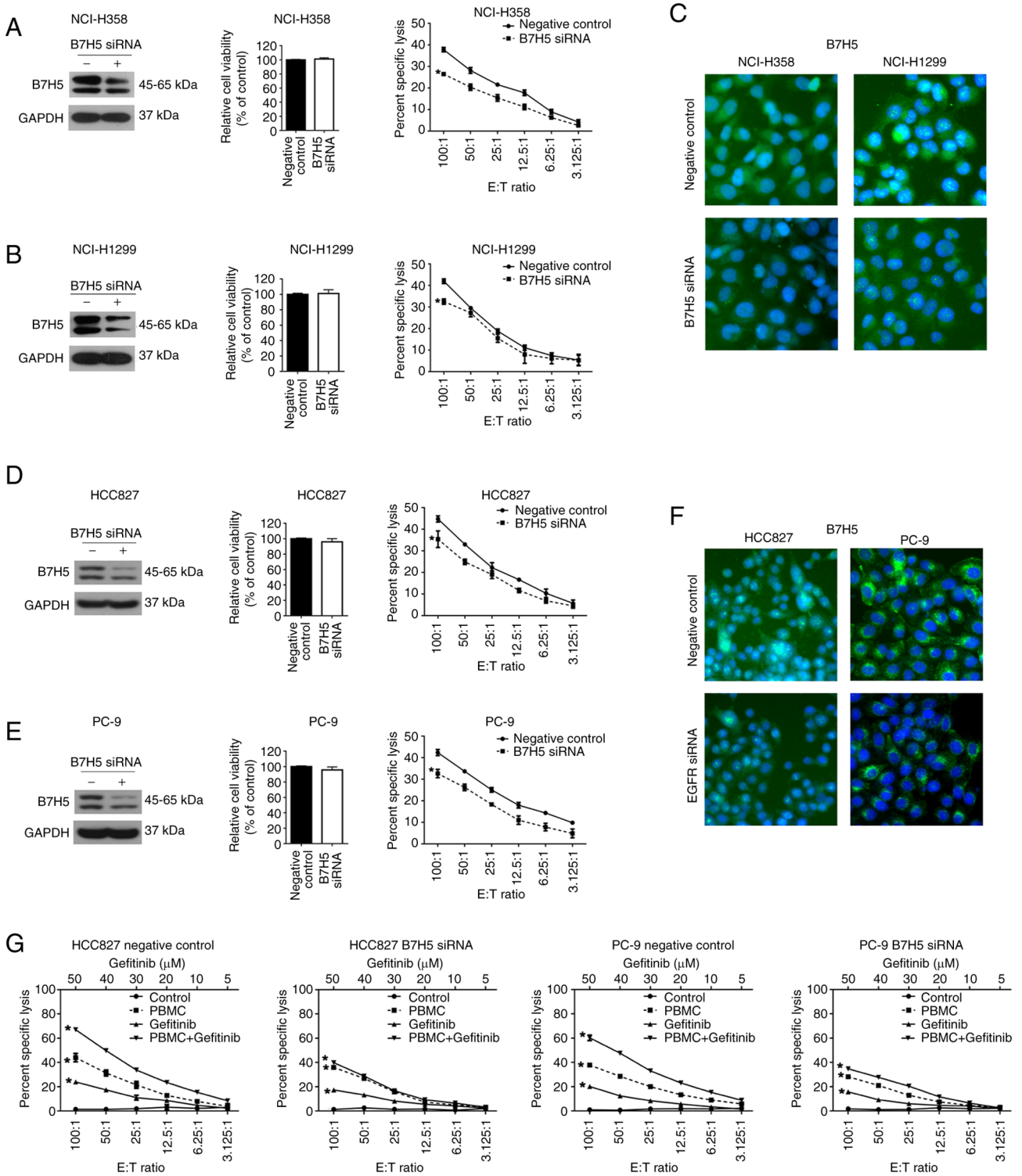


Figure 5. B7H5 affects the killing effect of gefitinib combined with PBMCs in EGFR-mutated NSCLC cells. (A and B) Cell Counting Kit-8 assay and cytotoxicity test models of mixed cell culture to determine the cell viability and killing effect of PBMCs after treatment with or without B7H5 siRNA in (A) NCI-H358 and (B) NCI-H1299 cells. (C) Expression of B7H5 in NCI-H358 and NCI-H1299 cells transfected with or without B7H5 siRNA by immunofluorescence analysis (magnification, x200; B7H5 expression: Green colour). (D and E) The mixed cell culture toxicity test model was used to test the efficacy of gefitinib against EGFR-mutated NSCLC cells prior to and after B7H5 siRNA interference in (D) HCC827 and (E) PC-9 cells. (F) Expression of B7H5 in HCC827 and PC-9 cells transfected with or without B7H5 siRNA by immunofluorescence analysis (magnification, x200). (G) Killing effect of gefitinib combined with PBMCs with and without transfection with B7H5 siRNA on EGFR-mutated NSCLC cells in HCC827 and PC-9 cells. \* $P < 0.05$  vs. Negative control (A-E); \* $P < 0.05$  vs. control (G). siRNA, small interfering RNA; E/T, effector/target; PBMC, peripheral blood mononuclear cell; NSCLC, non-small cell lung cancer.

*B7H5 increases the cytolytic activity of PBMCs in EGFR-mutated NSCLC cells after treatment with gefitinib.* It was then evaluated whether increased B7H5 expression is the

cause of the enhanced cytotoxicity in EGFR-mutated cells treated with gefitinib. First, cell viability was measured in NSCLC cell lines with wild-type EGFR (NCI-H358 and NCI-H1299) after



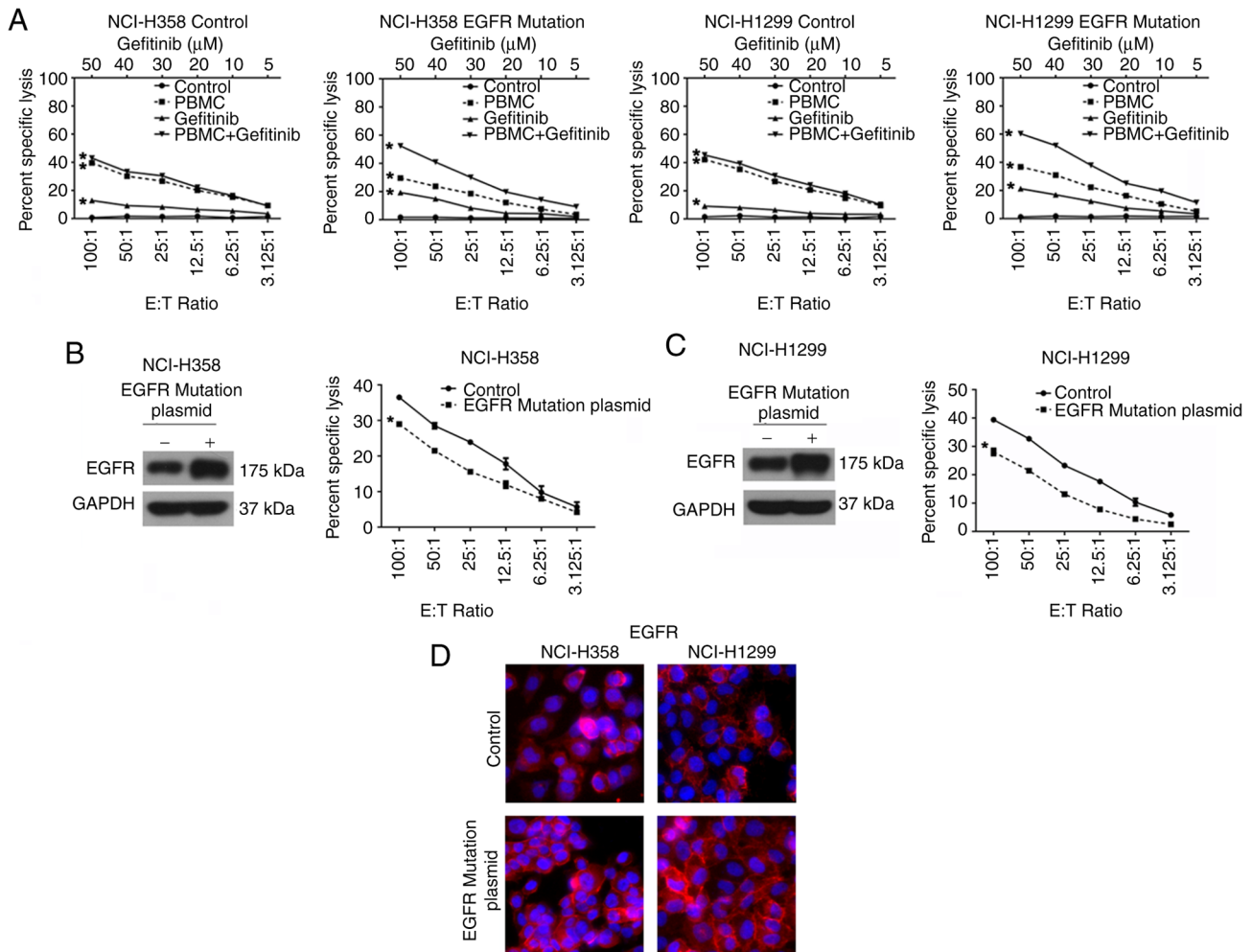


Figure 6. Gefitinib affects PBMC-mediated cytotoxicity in cells transfected with EGFR mutants and wild-type EGFR. (A) The killing effect of gefitinib combined with PBMCs was observed with and without transfection of the EGFR mutation plasmid. (B and C) Western blot analysis detected EGFR levels and the killing effect of PBMCs after transfection with or without the EGFR mutation plasmid in wild-type EGFR NSCLC (B) NCI-H358 and (C) NCI-H1299 cells. (D) Immunofluorescence analysis of EGFR expression (red colour) after transfection with or without the EGFR mutation plasmid in wild-type EGFR NSCLC cells (magnification,  $\times 200$ ). \* $P < 0.05$  vs. control. siRNA, small interfering RNA; PBMC, peripheral blood mononuclear cell; NSCLC, non-small cell lung cancer; E/T, effector/target.

transfection with B7H5 siRNA. As presented in Fig. 5A-C, the efficiency of siRNA interference of B7H5 was verified by western blot and immunofluorescence analyses. B7H5 knockdown had no significant effects on cell viability in these two cell lines. Of note, the PBMC-induced cytotoxicity decreased in NCI-H358 and NCI-H1299 cells after B7H5 siRNA treatment. Subsequently, B7H5 expression was reduced by siRNA silencing in EGFR-mutated NSCLC cell lines (HCC827 and PC-9). The knockdown effects were confirmed by western blot and immunofluorescence analysis. However, reduced expression of B7H5 in HCC827 and PC-9 cells had no effects on cell viability (Fig. 5D-F). PBMC-mediated toxicity in HCC827 and PC-9 cells was reduced when B7H5 expression was decreased (Fig. 5D and E). The results suggested that B7H5 expression is not related to the viability of NSCLC cells, but its expression is associated with the PBMC-mediated immune response. Given that gefitinib enhanced PBMC-mediated cytotoxicity in EGFR-mutated NSCLC cell lines, it was next investigated whether B7H5 is involved in this improved immunoregulation. When B7H5 expression was reduced in EGFR-mutated NSCLC

cells co-cultured with PBMCs, the gefitinib-enhanced toxicity was not different from that of the control (Fig. 5G). These findings indicated that gefitinib-induced immune toxicity toward EGFR-mutated NSCLC cells is mediated by upregulation of B7H5.

NCI-H358 and NCI-H1299 cells were then transfected with plasmids expressing either wild-type or mutant EGFR, and these cells were then cultured with PBMCs. Consistent with the results in EGFR-mutated cell lines, gefitinib significantly enhanced the PBMC-mediated cytotoxicity in cells transfected with EGFR mutants, but not in cells expressing wild-type EGFR (Fig. 6A-D).

*CD28H knockdown in PBMCs decreases PBMC-mediated cytotoxicity in EGFR-mutated NSCLC cells.* It has been previously reported that B7H5 is the ligand for CD28H and the interaction between B7H5 and CD28H stimulates the T cell response (16); therefore, the present study aimed to determine whether the B7H5/CD28H signaling pathway is involved in PBMCs-mediated cytotoxicity in EGFR-mutated NSCLC cells. Western blot and flow cytometric analyses confirmed

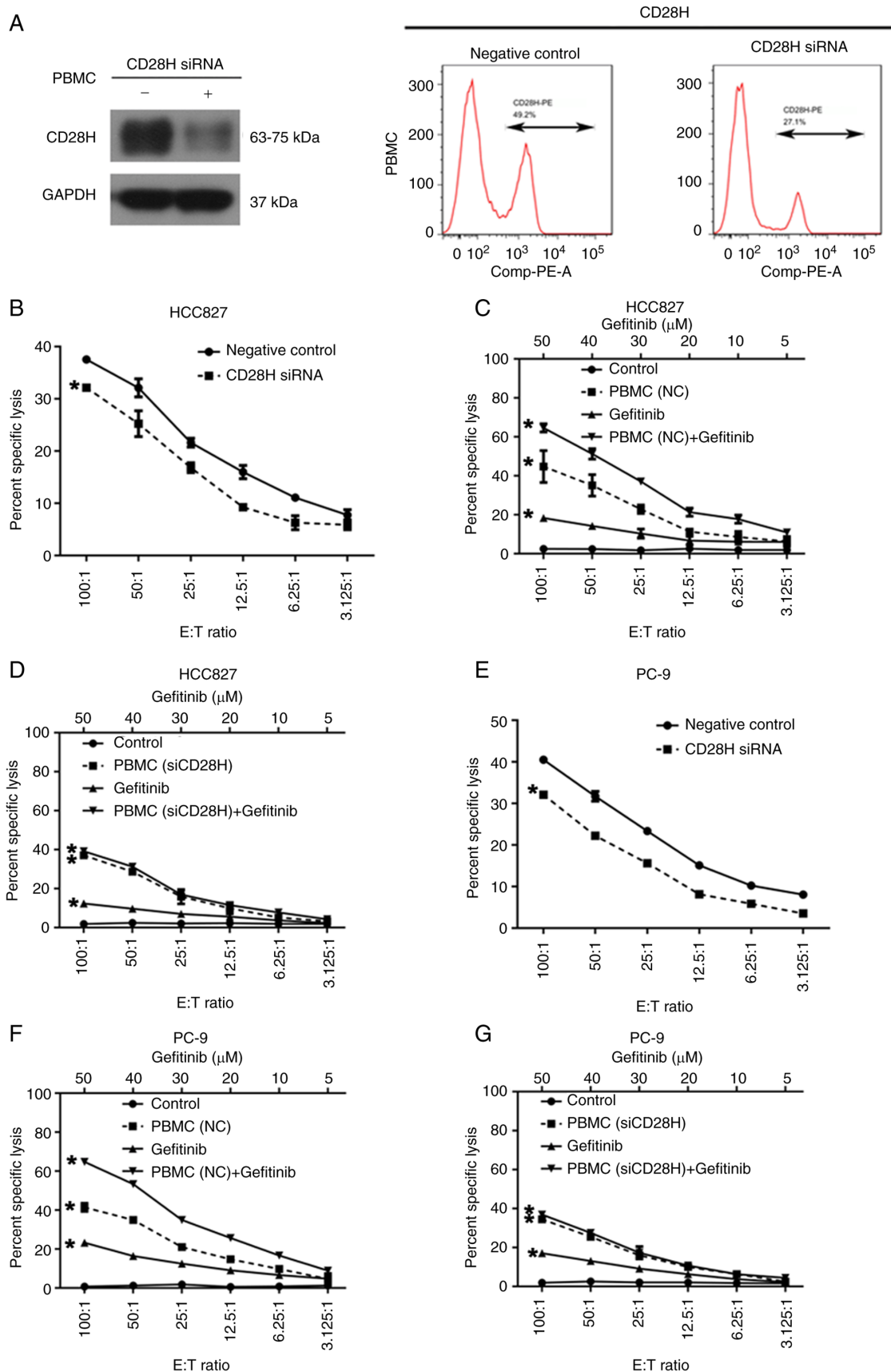


Figure 7. Interference with CD28 expression markedly decreases the killing activity against EGFR-mutant NSCLC cells. (A) CD28 expression was determined by western blot analysis and flow cytometry. (B) Mixed cell culture toxicity assay to determine the cytotoxic activity after transfection with or without CD28H siRNA; (C) combined efficacy of PBMCs and gefitinib, (D) combined efficacy of PBMCs and gefitinib with and without CD28H siRNA interference in EGFR-mutant NSCLC cells HCC827. (E) Mixed cell culture toxicity assay to determine the cytotoxic activity after transfection with or without CD28H siRNA; (F) combined efficacy of PBMCs and gefitinib, (G) combined efficacy of PBMCs and gefitinib with and without CD28H siRNA interference in EGFR-mutant NSCLC cells PC-9. \* $P < 0.05$  vs. Negative control. siRNA, small interfering RNA; PBMC, peripheral blood mononuclear cell; NSCLC, non-small cell lung cancer; E/T, effector/target; NC, negative control.

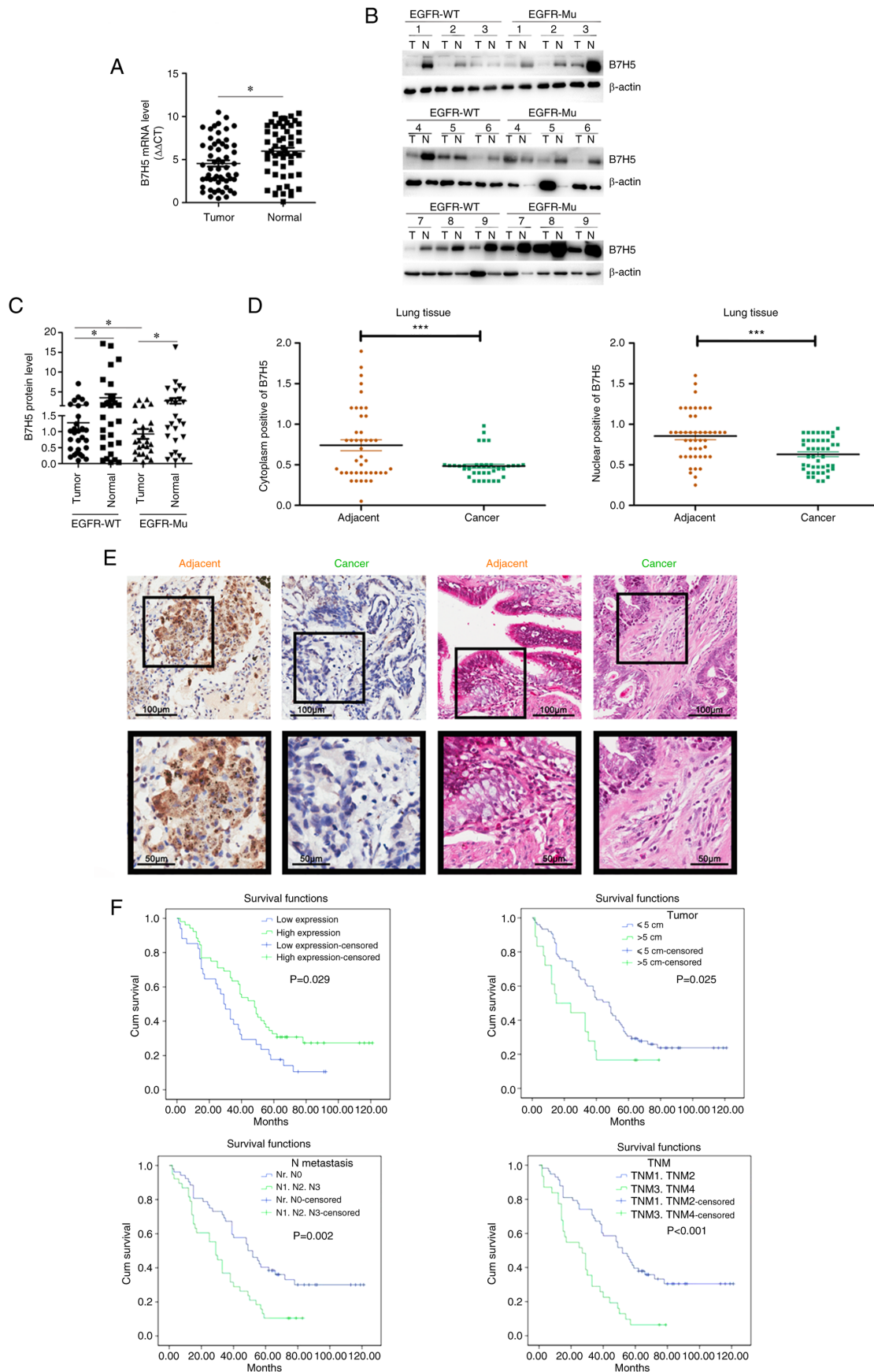


Figure 8. Tissue microarray analysis of differences in expression of B7H5 in cytoplasm between NSCLC and adjacent non-cancer tissue. The expression of B7H5 was determined by (A) reverse transcription-quantitative PCR and (B and C) western blot analysis with (B) representative blots and (C) quantitative protein levels provided. (D) Immunohistochemical analysis of the expression difference of B7H5 in the cytoplasm and nuclear of lung cancer and paracancerous tissues. (E) Immunohistochemical analysis of the expression difference of B7H5 in the cytoplasm and nuclear of lung cancer and paracancerous tissues (scale bars, 100 and 50 μm in upper panel and magnified windows below, respectively). (F) Overall survival of patients with NSCLC represented by Kaplan-Meier curves. \*P<0.05; \*\*\*P<0.001. NSCLC, non-small cell lung cancer; T, tumor tissue; N, normal tissue; WT, wild-type; Mu, mutant; Cum, cumulative.

that the expression of CD28H was reduced in PBMCs treated with CD28H siRNA (Fig. 7A). The cytotoxic activity of PBMCs against EGFR-mutated NSCLC cell lines prior to and after CD28H siRNA interference was compared in the *in vitro* NSCLC/PBMCs co-culture model. PBMC-mediated cell lysis in HCC827 and PC-9 cells decreased significantly when PBMCs were transfected with CD28H siRNA (Fig. 7B and C). Furthermore, after CD28H expression was inhibited in PBMCs, gefitinib treatment had no effect on the cytolysis of HCC827 and PC-9 cells co-cultured with PBMCs (Fig. 7D-G), suggesting that CD28H is required for gefitinib-enhanced PBMC cytotoxicity against EGFR-mutated NSCLC cells.

*Expression of B7H5 is lower in NSCLC tumors than that in normal tissue.* The expression of B7H5 was determined in lung tumor tissues and matched paracancerous tissues collected from 55 different patients, including 29 patients carrying wild-type EGFR and 26 patients carrying mutations in EGFR. The results indicated that B7H5 expression was lower in the tumor tissues compared with that in the matched paracancerous tissues in both the wild-type and mutated EGFR groups (Fig. 8A-C). Further tissue microarray analysis indicated that the expression of B7H5 was lower in lung tumor tissue compared with that in the adjacent tissue (Fig. 8D). Immunohistochemical analysis also indicated that the expression of B7H5 in lung cancer tissues was lower than that in adjacent tissues (Fig. 8E). Kaplan-Meier analysis suggested that patients with certain characteristics, such as low B7H5 and high B7H5 expression, tumor diameter >5 cm, TNM3/TNM4 or metastasis stages of N1, N2 or N3, had a shorter overall survival time (Fig. 8F).

## Discussion

EGFR is a glycoprotein belonging to the tyrosine kinase receptor family that is inextricably linked to the occurrence and development of various solid tumors, as well as tumor invasion and metastasis. EGFR signaling pathways are frequently abnormally activated or their members are highly expressed in cancer, which indicates a correlation between tumor progression and EGFR-associated gene expression. When ATP binds to the ATP binding site on the tyrosine kinase domain, an EGFR-TK inhibitor is able to block the phosphorylation and activation of EGFR tyrosine kinase, which delays EGFR signal transduction, restrains cell proliferation and accelerates apoptosis, thus inhibiting the tumor. Recently, immune cells in the TME have been identified as immunological effector cells that serve an important role in tumor recognition and immune defense to attack tumor cells. Thus, immunotherapy has an important role in the treatment of NSCLC. It may activate the immune system by killing tumor cells that have escaped previous immunological surveillance (21). The results of the present study confirmed that gefitinib was able to increase the cytotoxicity of PBMCs to kill EGFR-mutant NSCLC cells. Transfection with the mutated EGFR plasmid reduced the cytotoxicity of PBMCs in wild-type NSCLC cells; however, gefitinib treatment partially recovered the cytotoxicity of PBMCs in wild-type NSCLC cells expressing mutant EGFR. These results demonstrated that gefitinib was able to inhibit the overactivation of EGFR in EGFR-mutated NSCLC cells and enhance the toxicity of PBMCs against NSCLC cells.

Immune checkpoint therapy is a novel treatment that targets regulatory pathways in T cells to enhance anti-tumor immune responses. The B7/CD28 axis, the first discovered T-cell ligand/receptor complex, has been studied extensively (22). The B7/CD28 family has an important role in cancer pathogenesis. It has been reported that the B7-H5/CD28H pathway has co-inhibitory and co-stimulatory functions in immune signaling (15,17,23). High B7-H5 expression induced a more potent immune reaction following co-culture with T cells in pancreatic ductal adenocarcinoma (PDAC) cell culture and high expression of B7-H5 was able to improve the prognosis of patients with PDAC (16). The present findings indicated that the expression of B7H5 in EGFR-mutant NSCLC cells was upregulated after gefitinib treatment; however, B7H5 expression was not changed in wild-type NSCLC cells treated with gefitinib. For wild-type NSCLC cells, B7H5 was downregulated relative to the control group only after transfection with the EGFR mutation plasmid and it was upregulated after EGFR mutation combined with gefitinib treatment, leading to an enhanced killing effect of PBMCs on wild-type NSCLC cells. The results also indicated that reduced B7H5 expression by siRNA in NSCLC cells decreased the cytotoxicity of PBMCs. Furthermore, B7H5 knockdown suppressed the gefitinib-enhanced cytotoxicity by PBMCs in the EGFR-mutant NSCLC cells. These findings suggested that B7H5 expression is upregulated under gefitinib treatment, causing an improved immune response in PBMCs.

CD28 has been recognized as a major co-stimulatory receptor that specializes in priming pan-naïve T cells, and promoting both T cell division and cytokine production, particularly IL-2, in secondary lymphoid organs (7). CD28 signaling prevents T-cell anergy, the unresponsiveness status of T cells to antigen challenge (10,17). The results of the present study indicated that transfection of a CD28 siRNA into PBMCs blocked the gefitinib-induced high toxicity of PBMCs toward EGFR-mutated NSCLC. These results indicated that the B7H5/CD28H interaction is involved in the boosted immune response of PBMCs after EGFR-mutated cells were treated with gefitinib. Furthermore, our group is also considering examining and further screening how the mutant EGFR signal enters cells and specifically regulates B7H5 or what signaling pathways are involved. This will comprise a large number of signaling pathways and the workload is high, which will be the focus of research by our group in the future. Furthermore, deep research will be performed to verify whether mutant EGFR activates any specific signaling that is not affected by the wild-type protein.

In conclusion, the present study indicated that inhibition of mutated EGFR by gefitinib was able to activate the B7-H5/CD28H signaling pathway and increase the cytotoxicity of PBMCs against NSCLC cells. Furthermore, the expression of B7-H5 is associated with improved prognosis in NSCLC. The present findings indicated that the B7-H5/CD28H pathway may be a potential immunotherapeutic target in NSCLC.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

XW and WX conceived the study. HG, XZ, SX and TW performed the experiments. DX, YC and DC analyzed the data. XW wrote the manuscript. All authors have read and approved the final version of the manuscript. XW and WX confirm the authenticity of all the raw data.

## Ethics approval and consent to participate

This study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Huzhou University (Huzhou, China; approval no. 2022KYLL043). All participants and/or their legal guardian provided written informed consent prior to use of their tissues for scientific research.

## Patient consent for publication

All participants and/or their legal guardian provided written informed consent prior to use of their tissues for scientific research.

## Competing interests

The authors declare that they have no competing interests.

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