Knockdown of TRAF1 in NSCIC Contributes Gefitinib Sensitivity by Promoting G2/M Cell Cycle Arrest

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Research

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Abstract

Background: Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) achieves remarkable success in the treatment of patients with advanced non-small cell lung cancer (NSCLC) bearing activating EGFR mutations. However, EGFR-TKI resistance is still a obstacles in the clinical practice. TRAF1 as a member of TRAF family participates NSCLC progression. In our previous research found that TRAF1 played an important role in the progression of NSCLC.

Methods: In vivo and vitro, quantitative PCR (qPCR) and western blot (WB) were carried out to check the expression of TRAF1 in gefitinib-sensitive and -resistant NSCLC samples. The cell counting kit-8 (CCK8) and flow cytometry were used to measure cell proliferation, apoptosis and cycle arrest.

Results: Here we showed that TRAF1 over-expression is associated with clinical resistance to EGFR TKI. TRAF1 was significantly up-regulated in gefitinib-resistant cells. Knockdown of TRAF1 increased the sensitivity of gefitinib-resistant cell. Knockdown of TRAF1 induced cell apoptosis and cell cycle arrest.

Conclusions: Therefore, our results highlight the function of TRAF1 in the EGFT-TKI resistance.

Background

Lung cancer is the most leading cause of cancer-related deaths worldwide, with non-small cell lung cancer (NSCLC) being one of the most common types. The median overall survival for the traditional platinum-based chemotherapy in the first-line therapy advanced NSCLC is only 8–11 months (1). The criterion of treatment of patients with advanced NSCLC has shifted from chemotherapy to molecular-targeted cancer therapy and immune checkpoint inhibitor. EGFR signaling plays an important role in lung cancer proliferation, angiogenesis, and metastasis (2-3). For advanced NSCLC patients with activating EGFR mutations, the EGFR-TKIs are preferred. However, for the NSCLC patients with EGFR mutations accepting EGFR-TKIs treatment, the progression free survival (PFS) is less than one year (4-5). EGFR-TKI resistance is still a obstacles in the fight against lung cancer. Despite many efforts, illumination the mechanism of resistance to EGFR-TKIs is an ongoing challenges (6).

The tumor necrosis factor receptor-associated factor 1(TRAF1) is a member of TRAF family and plays an important role in activating the NF-κB pathway (7). In our previous study, we first reported that TRAF1 was significantly up-regulated in NSCLC tissues (8). The over-expression of TRAF1 was negatively associated with patient overall survival. Qiushi *et al* found that knockdown TRAF1 attenuated the NSCLC cells proliferation through the RAF–MEK–ERK pathway in the TRAF1 knockout (TRAF1 KO) mice model (9). The RAF–MEK–ERK pathway is a crucial part in the EGFR-TKI rsistence mechanism (10). The role of TRAF1 in the EGFR-TKI rsistence mechanism is unclear.

In the present study, we first reported that TRAF1 up-regulation is associated with clinical resistance to EGFR TKI. TRAF1 over expressed in gefitinib-resistant cells. Knockdown of TRAF1 increased the gefitinib-resistant cell sensitivity. TRAF1 promoted gefitinib-resistance through restraining cell apoptosis and cell

cycle arrest. Therefore, our results highlight that up-regulation of TRAF1 contributed NSCLC EGFT-TKI resistance.

Methods

Cell culture

The PC9 and HCC4006 cells were collected from the American Type Culture Collection (Manassas, VA, USA). Lung cancer cells were cultured and acquired gefitinib resistance according to procedures described in our previous study (11).

Lentivirus construction and infection

The TRAF1 short hairpin(sh) RNA was purchased from the RNAi Consortium (Broad Institute, Cambridge, MA, USA). We purchased negative control and shTRAF1 plasmids (GV112) from GeneChem (Shanghai, China). Lentivirus construction and infection were carried out according the procedures reported in our previous study (11).

Cell proliferation assay

The cell proliferation assay was performed by counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). For the cell proliferation assay, 1000 cells/well were seeded in 96-well plates and cultured 24h. According to the manufacturer's instructions, cells were incubated with 10ul CCK8 reagent for 1h at 37°C, then assayed at 450nm on a Multiskan microplate reader.

Detection of apoptotic cells by flow cytometry

The annexin V-FITC/PI staining kit was purchased from Thermo Fisher Scientific. A FACS Aria II flow cytometer (BD Biosciences) was used to detected cells. The procedures were performed according the manufacturer's instructions and our previous study (12).

Cell cycle analysis

For the cell cycle analysis, 5×10^5 PC9R cells per well were seeded in the 6-well plates. We incubated the PC9R cells with gefitinib. According the manufacturer's instructions, the cells were harvested, fixed and stained. The flow cytometry was used to analysis.

qPCR

The TRIzol reagent was obtained from Thermo Fisher Scientific. RT-PCR kit was purchased from Toyobo. The experimental procedures and results analysis were carried out according to the manufacturer's instructions.

PCR primers: TRAF1, 5'-CTACTGTTTTCCTTTACTTACTACACCTCAGA-3'

and 5'- ATCCAGACAACTGTTCAAACTGATG-3';

β-actin,5'-CTGGCACCCAGCACAATG-3'

and 5'-CCGATCCACACGGAGTACTTG-3'.

Western blotting

The anti-TRAF1 polyclonal antibody (sc1830) and β-actin antibody (A3854) were obtained from Santa Cruz Biotechnology and Sigma-Aldrich. The procedures of western blot (WB) were carried out according manufacturer's instructions and our previous studies (8).

Pleural effusion sample collection

The 50 patiens with EGFR TKIs sensitive and 45 patients with EGFR TKIs resistance were enrolled in the study at the Department of Pulmonary Medicine and Department of Oncology, Shengli Oilfield Central Hospital, Shandong Province, China. The study protocol and informed consent providing to the participants were verified by the institutional ethics committee. Clinic sample collection and data management were performed in conformity with guidelines. After collected the samples, in 1000 × G centrifugation for 10 min to obtain cell pellets. *TRAF1* mRNA level was quantitated by qPCR.

Statistical analysis

All experiments were carried out with triplicate samples. SPSS version 20 and GraphPad Prism version 5.0 were used for statistical computation. The principles of statistical analysis were consistent with our previous studies. $P < 0.05$ means statistically significant.

Results

TRAF1 is up-regulated in EGFR tyrosine kinase inhibitors resistance tissues compared with sensitive tissues.

To investigate the clinical significance of TRAF1 in EGFR resistance progression, we collected pleural effusions from 95 NSCLC patients. There are 50 patients were sensitive and 45 were resistant to EGFR tyrosine kinase inhibitors. The baseline characteristics of the two groups were well balanced(table 1). The cancer cell pellets were centrifuged from the pleural effusions. We examined the expression levels of the TRAF1 mRNA in the cancer cell pellets using quantitative PCR (qPCR). The result showed that TRAF1 was up-regulated in the resistant pleural effusions compared to sensitive pleural effusions (Figure1A). Furthermore, TRAF1 expression was frequently (12 of 15, 80%) increased in tumor pleural effusions cells from patients with acquired resistance to gefitinib compared with paired tumor pleural effusions cells obtained before gefitinib treatment. However, TRAF1 expression was decreased in 3 of 15 patients (Figure1B). The results presented that TRAF1 was frequently overexpressed in gefitinib resistance tissues compared with sensitive tissues.

TRAF1 is up-regulated in gefitinib-resistant cells.

Through exposing the PC9 and HCC4006 cells to increasing concentrations of gefitinib, we obtained the gefitinib-resistant PC9R and HCC4006R lung cancer cells, as previously reported (12). The qPCR and western blotting (WB) were carried out to compared TRAF1 expression in gefitinib-sensitive and -resistant cells. We found that TRAF1 was up-regulated in gefitinib-resistant cells as compared to gefitinib-sensitive cells (Figure 2A,B). This result suggested that TRAF1 play a role in gefitinib resistance.

TRAF1 significantly induces gefitinib resistance in vitro.

To better understand the function of TRAF1 in the gefitinib-resistance, PC9R and HCC4006R cells were infected with viral particles expressing either TRAF1 short hairpin (sh) RNA to silence *TRAF1* expression or negative control shRNA. qPCR and WB analyses showed that TRAF1 was knockdown in both cell lines (Figure 3A,B).The cell Counting Kit (CCK) 8 assay was carried out to evaluate the function of TRAF1 on the gefitinib-resistant cells. The result showed that knockdown of *TRAF1* repressed the viability of PC9R and HCC4006R cells by exposing lung cancer cells with different gefitinib concentrations (ranging from 0.1 to 20.0 μM) (Figure 3C,D).

Knockdown TRAF1 induces gefitinib-resistant cells apoptosis and cell cycle arrest.

To further confirm the function of TRAF1 on gefitinib resistance cells, the annexin V-FITC/PI double staining assay and flow cytometry analysis were carried out. Knockdown TRAF1 significantly increased the apoptosis rate of the PC9R cells when exposed in 1 μM gefitinib (Fig. 4A and B). We also found that knockdown TRAF1 significantly decreased the number of PC9R cells in S phase compared to the negative control. And the the number of cells in G2/M phase were significantly increased (Fig. 4C and D). The result showed knockdown TRAF1 promoted gefitinib inducing lung cancer cells apoptosis and cell cycle arrest.

Discussion

Gefitinb as the first-generation EGFR-TKIs inhibited EGFR signaling by blocking the intracellular TK domain of EGFR. The traditional therapy method for advanced NSCLC has shifted from chemotherapy to molecular-targeted cancer therapy and immune checkpoint inhibitor. EGFR-TKIs are preferred for advanced NSCLC with EGFR mutation (13). However, the efficient of the first-generation EGFR-TKIs was approximately 70-80 % for NSCLC patients with EGFR mutation, and even those who show partial response in the first-line treatment, eventually develop progressive disease after about one year (14). EGFR-TKI resistance is the main obstacle on the way conquering lung cancer.

The secondary mutation T790M in exon 20 of the EGFR gene was the main reason inducing first-line EGFR-TKI resistance. The MET proto-oncogene amplification and HGFover-expression are significant parts in the EGFR-TKI resistance(15-17). However, approximately 25 % of the EGFR-TKIs resistance cases cannot be explained through these mechanisms. In our study, we first found that TRAF1 up-regulation is

associated with clinical resistance to EGFR TKI resistence. TRAF1 was significantly up-regulated in gefitinib-resistant cells. Knockdown of TRAF1 increased the sensitivity of gefitinib-resistant cell. Therefore, our results highlight the function of TRAF1 in the mechanism of EGFT-TKI resistance.

The activation of EGFR signaling is required for the regulation of cancer cell division, survival, metastasis and cellular repair (18). The Ras/Raf/mitogen-activated protein kinase (MAPK) pathway is the main downstream signaling route. Other downstream signaling routes include Janus kinase (JAK)/signal transducer and activator of transcription (STAT), and the phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathways. The EGFR-KRAS-RAF-MEK-ERK cascade is also an important pathway for EGFR signaling regulated cancer cells proliferation (10, 19). In our previous study, we found that TRAF1 was overexpressed in the NSCLS tissues. This study found TRAF1 was up-regulated in gefitinib resistance samples compared to sensitive samples. Many reports indicate that TRAF1 and TRAF2 preferentially form the TRAF1: (TRAF2) heterodimeric complex that is involved in various cellular physiologies. TRAF2 has been verified to promote the BRAF protein degradation by acting as an E3 ligase in mediating ubiquitination. TRAF1 decreased the binding affinity between TRAF2 and BRAF (20, 21). In this way, *Qiushi WanG et al* revealed that knocking down TRAF1 expression in H1299 and H1975 lung cancer cells impaired BRAF phosphorylation and reduced BRAF total protein levels, which led to a decrease in BRAF downstream signaling. Knockdown TRAF1 blocked the BRAF–MEK–ERK cascades in NSCLC cells (9). Our findings found knockdown the TRAF1 increased the NSCLC cells sensitivity to gefitinib. The BRAF–MEK–ERK cascades may play an important role in the TRAF1 promoting gefitinib resistance.

Conclusions

We first report that TRAF1 over-expression is associated with clinical resistance to EGFR TKI. TRAF1 was significantly up-regulated in gefitinib-resistant cells. Knockdown of TRAF1 increased the sensitivity of gefitinib-resistant cell. Therefore, our results highlight that up-regulation of TRAF1 in the NSCLC promotesd EGFT-TKI resistance.

Declarations

Ethics approval and consent to participate

Experiments were approved by the Research Ethics Committee of Shengli Oilfield Central Hospital, (Shandong Province, China) and performed according to relevant guidelines and regulations. Written informed consent was obtained from all participating individuals.

Consent for publication

All authors have read the final version of the manuscript and are in agreement for publication upon acceptance.

Availability of data and material

The dataset used for analysis was available upon request.

Competing interests

Authors have no financial, professional, or personal conflicts to disclose.

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Authors' contributions

Tao Fang and Bingping Wang supervised the conduction of the entire project. Xiaoxing Wen, TingTing Zhang, Tao Feng performed the research, analyzed the data, and prepared the manuscript.

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Table

Table 1. The clinical characteristics of EGFR TKI-sensitive and –resistance lung adenocarcinoma patients

Clinicopathological Features	Number of cases	EGFR TKI-sensitive	EGFR TKI-resistance	*p* Value
Age (years)				
<65	55	30	25	0.409
≥65	40	20	20	
Gender				
Male	22	11	11	0.484
Female	73	39	34	
Smoking				
No-smokers	80	42	38	0.589
Smokers	15	8	7	
Tumor size				
T1/T2	61	35	26	0.152
T3/T4	34	15	19	
lymphatic metastasis				
Absent	40	24	16	0. 154
Present	55	26	29	
Clinical stage				
IV	95	50	45	

P value represents the probability from a Chi-square test for different number of EGFR TKI-sensitive and –resistance cases.

Figures

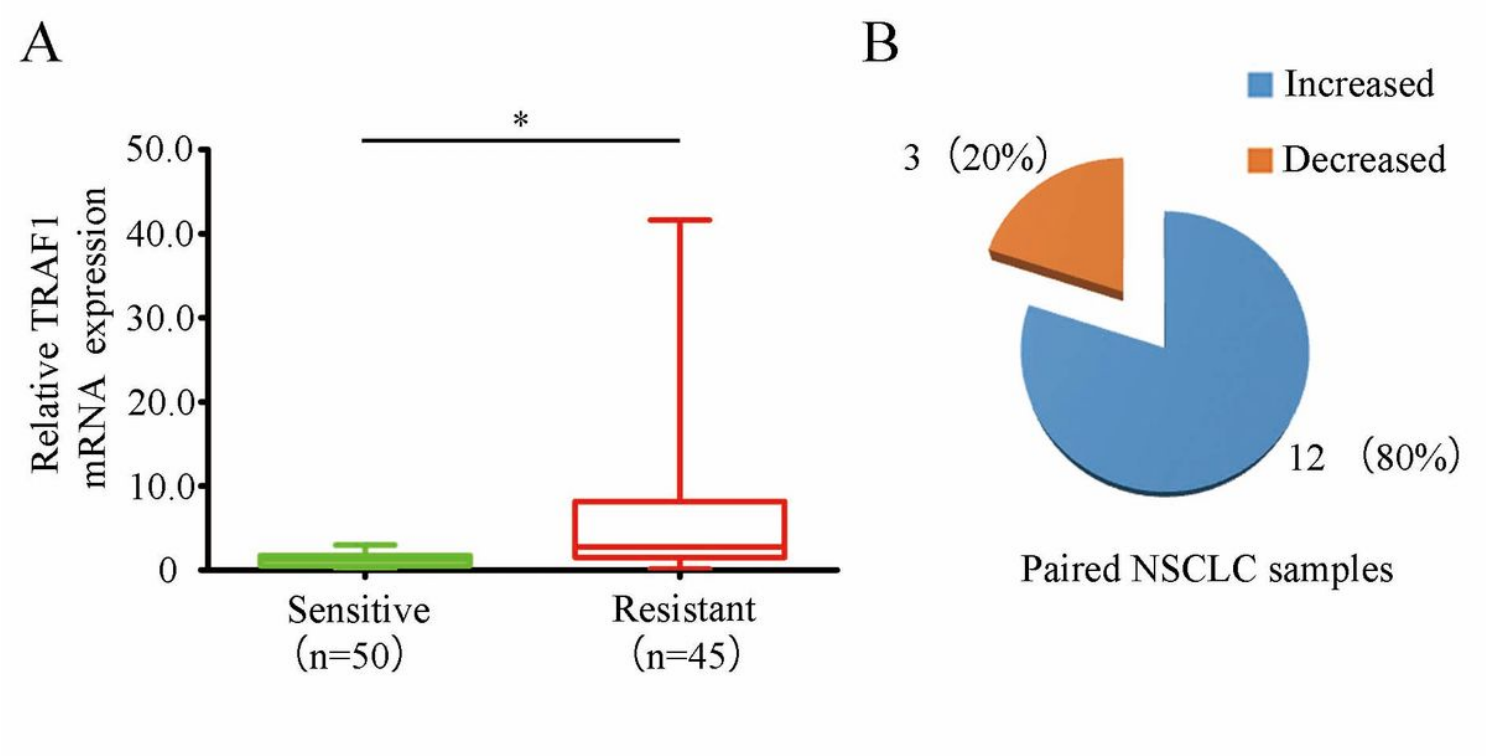

Figure 1

TRAF1 is up-regulated in EGFR tyrosine kinase inhibitors resistance tissues compared with sensitive tissues. (A) TRAF1 expression was determined using qPCR in pleural effusions from NSCLC patients who showed sensitive to the EGFR TKI (n=50) or resistant to EGFR TKI (n=45). (B) Statistical analysis of TRAF1 mRNA expression changed in paired NSCLC samples before gefitinib treatment and after acquiring resistance to gefitinib. * indicates a significant difference at $p < 0.05$ versus the negative control.

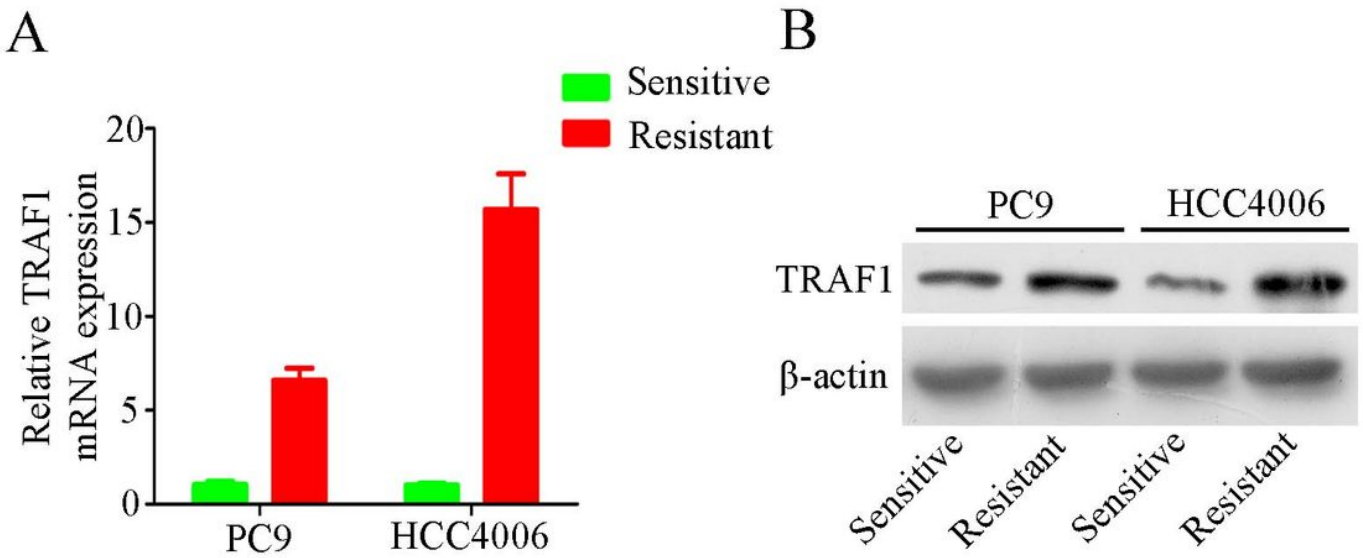

Figure 2

TRAF1 is up-regulated in gefitinib-resistant cells. (A) TRAF1 expression analysis in PC9R and HCC4006R cells by qPCR. (B) TRAF1 expression analysis in PC9R and HCC4006R cells by WB. *P < 0.05 vs. negative control.

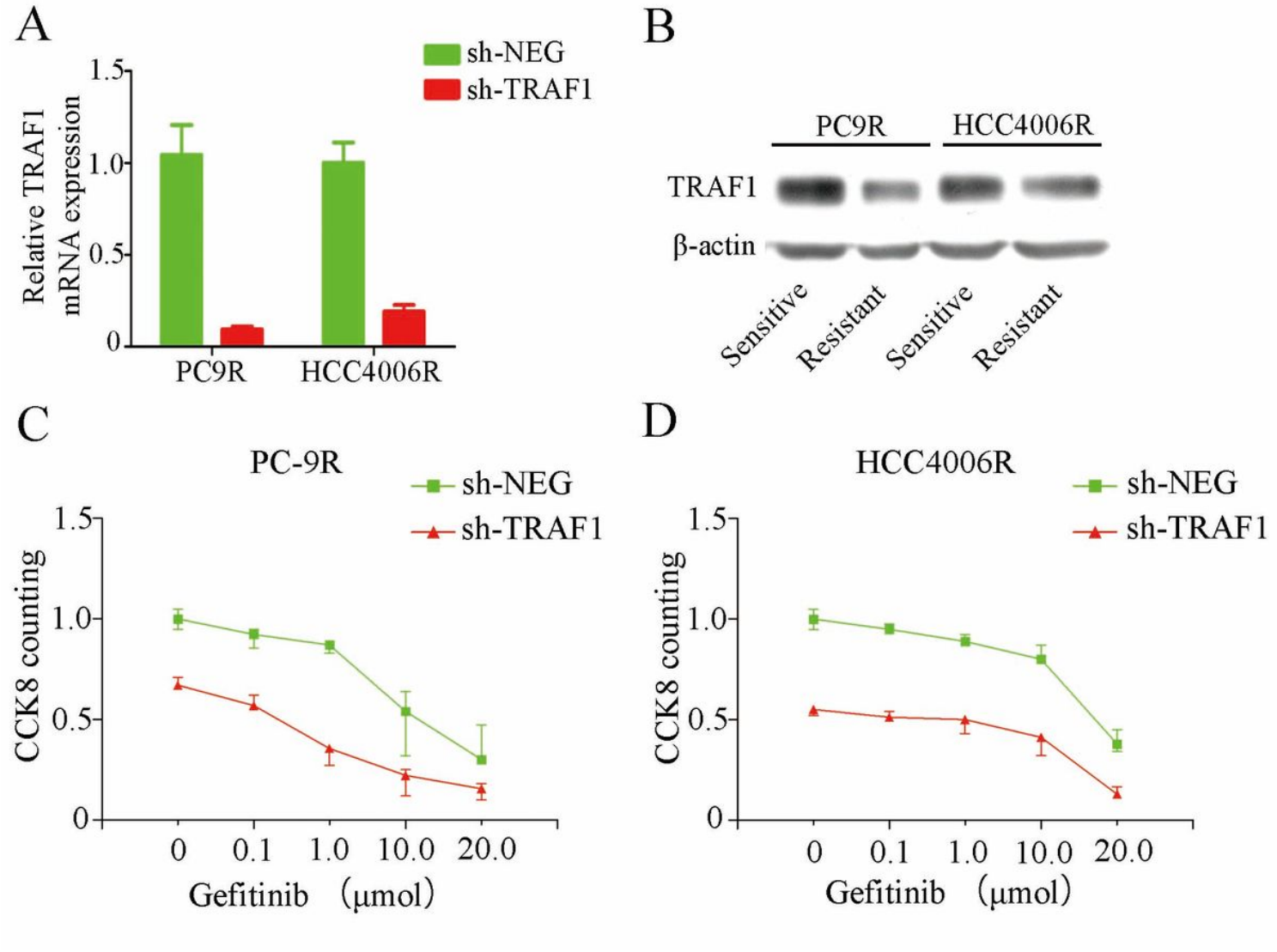

Figure 3

TRAF1 significantly induces gefitinib resistance in vitro. (A) The TRAF1 knockdown efficiency were checked by qPCR in PC9R and HCC4006R cells expressing shTRAF1 or negative control shRNA (shNEG). (B) WB analysis of TRAF1 expression in PC9R and HCC4006R cells expressing shTRAF1 or negative control shRNA (shNEG). (C, D) With different concentration of gefitinib, The PC9R and HCC4006R cells transfected with shTRAF1 or shNEG were checked by CCK8 assays.*P < 0.05 vs. negative control.

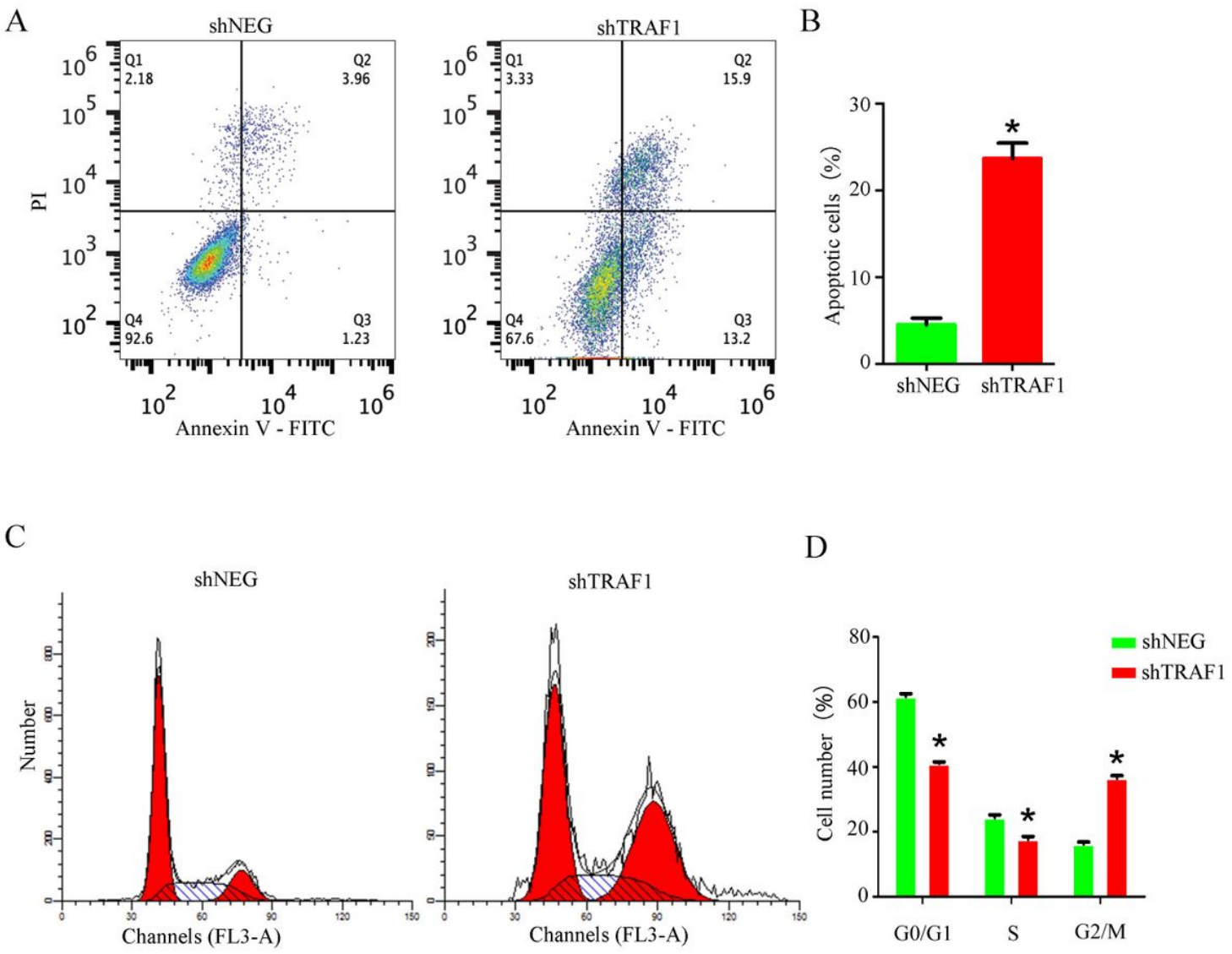

Figure 4

Knockdown TRAF1 induces gefitinib-resistant cells apoptosis and cell cycle arrest. (A, B) Transfected the PC9R cells with shTRAF1 or control and incubated cancer cells with 1 μM gefitinib. The flow cytometry was used to analyze cancer cells. Knockdown TRAF1 induces gefitinib-resistant cells apoptosis. (C, D) Transfected the PC9R cells with shTRAF1 or control and incubated cancer cells with 1 μM gefitinib. Flow cytometry was used to detect cancer cell cycle distribution.