

# Sensitivity to chemotherapeutics of NSCLC cells with acquired resistance to EGFR-TKIs is mediated by T790M mutation or epithelial-mesenchymal transition

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**Abstract.** Chemotherapy is one of the methods to treat patients with non-small cell lung cancer (NSCLC) developing resistance to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs), such as gefitinib. Previous studies revealed that the sensitivity to chemotherapy may depend on different cellular mechanisms of acquired EGFR-TKIs resistance. Thus, the present study aimed to investigate the sensitivity of distinct gefitinib-resistant NSCLC cell lines to chemotherapy in order to help select effective treatment regimens for patients with EGFR-TKI resistance. In the present study, we established two gefitinib-resistant cell lines (PC-9/ZD and PC-9/GR) with the human lung adenocarcinoma cell line PC-9 (carrying the delE746-A750 mutation in the EGFR gene). PC-9/ZD cell line expressed the T790M mutation, while PC-9/GR presented the phenotypes of epithelial to mesenchymal transition (EMT). PC-9/ZD cells were more sensitive to paclitaxel and docetaxel than PC-9 cells and knockdown of T790M decreased this sensitivity. In addition, PC-9/GR cells were less sensitive to chemotherapeutic drugs tested, including cisplatin, gemcitabine, pemetrexed, paclitaxel and docetaxel, compared to PC-9 and PC-9/ZD cells. CDH1 transfection reversed the EMT and restored the sensitivity to chemotherapy in PC-9/GR cells. Furthermore, PC-9 cells became resistant to chemotherapy after TGF- $\beta$ 1-induced EMT. The EMT in NSCLC cells significantly increased cancer stem cell (CSC) properties and tumorigenicity. Collectively, the present study revealed that

gefitinib-resistant NSCLC cells carrying the T790M mutation were sensitive to taxane chemotherapy, indicating that T790M is a useful biomarker for the selection of chemotherapy. EMT in NSCLC cells confers resistance to chemotherapy, which may be associated with enhanced CSC properties.

## Introduction

The majority (80-85%) of lung cancers are non-small cell lung cancer (NSCLC), which is the leading cause of cancer-related death (1). Epidermal growth factor receptor (EGFR) with primary gain-of-function mutations is a major molecular driver of NSCLC. EGFR-tyrosine kinase inhibitors (EGFR-TKIs), such as gefitinib and erlotinib, have been effective standard therapies for NSCLC with EGFR mutations. However, almost all patients with NSCLC develop drug resistance to EGFR-TKIs (1,2).

Multiple mechanisms underlying the resistance to EGFR-TKIs in NSCLC have been identified. These mechanisms include the T790M mutation (a threonine to methionine mutation in amino acid 790), c-MET amplification, activation of alternative signaling pathways and epithelial to mesenchymal transition (EMT) (1). EMT refers to epithelial cell transformation, loss of epithelial characteristics and morphological transformation to a stromal cell phenotype. Biochemically, EMT is characterized by the reduction of E-cadherin and the induction of vimentin (2). EMT is also an important mechanism in the acquired resistance (AR) to EGFR-TKIs. EMT is detected in ~5% of patients with secondary resistance to EGFR-TKIs (3).

Much effort has been directed toward overcoming the AR to EGFR-TKIs by targeting specific mechanisms of resistance. AZD9291 has been used to treat patients with AR to EGFR-TKIs with the T790M mutation. However, 50% of patients with AR to EGFR-TKIs without the T790M mutation need subsequent chemotherapy and predictive clinical factors to determine sensitivity to chemotherapy for these patients are still lacking (1,4).

Previous studies have revealed that chemotherapy efficacy may depend on the underlying mechanisms of AR to EGFR-TKIs. Rosell *et al* (5) observed that in patients with secondary T790M, tumors were more sensitive to

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chemotherapeutic drugs, thus prolonging progression-free survival (PFS) and overall survival (OS). In contrast, NSCLC cells with EMT and patients with EMT-positive tumors were less sensitive to chemotherapy or radiotherapy (6). Furthermore, Kuo *et al* (4), revealed that in patients with AR to EGFR-TKIs, PFS and OS were improved by taxanes, although the underlying mechanisms of EGFR-TKI resistance are unknown (4).

PC-9 is a NSCLC cell line sensitive to gefitinib. PC-9 cells have an EGFR-activating mutation, which is a 15-bp deletion in the EGFR exon 19 (7). In the present study, we used two PC-9 sublines, PC-9/ZD and PC-9/GR, which achieve AR through a secondary T790M mutation and development of EMT, respectively. We studied the correlation between sensitivity of NSCLC cells to chemotherapeutic drugs and different mechanisms of EGFR-TKI resistance. We revealed that the T790M mutation increased NSCLC sensitivity to chemotherapy, whereas EMT resulted in the opposite effect. Furthermore, we observed that EMT led to chemotherapeutic resistance by enhancing cancer stem cell (CSC) properties.

## Materials and methods

### *Cell culture and NSCLC sublines with AR to EGFR-TKIs.*

The human lung adenocarcinoma cell line PC-9 (carrying the delE746-A750 mutation in the EGFR gene) was purchased from The Shanghai Cell Collections of the Chinese Academy of Sciences (Shanghai, China). The gefitinib-resistant subline PC-9/ZD was donated by Dr Koizumi at the National Cancer Center Hospital (Tokyo, Japan). The gefitinib-resistant PC-9/GR subline was generated as previously described (7). The cancer cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (both from Sigma-Aldrich, St. Louis, MO, USA) at 37°C and 5% CO<sub>2</sub>.

### *Analysis of the EGFR gene mutations and c-MET amplification.*

DNA was prepared from cancer cells using the High Pure PCR Template Preparation kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's instructions. We used the amplification refractory mutation system (ARMS) kit (Zhensheng Biomed, Xiamen, China) to detect point mutations in the EGFR gene in exons 18, 19, 20 and 21. Amplification of c-MET was determined by fluorescence *in situ* hybridization (FISH) as previously described (7).

### *Cell Counting Kit-8 (CCK-8) cell viability assay.*

Cells in logarithmic growth phase were inoculated into 96-well plates at a density of  $1.5 \times 10^3$  cells/well. After attachment, the cells were treated with different concentrations of various drugs. The drugs tested included gefitinib ( $10^{-2}$ – $10^2$   $\mu$ mol/l), paclitaxel ( $10^{-4}$ – $10^4$  nmol/l), docetaxel ( $10^{-4}$ – $10^4$  nmol/l), pemetrexed ( $10^{-4}$ – $10^4$  nmol/l), cisplatin ( $10^{-2}$ – $10^2$   $\mu$ mol/l) and gemcitabine ( $10^{-4}$ – $10^4$  nmol/l) (Sigma-Aldrich). After 72 h, cell viability in each well was determined using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions.

### *In vitro cell migration and invasion assays*

**Scratch wound assay.** Cells at a logarithmic growth phase were inoculated in 12-well plates at  $5.0 \times 10^4$  cells/well. After reaching 80% confluency, the cells were starved overnight in

serum-free medium. Pipette tips (10  $\mu$ l) were used to generate three scratch lines in parallel in the cell monolayer. Images were captured at 0, 12, 24 and 48 h post wound under a phase-contrast microscope (Olympus, Tokyo, Japan). Each experiment was repeated at least three times.

**Invasion assays.** QCM™ 24-well Collagen-based Cell Invasion assay Transwell plates (EMD Millipore, Darmstadt, Germany) were used for invasion studies. Cells ( $5 \times 10^5$ ) were suspended in 200  $\mu$ l of serum-free medium and added to the upper chamber. RPMI-1640 medium (500  $\mu$ l) containing 10% newborn bovine serum was added to the lower chamber as a chemokine attractant. The cells that migrated into the lower chamber were stained with crystal violet for 30 min. Five fields per chamber were imaged using an Olympus fluorescence inverted microscope (Olympus).

**Western blot analysis.** Equal amounts of total protein were resolved by SDS-PAGE and blotted onto polyvinylidene fluoride (PVDF) membranes. The membranes were probed with primary antibodies against vimentin (1:1,500; no. SAB1305445), E-cadherin (1:1,000; no. SAB5500022), EGFR (1:1,000; no. SAB4300352), phosphorylated (p)EGFR (1:1,000; no. SAB4503760), AKT (1:1,000; no. SAB4500802), pAKT (1:1,000; no. SAB4504015), extracellular regulated kinase (ERK; 1:1,000; no. M5670), pERK (1:1,000; no. SAB4301578) and  $\beta$ -actin (1:400; no. SAB5500001) (all antibodies were rabbit anti-human monoclonal and were purchased from Sigma-Aldrich). After incubation with primary antibodies for 24 h, the blots were incubated with HRP-conjugated secondary antibody (1:2,000; no. A9169, goat anti-rabbit polyclonal antibody; Sigma-Aldrich). Protein band intensity was analyzed using Quantity One image analysis software (Bio-Rad Laboratories, Hercules, CA, USA).

### *Stable expression of E-cadherin and EGFR T790M-specific siRNA by lentiviral transfection.*

Stable overexpression of cadherin 1 (CDH1) and T790M-specific siRNA was performed as previously described (7). Four T790M specific siRNA sequences (2608, 2597, 2603 and 2600) described by Chen *et al* (8) were used to identify the best sequence to interfere with the expression of T790M, but minimally affect the expression of wild-type (WT) EGFR for further studies. The negative shRNA sequence was LV3-NC: 5'TTCTCCGAA CGTGTCACGTTTC3'.

Real-time PCR was employed to determine the expression level of EGFR mRNA using the following oligonucleotides: EGFR forward and EGFR reverse for the WT allele (9) and T790M forward, 5'TCCTCGATGAAGCCTACGTGAT3' and reverse, 5'CAAGCGACGGTCCTCCAAGTAG3' (10).

**CSC analysis.** The expression of CD133 and CD44, putative biomarkers for the NSCLC CSCs was determined by flow cytometry. Cells ( $10^6$ ) collected in a 1.5 ml EP tube were incubated with 100  $\mu$ l diluted fluorescent-conjugated antibodies against CD133 and CD44 (both from BD Biosciences, Franklin Lakes, NJ, USA), or in a control group with 100  $\mu$ l phosphate buffered solutions with albumin (PBA), on ice for 1 h in the dark. The cells were then washed three times with (PBS). Following centrifugation (600 x g, 4°C, 10 min), the precipitated

Table I. IC<sub>50</sub> values of various chemotherapeutic agents in NSCLCs with AR to EGFR-TKIs.

IC <sub>50</sub>	PC-9	PC-9/GR	PC-9/ZD
Cisplatin ( $\mu\text{mol/l}$ )	3.877 $\pm$ 1.346	20.283 $\pm$ 4.316 <sup>a</sup>	2.956 $\pm$ 1.685 <sup>b</sup>
Gemcitabine ( $\mu\text{mol/l}$ )	0.145 $\pm$ 0.724	2.647 $\pm$ 0.117 <sup>a</sup>	0.130 $\pm$ 0.028 <sup>b</sup>
Pemetrexed ( $\mu\text{mol/l}$ )	30.517 $\pm$ 4.69	399.530 $\pm$ 0.903 <sup>a</sup>	36.100 $\pm$ 4.615 <sup>b</sup>
Paclitaxel (nmol/l)	1.532 $\pm$ 0.777	16.710 $\pm$ 4.023 <sup>a</sup>	0.124 $\pm$ 0.231 <sup>a,b</sup>
Docetaxel (nmol/l)	5.226 $\pm$ 0.648	64.560 $\pm$ 8.454 <sup>a</sup>	0.800 $\pm$ 0.109 <sup>a,b</sup>

<sup>a</sup>Indicates statistically significant difference from the IC<sub>50</sub> values in PC-9 cells (P<0.05); <sup>b</sup>indicates statistically significant difference from the IC<sub>50</sub> values in PC-9/GR cells. IC<sub>50</sub>, inhibitory concentrations at 50%; NSCLC, non-small cell lung cancer; AR, acquired resistance; EGFR-TKIs, epidermal growth factor receptor-tyrosine kinase inhibitors.

cells were re-suspended in 300  $\mu\text{l}$  PBS, followed by flow cytometric analysis (BD, Biosciences, Franklin Lakes, NJ, USA).

**Side population assay.** Cells ( $10^6$ ) were incubated with (test group) or without (control group) 50  $\mu\text{mol/l}$  verapamil. Hoechst 33342 (Sigma-Aldrich) was added to a final concentration of 5  $\mu\text{g/ml}$  followed by addition of pyridine iodide at a final concentration of 2  $\mu\text{g/ml}$  to screen for dead cells. The cells were then subjected to flow cytometric analysis.

**In vitro tumor spheroid formation assay.** Cells at  $5 \times 10^3/\text{well}$  were inoculated in a 24-well culture plate and cultured in serum-free DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA) with (experimental group) or without (control group) 10 ng/ml basic fibroblast growth factor (bFGF) and 20 ng/ml epidermal growth factor (EGF) (both from Gibco, Grand Island, NY, USA). The medium was changed every three days. The culture was terminated after two weeks and tumor spheroids exceeding 75  $\mu\text{m}$  were counted.

**In vivo tumor formation assay.** Cells were sorted into four groups: i) CD133<sup>+</sup>, ii) CD133<sup>-</sup>, iii) CD44<sup>+</sup> and iv) CD44<sup>-</sup>. For each group of four female nude (NOD/SCID) mice,  $10^3$ ,  $10^4$ ,  $10^5$  and  $10^6$  cells were injected subcutaneously in the front flanks of the mice. Tumors exceeding 50 mm<sup>3</sup> were counted to determine the minimum number of inoculated cells required for tumor formation *in vivo*. The mice (~100) were purchased from Vital River Laboratories (Beijing, China). All animal handling and procedures were approved by the Animal Care and Use Committee of the General Hospital of Guangzhou Military Command of PLA.

**Statistical analysis.** Data are expressed as the mean  $\pm$  standard deviation. SPSS 20.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. The t-test was used to compare data between two groups. Single factor analysis of variance (ANOVA) was used to compare data among groups. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Characterization of NSCLC sublines with AR to gefitinib.** The sensitivity of PC-9/ZD and PC-9/GR cells to gefitinib was significantly lower compared to PC-9 cells (P<0.05), while the

sensitivity of PC-9/ZD and PC-9/GR to gefitinib was similar (P>0.05). The inhibitory concentrations at 50% (IC<sub>50</sub>) of growth in PC-9/ZD, PC-9/GR and PC-9 cells were 7.85 $\pm$ 0.21, 10.62 $\pm$ 0.19 and 0.05 $\pm$ 0.005  $\mu\text{mol/l}$ , respectively. PC-9/ZD cells carried the T790M mutation in addition to the delE746-A750 in exon 19. T790M was not detected in the PC-9/GR cells. No c-MET amplification was detected in any of the cell lines as determined by FISH assays (data not shown).

**Sensitivity of resistant sublines to various chemotherapeutic drugs.** Cytotoxicity assays revealed that PC-9/ZD cells had higher sensitivity to paclitaxel and docetaxel compared to PC-9 cells (P<0.05; Table I). PC-9/ZD and PC-9 cells were similar in sensitivity to cisplatin, gemcitabine and pemetrexed (P>0.05; Table I). PC-9/GR cells were less sensitive to all chemotherapeutic drugs tested, including cisplatin, gemcitabine, pemetrexed, paclitaxel and docetaxel, compared to PC-9 and PC-9/ZD cells (P<0.05; Table I).

**T790M increases sensitivity of PC-9 cells to chemotherapy.** A total of four siRNAs targeting T790M (2597, 2600, 2603 and 2608) and one control (scrambled sequence) were examined. We observed that siRNA-2603 minimally inhibited WT EGFR (Fig. 1A), but it had the greatest inhibitory effect on T790M EGFR (Fig. 1B). Western blot analysis demonstrated that siRNA-2603 had the least inhibitory effect on EGFR (Fig. 1C). Consequently 2603 and the empty vector control were transfected into PC-9/ZD cells to assess the effect on downstream EGFR signaling pathways. The transfection of 2603 did not affect the expression of AKT, p-AKT, ERK and p-ERK (P>0.05; Fig. 1D), indicating that 2603 had no effect on the AKT and ERK pathways. The sensitivity of 2603-treated PC-9/ZD cells to gefitinib was significantly higher compared to the control. The IC<sub>50</sub> value of gefitinib was 0.016  $\mu\text{mol/l}$  vs. 1.91  $\mu\text{mol/l}$  (P<0.05, data not shown).

Treatment with 2603 markedly decreased the IC<sub>50</sub> values in PC-9/ZD cells from 15.762 $\pm$ 1.673 to 0.124 $\pm$ 0.231  $\mu\text{mol/l}$  (P<0.05) for paclitaxel and from 21.110 $\pm$ 2.165 to 0.800 $\pm$ 0.109 nmol/l for docetaxel (P<0.05), indicating that knockdown of T790M EGFR significantly reduced sensitivity to taxanes (data not shown).

**PC-9/GR cells display EMT properties.** Western blot analysis revealed that E-cadherin was decreased and vimentin and

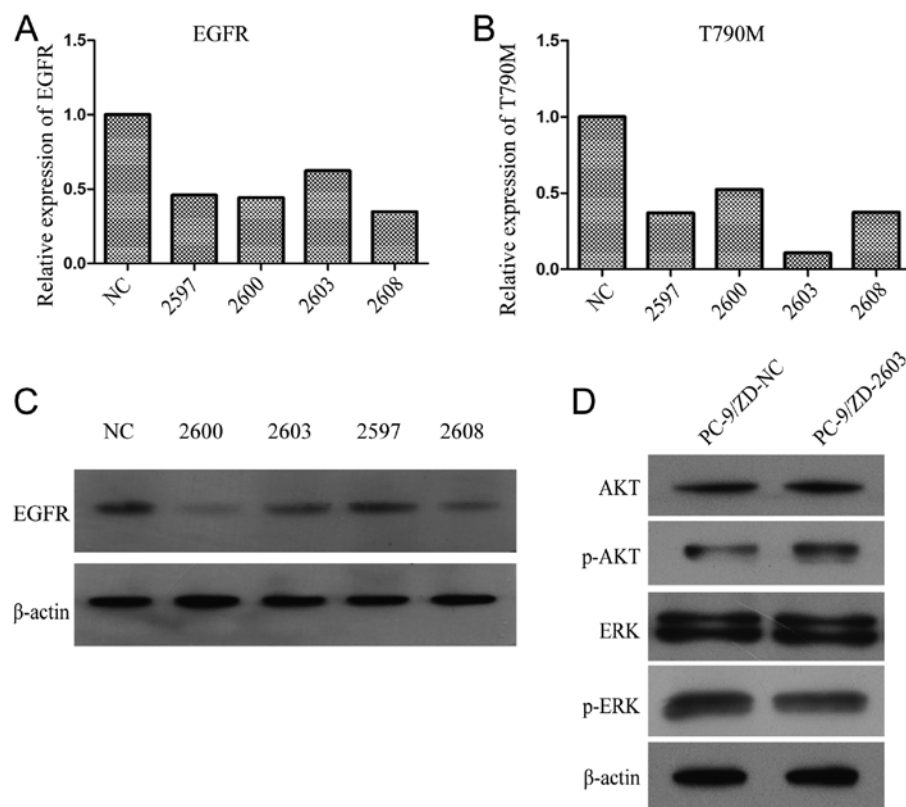


Figure 1. siRNA-mediated knockdown of T790M. Relative expression of (A) EGFR and (B) T790M in PC-9 cells, as determined by real-time PCR after transfection of siRNA (2608, 2597, 2603, 2600 and NC) expressing lentiviral vectors. (C) Western blot analysis of total EGFR and  $\beta$ -actin protein in PC-9 cells after transfection of siRNA as indicated. (D) Western blot analysis of ERK, p-ERK, AKT, p-AKT and  $\beta$ -actin in PC-9/ZD cells after transfection with siRNA NC and 2603 expressing lentiviral vectors.

Table II.  $IC_{50}$  values of various chemotherapeutic drugs in PC-9 cells before and after treatment with TGF- $\beta$ 1.

$IC_{50}$	PC-9	PC-9/TGF- $\beta$ 1
Cisplatin ( $\mu$ mol/l)	3.877 $\pm$ 1.346	15.617 $\pm$ 0.811 <sup>a</sup>
Gemcitabine ( $\mu$ mol/l)	0.145 $\pm$ 0.724	1.667 $\pm$ 0.101 <sup>a</sup>
Pemetrexed ( $\mu$ mol/l)	30.517 $\pm$ 4.69	246.197 $\pm$ 4.883 <sup>a</sup>
Paclitaxel (nmol/l)	1.532 $\pm$ 0.777	10.377 $\pm$ 0.891 <sup>a</sup>
Docetaxel (nmol/l)	5.226 $\pm$ 0.648	36.110 $\pm$ 3.360 <sup>a</sup>

<sup>a</sup>indicates statistically significant difference from the  $IC_{50}$  values in PC-9 cells ( $P < 0.05$ ).

Table III.  $IC_{50}$  values of chemotherapeutic agents in PC-9/GR cells after the overexpression of E-cadherin by CDH1 transfection.

$IC_{50}$	PC-9/GR-CDH1	PC-9/GR-control
Cisplatin ( $\mu$ mol/l)	3.987 $\pm$ 0.818 <sup>a</sup>	17.28 $\pm$ 0.445
Gemcitabine ( $\mu$ mol/l)	0.267 $\pm$ 0.098 <sup>a</sup>	3.546 $\pm$ 0.120
Pemetrexed ( $\mu$ mol/l)	35.497 $\pm$ 2.075 <sup>a</sup>	348.856 $\pm$ 1.875
Paclitaxel (nmol/l)	2.484 $\pm$ 0.908 <sup>a</sup>	19.429 $\pm$ 0.937
Docetaxol (nmol/l)	7.838 $\pm$ 2.885 <sup>a</sup>	73.676 $\pm$ 2.840

<sup>a</sup>indicates statistically significant difference from the  $IC_{50}$  value in PC-9/GR control cells ( $P < 0.05$ ).

N-cadherin were increased in PC-9/GR cells, but not in PC-9/ZD cells, indicating that PC-9/GR cells but not PC-9/ZD cells acquired the EMT phenotype ( $P < 0.05$ , Fig. 2A). In addition, PC-9/GR cells exhibited mesenchymal morphology, with cells being long, polarized and sparsely connected (Fig. 2B). Furthermore, PC-9/GR cells undergoing EMT exhibited higher migration and invasion abilities than PC-9 cells without EMT ( $P < 0.05$ ), whereas no significant difference of the migration and invasion abilities was observed between PC-9/ZD and PC-9 cells ( $P > 0.05$ ; Fig. 2C-F).

*EMT decreases sensitivity to chemotherapy in AR-cell lines.* Application of exogenous TGF- $\beta$ 1 (10 ng/ml) to parental PC-9

cells successfully induced EMT after 72 h and decreased the sensitivity to gefitinib. The sensitivity of PC-9 cells treated with exogenous TGF- $\beta$ 1 (10 ng/ml) to different chemotherapeutic agents was assessed 72 h after treatment. TGF- $\beta$ 1 significantly inhibited the chemotherapeutic effect of gemcitabine, paclitaxel, docetaxel and cisplatin, as evidenced by decreased  $IC_{50}$  values ( $P < 0.05$ ; Table II).

PC-9/GR cells that overexpressed E-cadherin following CDH1 transfection had significantly increased sensitivity to gemcitabine, paclitaxel, pemetrexed, docetaxel and cisplatin, as evidenced by the increased  $IC_{50}$  values ( $P < 0.05$ , Table III).

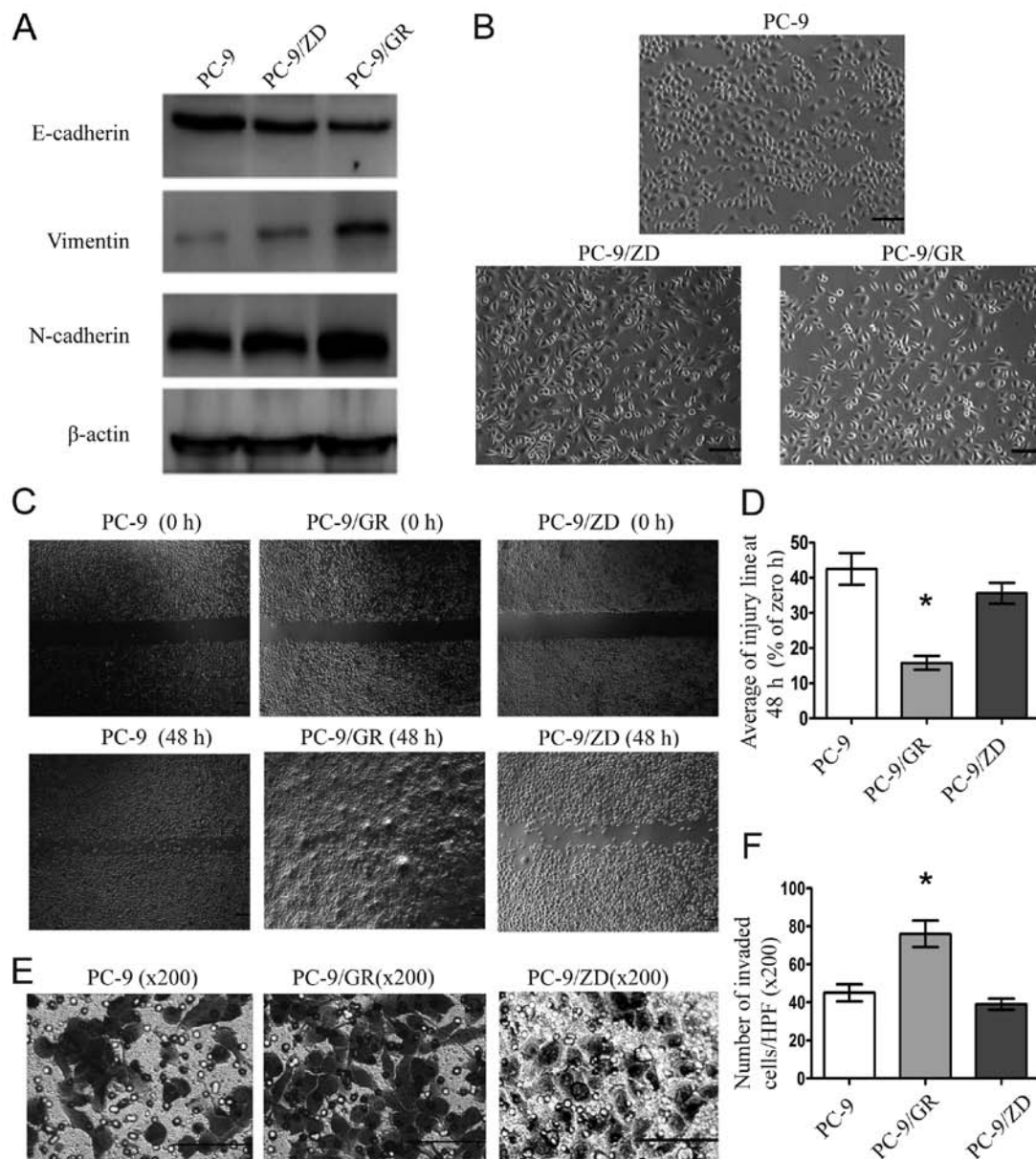


Figure 2. PC-9/GR cells display EMT. (A) Western blot analysis revealed that E-cadherin decreased and vimentin and N-cadherin increased in PC-9/GR cells, but not in PC-9/ZD cells, compared to PC-9 cells. (B) Representative fields of PC-9, PC-9/ZD and PC-9/GR cells under phase-contrast microscopy. (C) Representative images of scratch assays of PC-9, PC-9/GR and PC-9/ZD cells at 0 and 48 h post-scratch wound. (D) Quantitative results of scratch assay displaying average injury in PC-9, PC-9/ZD and PC-9/GR cells at 48 h post-scratch wound. (E) Representative fields of invasion assays in PC-9, PC-9/ZD and PC-9/GR cells under phase-contrast microscopy. Cells were stained with crystal violet. (F) Number of invaded PC-9, PC-9/ZD and PC-9/GR cells. \* $P < 0.05$  vs. PC-9 groups ( $n = 3$ ). Scale bar, 200  $\mu\text{m}$ .

*EMT is associated with increased CSCs in NSCLC sublines.* Previous studies have used CD44 and CD133 as markers for CSCs in NSCLC cells (11-13). Thus, we determined the expression of CD44 and CD133 in PC-9 cell lines by flow cytometry. The percentage of CD133<sup>+</sup> cells in the PC-9 cell line was 0.2% (Fig. 3A) and the percentage of CD44<sup>+</sup> cells was 94.1% (Fig. 3B). To further confirm whether CD44 and CD133 can be used as CSC surface markers in PC-9 derived cells, we first separated PC-9/GR cells into CD133<sup>+</sup>, CD133<sup>-</sup>, CD44<sup>+</sup> and CD44<sup>-</sup> cell groups using MACS and assessed their tumor formation activity both *in vitro* and *in vivo*. The number of spheroids formed in the *in vitro* assays was significantly higher in CD133<sup>+</sup> cells ( $452 \pm 10.60$ ) compared to CD133<sup>-</sup> cells ( $4.67 \pm 0.88$ ) ( $P < 0.05$ , Fig. 3C). *In vivo*, tumorigenicity in

CD133<sup>+</sup> cells was also significantly higher compared to CD133<sup>-</sup> cells (Fig. 3D). The CD133<sup>+</sup> cells required only  $10^3$  cells to form tumors, while the CD133<sup>-</sup> cells did not form tumors even when injected with  $10^6$  cells (Fig. 3D). These results indicated that CD133<sup>+</sup> cells had a strong self-renewal and differentiation ability. Thus, CD133<sup>+</sup> can be used as a CSC marker in PC-9-derived cells. However, there was no significant difference between CD44<sup>+</sup> and CD44<sup>-</sup> cells in tumor formation capability both *in vitro* and *in vivo* ( $P > 0.05$ , Fig. 3E and F).

PC-9/GR cells expressed a much higher percentage of CD133<sup>+</sup> compared to PC-9 and PC-9/ZD cells ( $P < 0.05$ , Fig. 4A). The percentage of CD133<sup>+</sup> cells was  $0.20 \pm 0.02\%$  in PC-9,  $0.50 \pm 0.04\%$  in PC-9/ZD and  $3.00 \pm 0.22\%$  in PC-9/GR cells. Side population assays revealed that the number of cells in

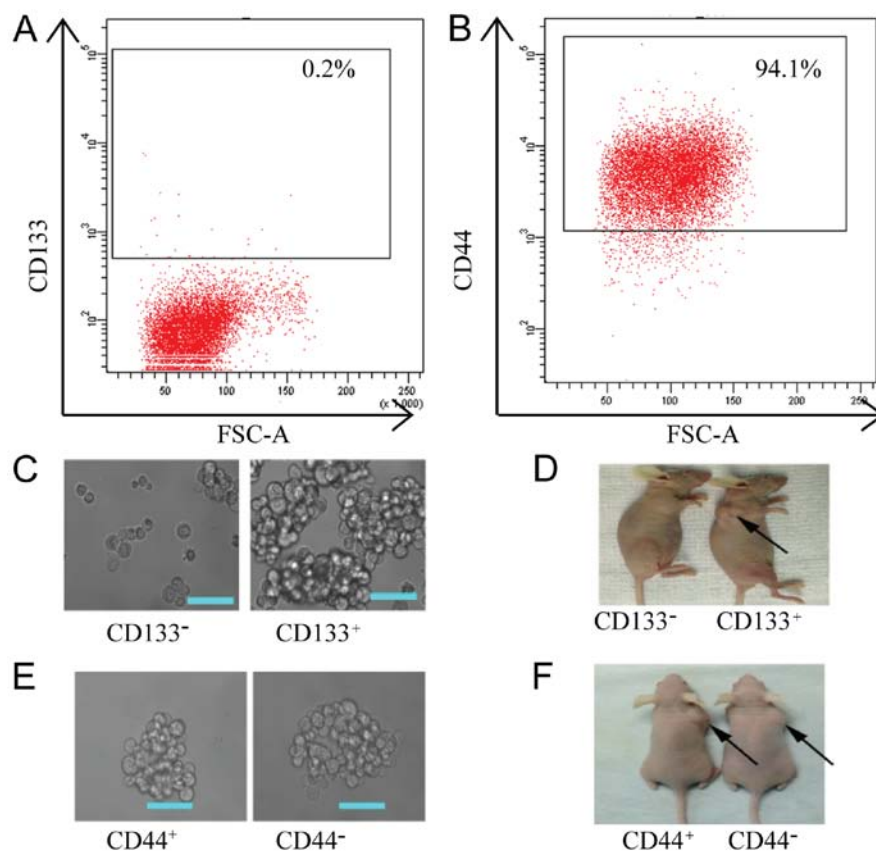


Figure 3. CD133<sup>+</sup> PC-9 cells displayed CSC properties. Flow cytometric analysis of PC-9 cells stained with (A) CD133 and (B) CD44. The percentages of CD133<sup>+</sup> and CD44<sup>+</sup> cells are displayed. (C) Spheroid formation assay revealed that CD133<sup>+</sup> PC-9 cells had a higher ability to form spheroids compared to CD133<sup>-</sup>. (D) *In vivo* tumor formation assays demonstrated that CD133<sup>+</sup> PC-9 cells had a higher ability to form tumor compared to CD133<sup>-</sup> in nude (NOD/SCID) mice. (E) Spheroid formation assay revealed that CD44<sup>+</sup> and CD4<sup>-</sup> PC-9 cells had a similar ability to form spheroids. (F) *In vivo* tumor formation assays revealed that CD44<sup>+</sup> and CD44<sup>-</sup> PC-9 cells had a similar ability to form tumors in nude mice.

the PC-9/GR cell line was increased ( $1.50 \pm 0.24\%$ ) compared to the PC-9 and PC-9/ZD cell lines ( $P < 0.05$ , Fig. 4B). Furthermore, the side-population cells were essentially eliminated by verapamil. The tumor-forming ability of PC-9/GR cells was significantly higher compared to PC-9 and PC-9/ZD cells ( $P < 0.05$ , Fig. 4C).

These results indicated that PC-9/GR cells with EMT displayed more prominent stem cell characteristics. To further demonstrate the effect of EMT on CSCs, we repeated the assays by overexpressing CDH1 in the PC-9/GR cells to reverse EMT, as well as by treating PC-9 cells with TGF- $\beta$ 1 to induce EMT. The percentage of CD133<sup>+</sup> and side population cells significantly decreased when PC-9/GR cells were transfected with CDH1 ( $P < 0.05$ , Fig. 5A and B). Furthermore, the number of both CD133<sup>+</sup> and side population cells increased in PC-9 cells in response to TGF- $\beta$ 1 (Fig. 5A and B).

**TGF- $\beta$ 1 induces EMT of NSCLC cells.** To demonstrate the EMT of the NSCLC cells, PC-9 cells were treated with a potent EMT inducer TGF- $\beta$ 1 at different concentrations ranging from 0.1 to 100 ng/ml. As displayed in Fig. 6, vehicle-treated PC-9 cells maintained a flattened shape which is a prominent feature of the epithelial phenotype. In contrast, TGF- $\beta$ 1-treated cells exhibited a spindle-shaped morphology and 10 ng/ml of TGF- $\beta$ 1 induced the most pronounced mesenchymal phenotype while having no effect on cell viability.

These results indicated that TGF- $\beta$ 1 induces EMT in a dose-dependent manner.

## Discussion

In the present study, we investigated the association between sensitivity of gefitinib-resistant NSCLC cell lines to chemotherapeutic drugs and the underlying molecular mechanisms of resistance to gefitinib, using two distinct PC-9 derived gefitinib-resistant sublines, PC-9/ZD and PC-9/GR. We confirmed that increased sensitivity of the PC-9/ZD cell line to taxanes was associated with the T790M mutation and decreased sensitivity of the PC-9/GR cell line to various chemotherapeutics was associated with EMT. Furthermore, we revealed that the promotion of CSC properties may be one of the mechanisms for the increased resistance to chemotherapeutics in the EMT phenotype in NSCLC cells.

T790M accounts for ~50% of AR cases in patients with NSCLC. After prolonged exposure to EGFR-TKIs, the T790M mutant allele becomes enriched, resulting in AR to EGFR-TKIs (1). Rosell *et al* (5) reported that among AR NSCLC patients, T790M-positive patients had better outcomes (longer PFS and OS) than T790M-negative patients when treated with chemotherapy, indicating that tumors with AR due to the T790M mutation are more sensitive to chemotherapy. Kuo *et al* (4) observed that patients with AR to EGFR-TKIs



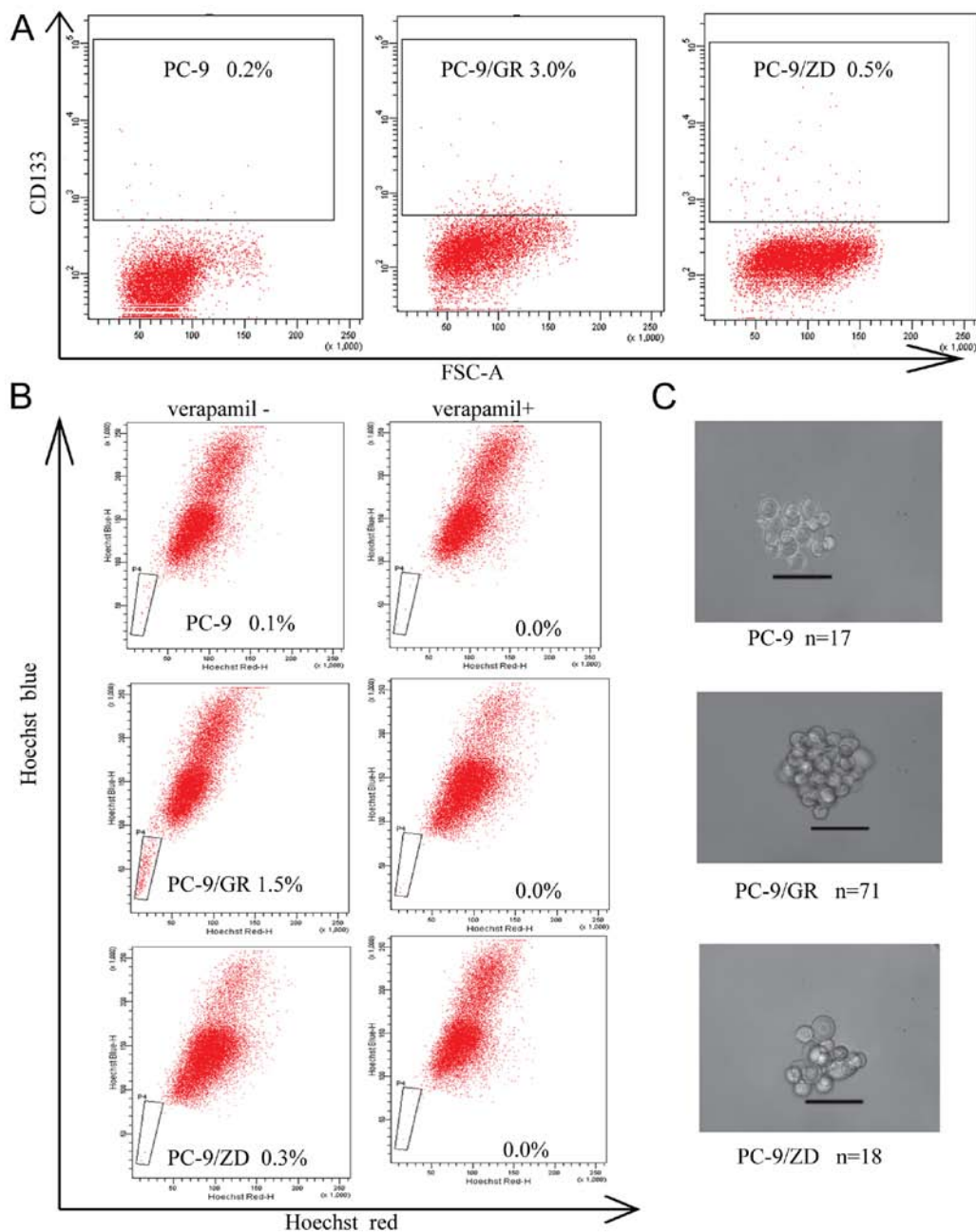


Figure 4. Flow cytometric analysis of putative CSC marker (CD133 and CD44) expression and side-population in PC-9, PC-9/ZD and PC-9/GR cells. (A) Flow cytometry indicated that PC-9/GR cells expressed a higher percentage of CD133<sup>+</sup> cells compared to PC-9 and PC-9/ZD cells. (B) Side population analysis by flow cytometry revealed that the percentage of side-population in PC-9/GR cells was higher compared to PC-9 and PC-9/ZD cells. The side-population cells were nearly eliminated by verapamil treatment. (C) Representative images of *in vitro* spheroid formation assays in PC-9, PC-9/ZD and PC-9/GR cells, as indicated. The number of spheroids of each cell line is displayed.

achieved better response rates and longer PFS and OS in response to taxane chemotherapy. Although the underlying mechanisms remained unknown in their study, the study indicated that the improved outcomes following taxane chemotherapy were due to higher sensitivity of T790M positive tumors.

In the present study, we first revealed the relationship between the T790M mutation and the sensitivity to chemotherapy *in vitro*. We identified that PC-9/ZD cells with the T790M mutation were resistant to gefitinib and had a higher sensitivity to taxanes (paclitaxel and docetaxel). Our findings were different from the ones of the study by Cheng *et al* (12),

which revealed no significant change in sensitivity to paclitaxel before and after AR to gefitinib. The discrepancy is probably due to differences in the ratio of the T790M allele to the WT allele. Our resistant PC-9/ZD cells were pure and were derived from a single T790M mutant cell, while only 30% of the cells used by Cheng *et al* were T790M positive. To further confirm the correlation between the T790M mutation and sensitivity to chemotherapy, we used siRNA to specifically knock down T790M (8) and found that sensitivity of the PC-9/ZD cells to paclitaxel and docetaxel decreased, indicating that the T790M mutation is a useful marker for taxane chemotherapy sensitivity.

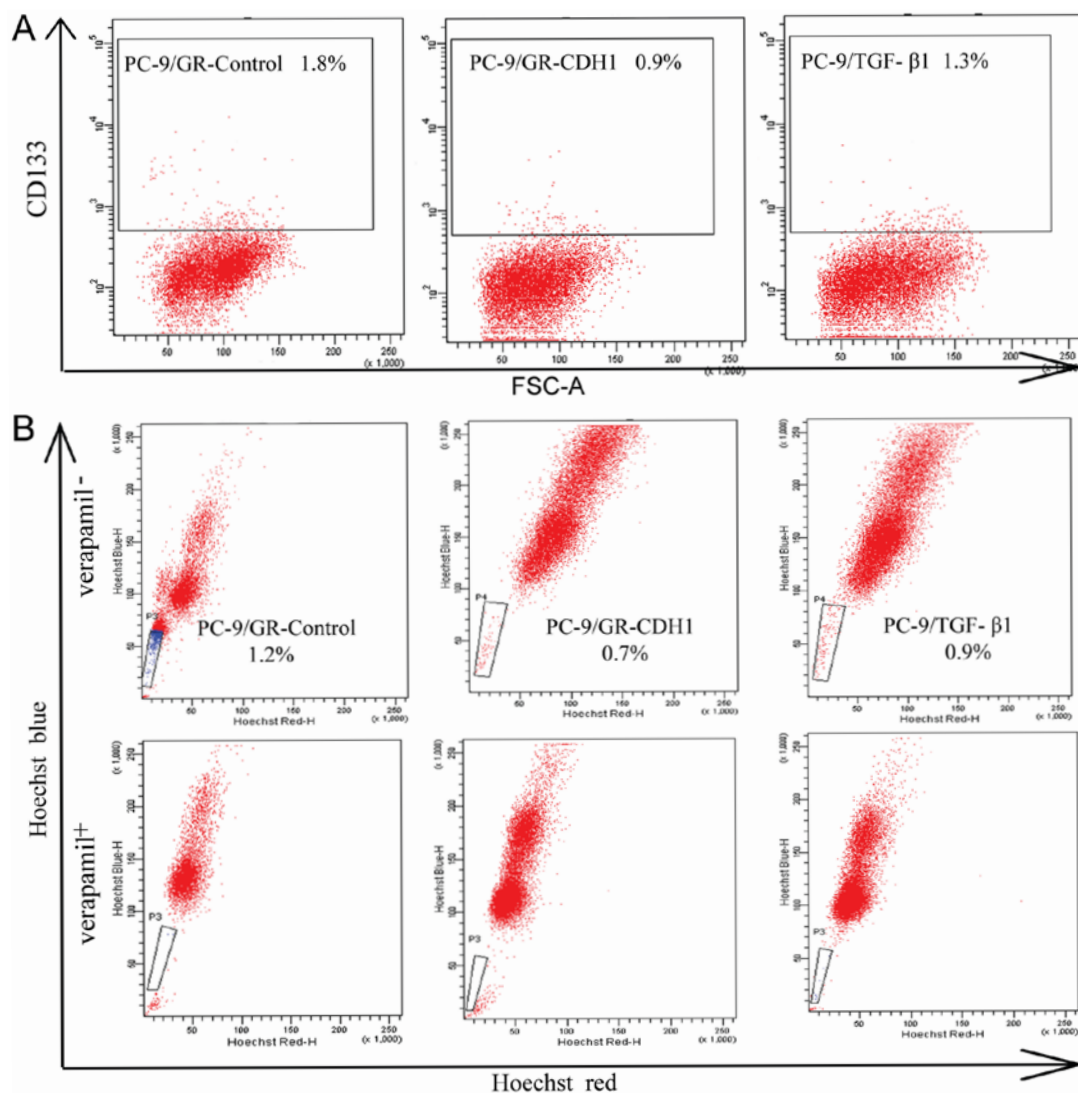


Figure 5. Flow cytometric analysis of the CSC properties of PC-9 cells treated with TGF- $\beta$ 1 and PC-9/GR cells transfected with CDH1. (A) Flow cytometry revealed that the expression of CD133<sup>+</sup> in PC-9/GR cells decreased when CDH1 was overexpressed and the expression of CD133<sup>+</sup> in PC-9 cells increased in response to TGF- $\beta$ 1 after 72 h. (B) Flow cytometry side population assays revealed that the number of side population in PC-9/GR cells decreased when CDH1 was overexpressed. The side population in PC-9 increased in response to TGF- $\beta$ 1 after 72 h.

Numerous previous studies have revealed that EMT contributes to AR to EGFR-TKIs in NSCLC (2,14-16). It has also been reported that NSCLC patients with EMT-positive tumors are resistant to chemotherapy and such patients have poor prognoses (2,6,13). In the present study, we revealed that PC-9/GR cells with EMT were significantly less sensitive to chemotherapeutic agents (gemcitabine, paclitaxel, pemetrexed, docetaxel and cisplatin) compared to parental PC-9 cells. Furthermore, we revealed that TGF- $\beta$ 1-induced EMT in PC-9 cells conferred resistance to chemotherapy. This is consistent with the study by Shintani *et al* (6) who found that the sensitivity of cells to the chemotherapeutic drugs cisplatin and paclitaxel decreased after TGF- $\beta$ 1-induced EMT in NSCLC cells, indicating that EMT plays an important role in tumor cell chemotherapeutic resistance (6). Furthermore, we demonstrated that when EMT in PC-9/GR cells was reversed by the overexpression of CDH1 the cells became less resistant to chemotherapeutic agents. These results further confirmed that EMT in NSCLC cells is directly associated with chemotherapeutic resistance. Previous studies have also

demonstrated that EMT in other types of tumor cells, such as pancreatic cancer (17), colon carcinoma (18) and ovarian cancer (19), lead to chemotherapeutic resistance.

Previous studies have revealed that EMT promotes the formation of cells with the CSC phenotype (20-22). The expression profiles of stem cell-related genes, including Oct4, Wnt, Notch, Hedgehog and Nanog (23,24), are altered in the process of EMT. This alteration is associated with the proliferation of CSCs, leading to increased resistance to chemotherapy (25). The present study revealed that PC-9/GR cells with EMT had an increase in the number of CD133<sup>+</sup> and side population cells. These cells had tumor stem cell characteristics, indicating that the increased resistance to chemotherapeutic drugs in EMT-positive cells may be related to the increase in CSCs. Shien *et al* (20) also revealed that in cells with AR to EGFR-TKIs, CSC characteristics of the sub-groups led to resistance to subsequent chemotherapy. We further confirmed the correlation between EMT and CSCs. We revealed that the number of CSCs was increased in the PC-9 cells in response to TGF- $\beta$ 1-induced EMT. This finding indicated that the production



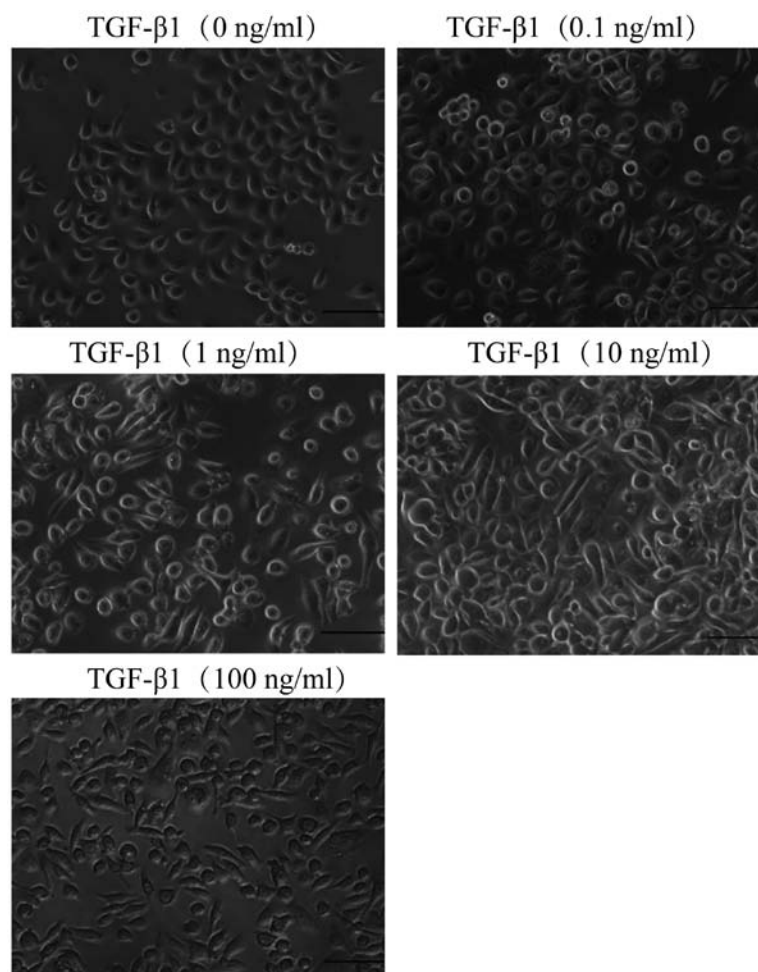


Figure 6. TGF- $\beta$ 1 induces EMT in NSCLC cells. PC-9 cells were treated with a vehicle or TGF- $\beta$ 1 at different concentrations as indicated for 72 h and microphotographs were captured under a light microscope. Scale bar, 200  $\mu$ m.

of CSCs may be one of the contributing factors to the resistance of EMT-positive cells to chemotherapy. However, it is worth noting that only 3.0% of the PC-9/GR cells were CSCs in the present study. Thus, further investigation is necessary to determine if CSCs are a primary factor in chemotherapy resistance.

NSCLC patients with EGFR-activating mutations initially respond very well to EGFR-TKIs, such as gefitinib and erlotinib, when administered as first-line treatments (2). However, all patients eventually develop resistance to EGFR-TKIs (1,2). Thus, it is critical to develop treatments that can overcome EGFR-TKI resistance by targeting the underlying mechanisms responsible for the resistance (26-29). The majority of NSCLC patients develop resistance that is mediated by the T790M mutation. Thus, much effort has been made to develop a third generation of EGFR-TKIs that target both the activating mutations, such as deletion in exon 19, and T790M. Third generation inhibitors include AZD9291, HM61713 and CO-1686. These inhibitors have shown early promise in clinical trials, with objective response rates ranging from 58-66% (2). However, there is no doubt that NSCLC patients will quickly develop resistance, since resistance to CO-1686 has already been demonstrated by an EMT mechanism (2). To target the mechanisms associated with EMT, histone deacetylase (HDAC) inhibitors and MAPK/ERK kinase (MEK) inhibitors are being developed to delay the onset or reverse EMT (2). Such efforts

have only achieved limited success, therefore subsequent chemotherapy remains an important choice of treatment after AR to EGFR-TKIs. In the present study, we confirmed that NSCLC cells with AR mediated by T790M had higher sensitivity to taxanes and those mediated by EMT were resistant to various chemotherapeutic drugs including taxanes and non-taxanes. These results provide a rationale for the selection of appropriate chemotherapeutic regimens to maximally benefit patients with intrinsic or acquired EGFR-TKI resistance.

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#### Competing interests

The authors declare that they have no competing interests.

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