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조 선 대 학 교 대 학 원

의 학 과

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국문초록

T790M 변이를 가진 비소세포폐암에서 gefitinib 내성극복에 대한 simvastatin의 영향

황 기 은

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비소세포폐암에서 epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs)의 초기 임상적 성적들은 실망스런 결과들을 보였으나 비흡연자, 여자, 선암, 아시아의 선택적 환자들에게서 좋은 임상적 결과들을 보여주고 있다. 하지만 EGFR-TKIs에 반응이 있었던 환자에서 수개월에서 수년 후 획득내성이 발생하여 이를 극복하고자 하는 치료제의 개발이 시급한 상황이다.

Statin은 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase의 억제를 통해 항콜레스테롤 효과를 보이는 약제로 이미 항암효과를 보임이 증명되었다. 이 연구는 EGFR의 T790M 획득내성을 가진 비소세포폐암주에서 simvastatin이 mechanism-based approach를 통해 EGFR-TKIs의 내성을 극복할 수 있는지 알아보고자 하였다.

T790M 획득내성을 가진 비소세포폐암주에서 gefitinib과 simvastatin 병합요법시 caspase 의존적인 세포고사가 유도됨을 확인하였다. 또한 병합요법시 AKT/β-catenin activity와 target protein인 cyclin D1과 survivin 역시 감소됨을 확인하였으며, insulin과 AKT overexpression 시에는 p-β-catenin, survivin 발현이 증가됨을 확인하였다. 그러나 AKT에 대한 siRNA나 LY294002을 통한 AKT 억제시 p-β-catenin, survivin 발현이 감소되어 AKT pathway가 target gene인 survivin의 발현을 직접적으로 조절

하고 있음을 알 수 있다. Gefitinb과 simvastain의 병합요법에 의해 유도된 세포고사에 survivin의 직접적인 영향을 확인하기위해 survivin siRNA를 처리한 경우 세포고사가 의미있게 증가하였으며 반대로 survivin up-regulation시 세포고사가 감소함을 확인하였다.

결론적으로 simvastatin이 T790M 획득내성을 가진 비소세포폐암에서 AKT/β-catenin 신호전달계에 의존적인 survivin의 down-regulation을 통해 EGFR-TKIs에 의한 내성을 극복할 수 있음을 확인하였다.

ABSTRACT

Effect of Simvastatin on Gefitinib resistance in Non-small Cell Lung Cancer with the T790M Mutation

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Although non-small cell lung cancer (NSCLC) tumors with activating mutations in the epidermal growth factor receptor (EGFR) are highly responsive to EGFR tyrosine kinase inhibitors (TKIs) including gefitinib and erlotinib, development of acquired resistance is almost inevitable. Statins show antitumor activity, but it is unknown whether they can reverse EGFR-TKIs resistance in NSCLC with the T790M mutation of EGFR. This study investigated overcoming resistance to EGFR-TKI using simvastatin. We demonstrated that addition of simvastatin to gefitinib enhanced caspase-dependent apoptosis in T790M mutant NSCLC cells. Simvastatin also strongly inhibited AKT activation, leading to suppression of β-catenin activity and the expression of its targets, survivin and cyclin D1. Both insulin treatment and AKT overexpression markedly increased p-β-catenin and survivin levels, even in the presence of gefitinib and simvastatin. However, inhibition of AKT by siRNA or LY294002 treatment decreased p-β-catenin and survivin levels. To determine the role of

survivin in simvastatin-induced apoptosis of gefitinib-resistant NSCLC, we showed that the proportion of apoptotic cells following treatment with survivin siRNA and the gefitinib - simvastatin combination was greater than the theoretical additive effects, whereas survivin up-regulation could confer protection against gefitinib and simvastatin-induced apoptosis. Similar results were obtained in erlotinib and simvastatin-treated HCC827/ER cells. These findings suggest that survivin is a key molecule that renders T790M mutant NSCLC cells resistant to apoptosis induced by EGFR-TKIs and simvastatin. Overall, these data indicate that simvastatin may overcome EGFR-TKI resistance in T790M mutant NSCLCs via an AKT/β-catenin signaling-dependent down-regulation of survivin and apoptosis induction.

Ⅰ. Introduction

The efficacy of epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) has been demonstrated in treatment of non-small cell lung cancers (NSCLCs). Although they sometimes induce a remarkable or durable response, all patients ultimately develop acquired resistance to EGFR-TKIs after varying periods of time [1]. The resistance to the EGFR-TKIs in EGFR-mutant NSCLC patients is acquired through two different mechanisms: ~50% have tumors with a secondary EGFR mutation in threonine 790 (T790M) and ~20% have tumors that show an amplification of the proto-oncogene MET [2,3]. Irreversible EGFR-TKIs, including BIBW2992, have been found to be effective in inhibiting the growth of NSCLC cells with the T790M mutation of EGFR both *in vitro* and *in vivo* [4-6]. To date, however, the efficacy of such inhibitors has not been verified in clinical studies, and it remains unclear whether such inhibitors can overcome T790M-mediated resistance when administered at a pharmacological dose [7]. Thus, there is a need to explore alternative therapeutic methods to overcome these resistance mechanisms.

Statins are widely used as lipid-lowering agents that inhibit the rate-limiting step of the mevalonate pathway, which is catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase [8]. Statins have been studied for their anticarcinogenic and apoptotic properties, which include impairment of protein prenylation and interference with the formation of cholesterol-rich lipid microdomains called lipid rafts within the cell membrane [9], in various cancer cell types, including NSCLCs and carcinomas of the colon, prostate, breast, and skin [10,11]. Both of these processes are critical for the function of EGFR and the activity of numerous proteins important for EGFR signaling, such as Ras

[12]. Apart from these signal transduction pathways, we have previously demonstrated that the ability of simvastatin to retard tumor growth and progression by inducing apoptosis is related to an AKT signaling-dependent down-regulation of survivin in A549 lung cancer cells [13]. Some studies have shown that the combination of gefitinib and lovastatin exerts a synergistic cytotoxicity and enhances EGFR inhibition in squamous cell head and neck carcinomas, NSCLCs, and colon carcinoma cell lines [14-16].

Survivin is the smallest member of the inhibitor of apoptosis protein (IAP) family and has been shown to inhibit apoptosis through direct inhibition of caspase-3 and caspase-7, which act as terminal effectors in the apoptotic protease cascade [17-19]. Survivin is highly expressed in transformed cells and in most human cancers, including lung, breast, pancreatic, and colon carcinomas, soft tissue sarcomas, brain tumors, and hematologic malignancies, among others [20-23]. Overall, increased survivin expression is also associated with an increased risk of recurrence, lymph node invasion, and metastasis [24, 25]. However, interference with survivin function reduces cancer cell growth, induces apoptosis, and sensitizes cancer cells to radiation or chemotherapy [26,27]. Okamoto et al. [28] reported that down-regulation of survivin plays a pivotal role in gefitinib-induced apoptosis in EGFR mutation-positive NSCLC cells. Persistent survivin expression might therefore be expected to result in resistance to gefitinib in NSCLC cell lines with the T790M mutation of EGFR.

In this study, we hypothesized that simvastatin may overcome EGFR-TKIs resistance in NSCLCs with the T790M mutation of EGFR by modulating the AKT-survivin pathway. We investigated whether the antitumor activity of EGFR-TKIs can be potentiated by simvastatin in NSCLCs with the T790M mutation of EGFR. Functional and biochemical studies were performed *in vitro* to test the effect of EGFR-TKIs, simvastatin, and EGFR-TKIs plus simvastatin

on cell proliferation and apoptosis. We also investigated whether simvastatin has an effect on AKT signaling and the modulation of survivin activity in NSCLC cell lines with the T790M mutation of EGFR.

Ⅱ. Materials and Methods

A. Materials

RPMI 1640, fetal bovine serum (FBS), and antibiotics were obtained from GIBCO BRL Co (Grand Island, NY). Gefitinib was obtained from Kemprotec, while simvastatin, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), propidium iodide (PI), bicinchoninic acid, dimethyl sulfoxide (DMSO), insulin, and LY294002 were purchased from Sigma-Aldrich (St. Louis, MO). Primary antibodies against the following targets were used: caspase-3, -8, and -9; poly(ADP-ribose) polymerase (PARP) (Santa Cruz Biotechnology, Santa Cruz, CA); and EGFR; phospho-EGFR; serine/threonine protein kinase (AKT); phospho-AKT; extracellular signal-regulated protein kinase (ERK1/2); phospho-ERK1/2; JNK; phospho-JNK; survivin; phospho-b-catenin; cyclin D1; GAPDH (Cell Signaling Technology, Beverly, MA). Anti-rabbit IgG-conjugated horseradish peroxidase (HRP) antibodies and the enhanced chemiluminescent (ECL) kit were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK).

B. Cell Culture and Viability Test

The NSCLC cell lines HCC827 [EGFR exon 19 deletion] and H1975 [EGFR exon 21 mutation (L858R) and exon 20 mutation (T790M)] were obtained from the American Type Culture Collection and grown in RPMI 1640 containing 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 10% FBS, and were maintained in the log phase in a humidified atmosphere of 5% CO_2 and at 37°C. Cell viability was determined by measuring the mitochondrial conversion of MTT

to a colored product. Following treatment with the specified drugs, cell viability was determined by adding MTT to the cell suspension for 4 h. After three washes with phosphate-buffered saline (PBS; pH 7.4), the insoluble formazan product was dissolved in DMSO. The optical density (OD) of each well was then measured using a microplate reader (Titertek Multiskan; Flow Laboratories, North Ryde, Australia) at 590 nm. The OD resulting from formazan production in control cells was considered as 100% cell viability, and all other measurements were expressed as a percentage of the control cell value.

C. Annexin V Assay for the Assessment of Apoptosis

The NSCLC cell lines undergoing early/late apoptosis were analyzed by annexin V-FITC and PI staining. In all, 2.5×10^5 cells in the exponential growth phase were seeded in 60-mm^2 dishes. Cells were left untreated or incubated with specified drugs for the indicated times at 37°C. Both adherent and floating cells were collected and analyzed by the Annexin V assay, according to the manufacturer's instructions. Pelleted cells were briefly washed with PBS and resuspended in an Annexin-binding buffer (BD Pharmingen). Cells were then incubated with Annexin V-phycoerythrin and propidium iodide for 15 min at room temperature. After incubation, the stained cells were analyzed using a FACScan system equipped with Cell Quest software (Becton Dickinson, San Jose, CA). Cells with no drug treatment were used as controls.

D. Western Blotting

Cells were harvested and lysed using radioimmunoprecipitation assay buffer (50 mM Tris-Cl [pH, 7.4], 1% NP40, 150 mM NaCl, 1 mM EDTA, 1 mM

phenylmethylsulfonyl fluoride, 1 μg/mL each of aprotinin and leupeptin, and 1 mM Na_3VO_4). After centrifugation at 12,000 × *g* for 30 min, the supernatant was collected, and the protein concentration was determined with the use of the Bradford reagent (Bio-Rad). Equal amounts of protein were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and subsequently transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk in TBS-T (25 mM Tris [pH, 7.6], 138 mM NaCl, and 0.05% Tween-20) for 1 h and probed with primary antibodies (at 1:1000 - 1:5000). After a series of washes, membranes were further incubated with secondary antibody (at 1:2000 - 1:10,000) conjugated with HRP. Detection of the immunoreactive signals was carried out using an ECL detection system (Amersham Co., England).

E. Gene Silencing

Transcriptional expression was specifically suppressed by the introduction of 21-nucleotide duplex siRNA, which targets nucleotides of the AKT or survivin mRNA coding sequence [29]. Briefly, cells (10^5 cells/well) were plated in 6-well plates and transiently transfected with 2 μg per well of AKT or survivin siRNA (Cell Signaling Technology) mixed with the X-tremeGENE siRNA transfection reagent (Roche Applied Science, Penzberg, Germany) according to the manufacturer's directions. Silencer Negative Control siRNA (Roche Applied Science) was used as a negative control and introduced into the cells using the same protocol.

F. Establishment of Cells Stably Overexpressing AKT or Survivin

A human survivin cDNA was obtained by reverse transcription - PCR of RNA derived from A549 cells with the following primers based on GenBank accession number U75285 [30]. A human AKT cDNA was obtained by reverse transcription - PCR of RNA derived from A549 cells with the following primers based on GenBank accession number NM-001014431.1 (Forward, 5′-CGGAATTCATGAGCGACGTGGCTATTG-3′ : Reverse, 5′-CCGCTCGAGGGCCGTGCCGCTGGCCGAG-3′). PCR products were digested with EcoRI and XhoI and ligated into pcDNA3-myr-myc.

G. Statistical Analysis

Each experiment was performed at least three times, and all values are represented as the means ± SD of triplicate samples. The Student's *t*-test was used to determine the statistical significance of the results. *P*-values < 0.05 were considered statistically significant.

Ⅲ. Results

A. Effect of Gefitinib on EGFR Signaling Pathways in EGFR Mutation-positive NSCLC Cell Lines

To establish an *in vitro* cell line model system for acquired resistance to the first-line molecular target therapy involving gefitinib treatment, we chose to use NSCLC HCC827 cells that contained an EGFR exon 19 in-frame deletion (delE746-A750). Prolonged treatment of HCC827 cells with gefitinib eventually led to the emergence of clones harboring the T790M secondary mutation, which confers acquired resistance to gefitinib. HCC827 cells were continuously exposed to escalating concentrations of gefitinib. The starting gefitinib dose was 20 nM, which was the dose causing the inhibition of 50% of cancer cell growth (IC_{50}). After exposure to gefitinib, they were washed and cultured in drug-free medium until the population of cells reached approximately 80% confluence. The viable cells were continuously exposed to increasing dosages and these dosages were sequentially increased to a final concentration of 2 μM gefitinib. The viable cells exhibited a 100-fold increase in resistance to the growth-inhibitory effect of gefitinib as determined by the MTT assay, and the resistant phenotype remained stable for at least six months under drug-free conditions. Figure 1A shows that HCC827/GR cells have similar viability to H1975 cells, NSCLC cells that are resistant to gefitinib. We next examined the effects of gefitinib on EGFR signaling pathways in a subset of EGFR mutation-positive NSCLC cell lines (HCC827, HCC827/GR, and H1975) by immunoblot analysis (Figure 1B). In HCC827 cells, gefitinib induced the dephosphorylation of EGFR as well as that of AKT and ERK. In addition, gefitinib induced downregulation of survivin expression in a dose-dependent

fashion. However, in HCC827/GR and H1975 cells, gefitinib had no effect on EGFR, AKT, and ERK phosphorylation, or on survivin expression. These data indicate that the resistance of some EGFR mutation-positive cells to gefitinib-induced apoptosis may be associated with persistent activation of the AKT-survivin pathway.

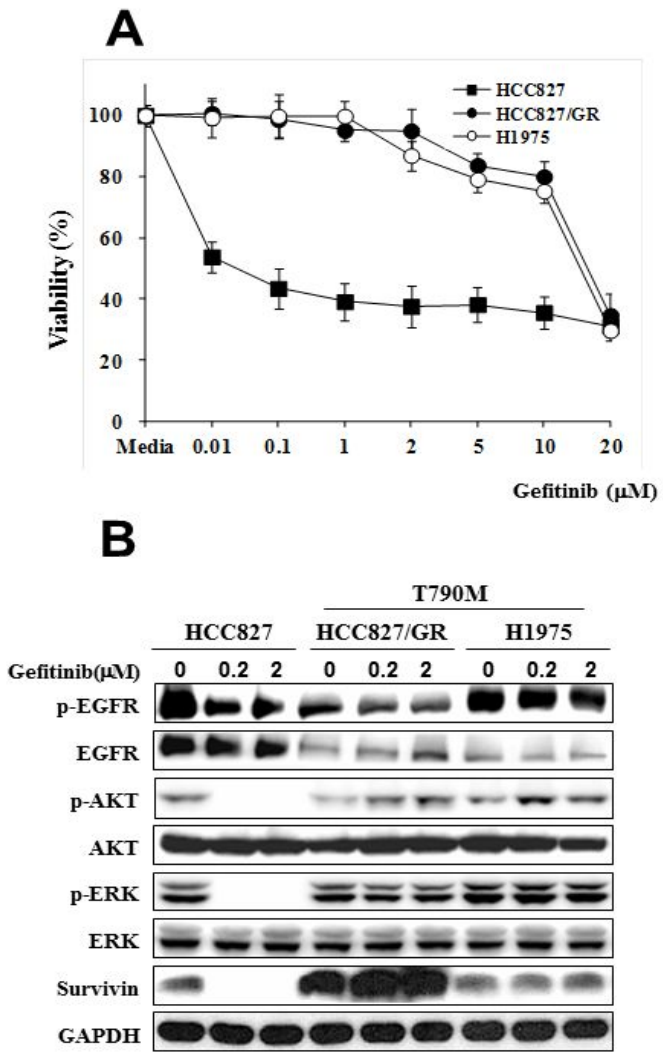


Figure 1. Effect of gefitinib on EGFR downstream pathways in EGFR mutation-positive NSCLC cell lines. (A) Effect of gefitinib on the growth of NSCLC cell lines. HCC827, HCC827/GR, or H1975 cells were treated with increasing concentrations of gefitinib and the viable cell numbers were analyzed using the MTT assay. Points show the mean of triplicates from experiments that were repeated a total of three times with similar results; bars, SD. (B)

Cells were incubated in the presence of the indicated concentrations of gefitinib for 48 h. Cell lysates were then prepared and subjected to immunoblot analysis with antibodies to phosphorylated (p) or total forms of EGFR, AKT, or ERK, to survivin, or to GAPDH (loading control). Immunoblots are representative of at least three independent experiments.

B. The Combination of Gefitinib and Simvastatin Enhances Caspase-dependent Apoptosis in NSCLC Cells with the T790M Mutation

We first examined the effect of the combination of simvastatin with gefitinib on the viability of NSCLC (HCC827/GR and H1975) cells with the T790M mutation. As shown in Figure 2A, the combination of gefitinib and simvastatin produced a synergistic inhibitory effect on the growth of both HCC827/GR and H1975 cells in a dose-dependent fashion. To examine whether the observed growth inhibition was due to enhanced apoptosis, the proportion of apoptotic cells was determined using annexin V-propidium iodide (PI) staining. Annexin V staining showed that the combination of gefitinib and simvastatin significantly enhanced apoptosis compared with individual treatment with either drug. As shown in Figure 2B, the combination of gefitinib and simvastatin induced apoptosis to a significantly greater extent in both HCC827/GR and H1975 cells compared with either monotherapy. To further elucidate the mechanism of apoptosis induced by gefitinib and simvastatin, cell lysates were evaluated by immunoblotting (Figure 2C). Our results showed that the combination of gefitinib and simvastatin enhanced the expression of the processed 85-kDa isoform of PARP, which is known to play a major role in apoptosis evasion. Moreover, combination of gefitinib and simvastatin led to a marked increase in the expression of caspase-3, -8, and -9. These results indicate that gefitinib and simvastatin play a major role in enhancing caspase-dependent apoptosis in NSCLC cells with the T790M mutation.

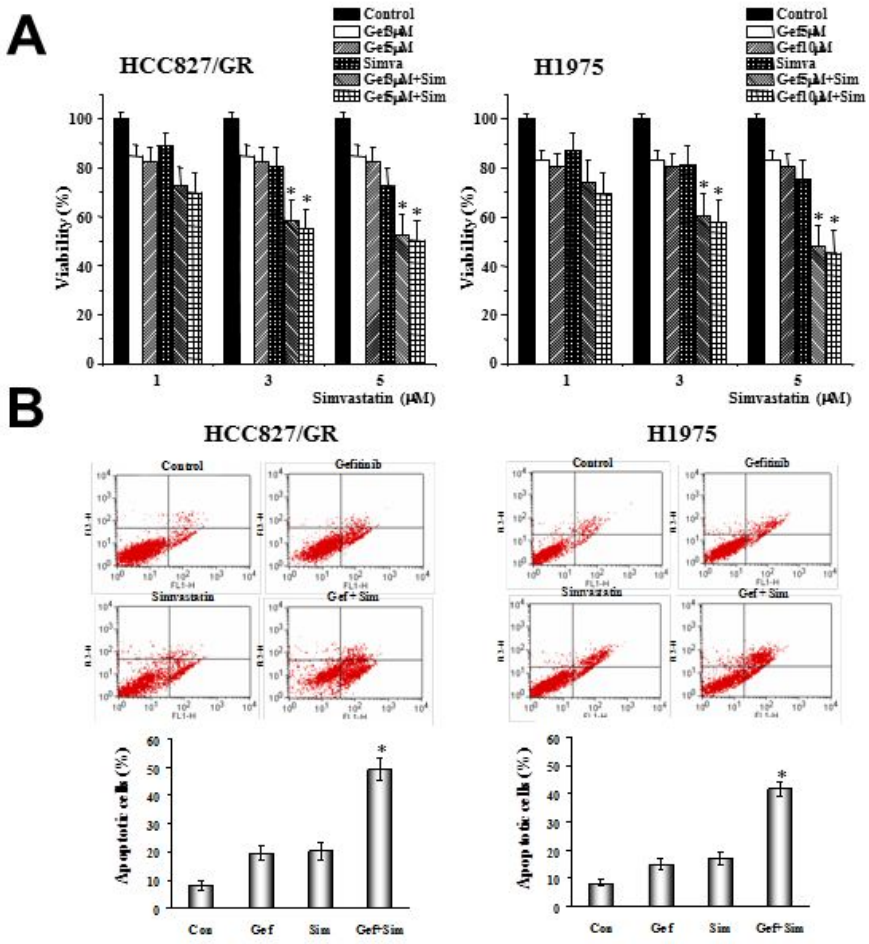

A
HCC827/GR
H1975
Control
Gef5μM
Gef10μM
Simva
Gef5μM+Sim
Gef10μM+Sim
Viability (%)
Simvastatin (μM)
B
HCC827/GR
H1975
Control
Gefitinib
Simvastatin
Gef+Sim
Apoptotic cells (%)
Con
Gef
Sim
Gef+Sim
C
HCC827/GR
H1975
Gefitinib
Simvastatin
Cleaved PARP
Cleaved caspase3
Cleaved caspase8
Cleaved caspase9
GAPDH

Figure 2. Effect of combination treatment with gefitinib and simvastatin on apoptosis of NSCLC cells with the T790M mutation. (A) HCC827/GR and H1975 cells were treated with different concentrations of gefitinib in the absence or presence of simvastatin for 48 h, and viability was then measured using the MTT assay. The viability of control cells was set at 100%, and cell survival relative to controls is presented. The data represent the mean ± S.D. of three independent experiments. $^{*}P < 0.05$ compared to the control. (B) Cells were incubated with 3-μM gefitinib and/or 5-μM simvastatin for 48 h, and apoptosis was evaluated by green fluorescent protein-annexin V + propidium iodide. Columns representing the flow cytometry data are presented on the right. Bars represent the mean ± S.D. of three independent experiments. $^{*}P < 0.05$, for the combination of gefitinib and simvastatin versus either control or one drug alone. (C) Cells were treated with gefitinib and simvastatin, alone and in combination, for 48 h, after which the cell lysate was subjected to 12% SDS-PAGE to measure the expression of PARP and caspase-3, -8, and -9.

C. Combination of Gefitinib and Simvastatin Inhibited the Expression of AKT/β-catenin and its Target Genes in NSCLC Cells with the T790M Mutation

To better understand the increased sensitivity of lung cancer cells to treatment with the combination of gefitinib and simvastatin, we next examined the role of AKT/β-catenin signal transduction pathways in the modulation of apoptosis. The status of AKT, β-catenin, cyclin D1, and survivin were evaluated in both HCC827/GR and H1975 cells treated with gefitinib and/or simvastatin for 48 h (Figure 3A). Our results showed that the combination of gefitinib and simvastatin resulted in a significant attenuation of AKT and β-catenin phosphorylation without affecting the phosphorylation of GSK-3β at Ser9. Moreover, the combination of gefitinib and simvastatin inhibited protein levels of its downstream targets, including survivin and cyclin D1, compared to cells treated with either gefitinib or simvastatin alone. It has been shown that AKT can phosphorylate β-catenin at Ser552, and enhances its transcriptional activity [31]. As shown in Figures 3B and 3C, inhibition of AKT by siRNA-mediated knockdown or treatment with the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 decreased the levels of p-AKT and p-β-catenin. In addition, the expression patterns of survivin and cyclin D1 changed in line with these phosphorylated signaling molecules. In contrast, we pharmacologically activated AKT by stimulating cells with insulin or introduction of constitutively activated AKT constructs into cells. As shown in Figures 3D and 3F, both insulin treatment and AKT overexpression markedly increased p-AKT and p-β-catenin levels, even in the presence of gefitinib and simvastatin. In addition, expression of survivin and cyclin D1 were also markedly elevated compared with the combination treatment.

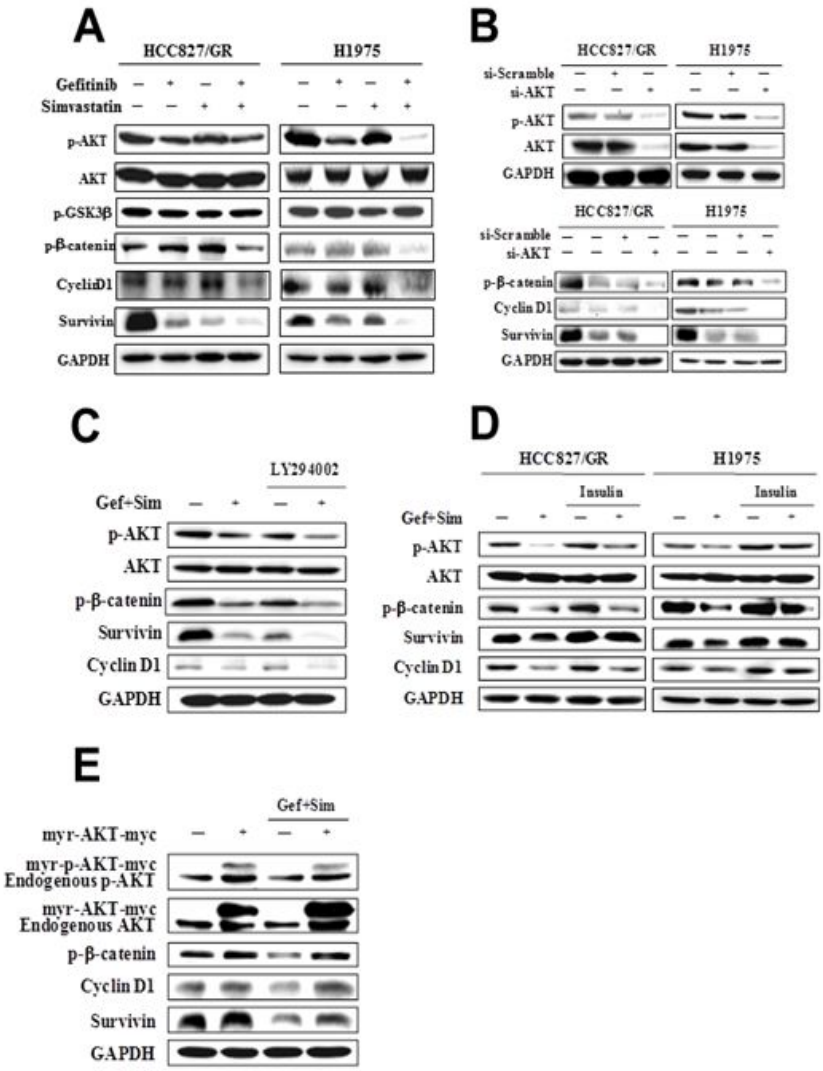


Figure 3. Gefitinib and simvastatin-induced inhibition of β-catenin through AKT. (A) Effect of gefitinib and simvastatin on AKT/β-catenin pathway. HCC827/GR and H1975 cells were treated with gefitinib and simvastatin, alone and in combination, for 48 h, after which the cell lysate was subjected to 12% SDS-PAGE to measure the expression of p-AKT, p-GSK 3β, p-β-catenin, cyclin D1, and survivin. (B and C) Effect of AKT inhibition on β-catenin

signaling. HCC827/GR and H1975 cells were transfected with AKT siRNA or pretreated with the PI3-kinase inhibitor LY294002, and then further incubated in the presence of gefitinib and/or simvastatin for 48 h. The cell lysates of each group were prepared and probed for p-β-catenin, cyclin D1, and survivin by western blot. GAPDH was used as a loading control. (D and E) Effect of AKT reactivation on gefitinib and simvastatin-induced inhibition of β-catenin pathway. Cells were pretreated with insulin solution for 2 h or transiently transfected with constitutively activated AKT constructs, followed by incubation with gefitinib and/or simvastatin for 48 h. The cell lysates were routinely prepared, and alterations in p-β-catenin as well as its downstream targets in these cells were determined by western blot.

D. Down-regulation of Survivin Enhanced Apoptosis Induced by Gefitinib and Simvastatin

To determine the role of survivin in apoptosis induced by gefitinib and simvastatin, we knocked down the survivin level by introducing siRNA for survivin. Figure 4A shows that the introduction of survivin siRNA abrogated survivin protein expression at 48 h after transfection. No reduction in survivin protein was observed in cells transfected with the scrambled RNA, which contained the same number of individual nucleotides as the survivin siRNA. We then examined the sensitivity of survivin siRNA-transfected cells to apoptosis induced by gefitinib and simvastatin. We spread an equal number of viable survivin siRNA-transfected and non-silencing siRNA-transfected cells at 48 h after siRNA transfection. After an additional 48 h of incubation, the cells were treated with gefitinib and simvastatin for 36 h, and the cell lysate was used to carry out western blotting. Both survivin siRNA and the gefitinib - simvastatin combination enhanced the expression of the processed 85-kDa isoform of PARP, caspase-3, -8, and -9. In addition, the proportion of apoptotic cells was determined using annexin V-PI staining. As shown in Figure 4B, introduction of survivin siRNA induced apoptosis without gefitinib and simvastatin treatment. However, the proportion of apoptotic cells induced by survivin siRNA and the gefitinib - simvastatin combination was greater than the theoretical additive effects. Together, these data indicate that down-regulation of survivin could enhance apoptosis induced by gefitinib and simvastatin.

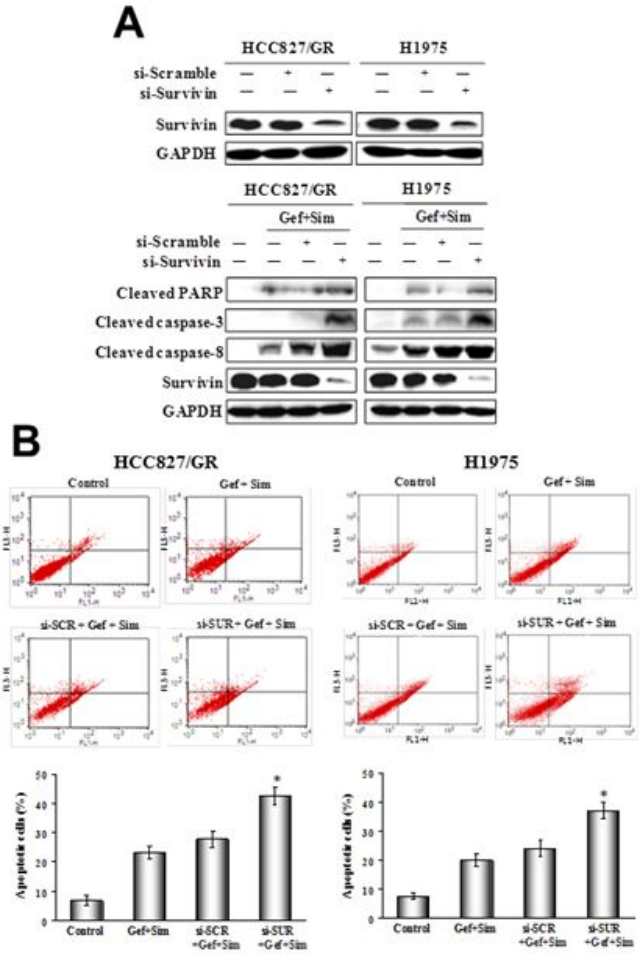


Figure 4. Effect of survivin depletion on apoptosis induced by gefitinib and simvastatin. (A) HCC827R and H1975 cells were transfected with siRNA specific for survivin, and survivin protein expression was assessed by immunoblot analysis at 48 h after transfection. Scrambled RNA containing the same number of each nucleotide as the survivin siRNA was used as the transfection control. Transfected cells incubated in complete medium with or without gefitinib and simvastatin for 36 h. Cell lysates were then prepared and subjected to immunoblot analysis with antibodies to PARP, to caspase-3 and

-8, or to survivin. Bands corresponding to the cleaved forms of PARP and caspase-3 and -8 are indicated. (B) Apoptosis was evaluated by green fluorescent protein-annexin V + propidium iodide. Columns representing the flow cytometry data are presented in the upper part. Bars represent the mean ± S.D. of three independent experiments. $^{*}P < 0.05$, for survivin siRNA and combination of gefitinib and simvastatin versus either scrambled siRNA and combined drugs or combined drugs alone.

E. Up-regulation of Survivin Attenuated Apoptosis Induced by Gefitinib and Simvastatin

We next examined whether survivin up-regulation could confer protection against apoptosis induced by gefitinib and simvastatin. We introduced the survivin gene expression vector pcDNA3-myc-survivin into HCC827/GR and H1975 cells. The survivin gene transfectants expressed myc-tagged survivin protection in addition to endogenous survivin protection. From the western blot results, it could be observed that survivin overexpression decreased the expression of the cleavage of PARP, caspase-3, -8, and -9, even in the presence of gefitinib and simvastatin (Figure 5A). We then evaluated the proportion of apoptotic cells in the survivin gene transfectants by using annexin V-PI staining after treating the cells with gefitinib and simvastatin for 48 h. As shown in Figure 5B, treatment of control vector transfectants with gefitinib and simvastatin for 48 h resulted in 35.2% more apoptotic cells than in the untreated HCC827/GR cells. However, in the survivin gene transfectants, gefitinib and simvastatin decreased the number of apoptotic cells by only 18.7% in HCC827/GR cells. These results suggest that survivin is a key molecule that renders the NSCLC cells with the T790M mutation resistant to apoptosis induced by gefitinib and simvastatin.

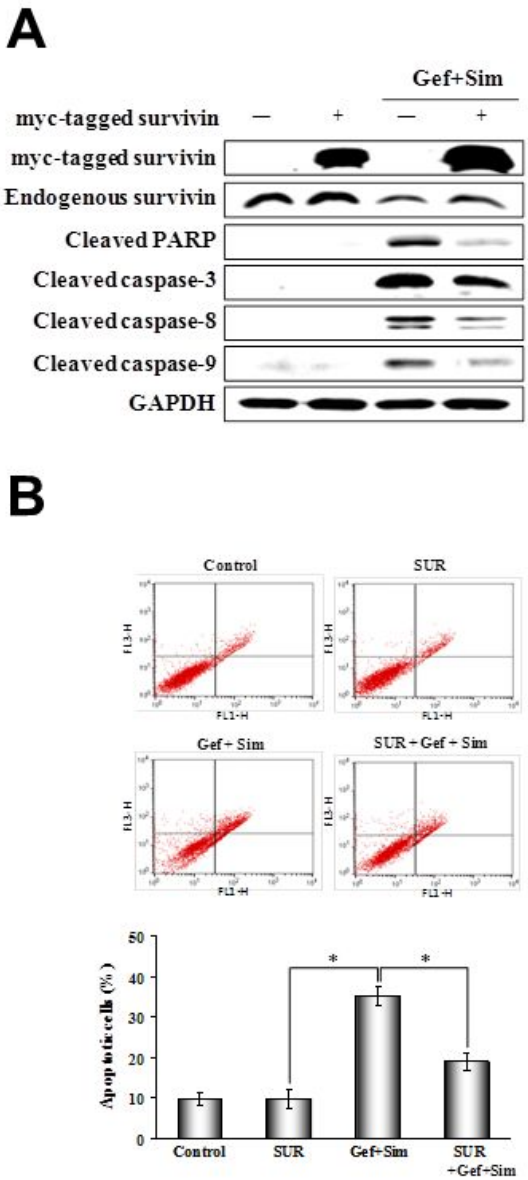
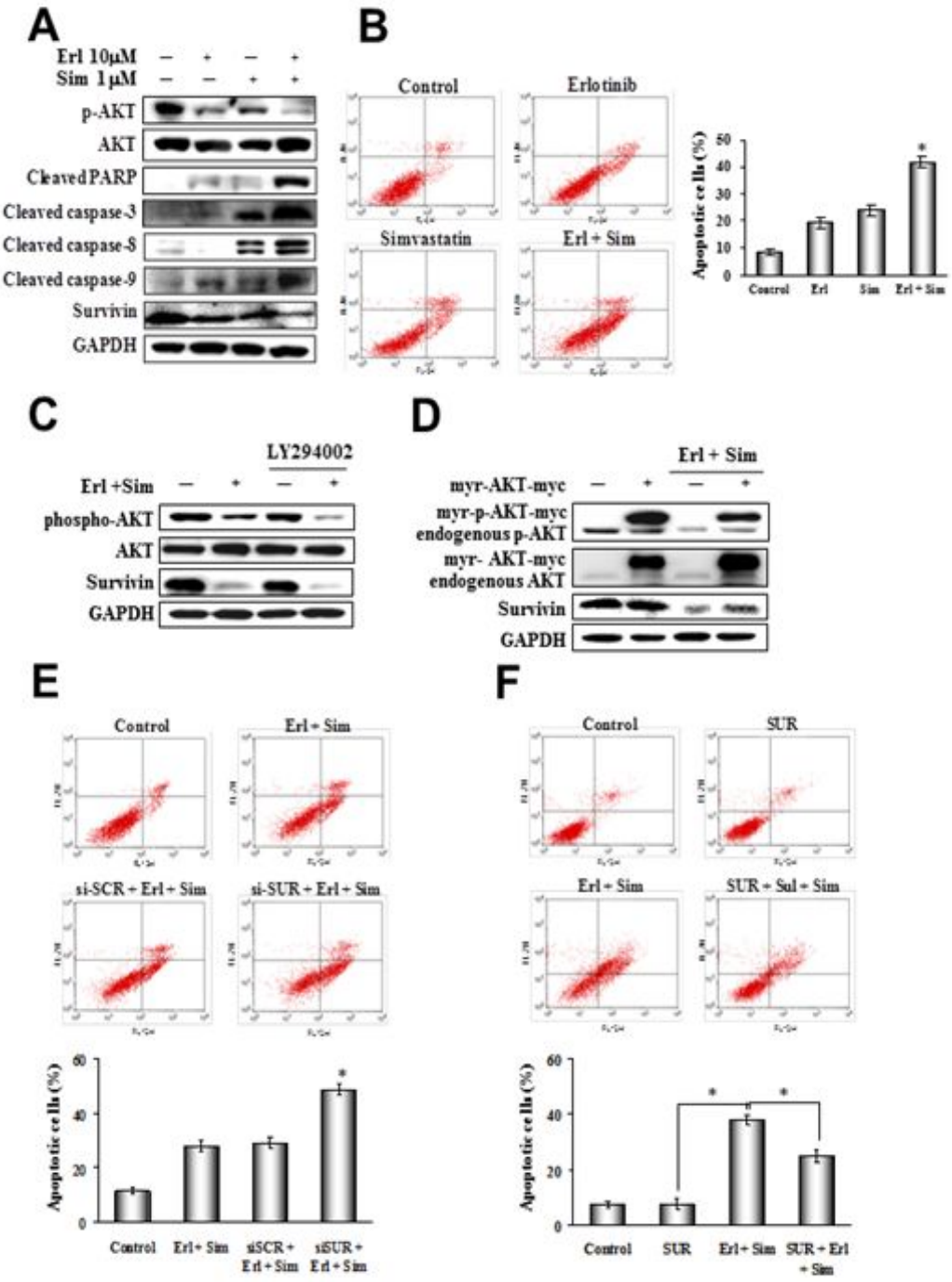


Figure 5. Effect of survivin overexpression on apoptosis induced by gefitinib and simvastatin in HCC827/GR cells. (A) Cells were transfected with the survivin gene expression vector pcDNA3-myc-survivin, and survivin protein expression was assessed by immunoblot analysis at 48 h after transfection. Transfected

cells were incubated in complete medium with or without gefitinib and simvastatin for 36 h, after which cell lysate was subjected to 12% SDS-PAGE to measure the expression of PARP and caspase-3, -8, and -9. (B) Apoptosis was evaluated by green fluorescent protein-annexin V + propidium iodide. Columns representing the flow cytometry data are presented in the upper part. The data represent the mean ± S.D. of three independent experiments. $^{*}P < 0.05$ for the indicated comparisons.

F. Combination of Erlotinib and Simvastatin Augmented their Apoptotic Potential via AKT/β-catenin Signaling-dependent Down-regulation of Survivin in NSCLC Cells with the T790M Mutation

To examine whether results similar to those observed for the gefitinib - simvastatin combination would be observed for erlotinib, another EGFR-TKI, we obtained HCC827/ER cells. As shown in Figures 6A and 6B, the combination of erlotinib and simvastatin induced apoptosis to a significantly greater extent in HCC827/ER cells compared with either monotherapy. Inhibition of AKT by LY294002 treatment caused inhibition of p-AKT and survivin. However, it could be observed that AKT overexpression increased p-AKT and survivin levels even in the presence of erlotinib and simvastatin (Figures 6C and 6D). To determine the role of survivin in apoptosis induced by erlotinib and simvastatin, we knocked down survivin using survivin siRNA or overexpressed it with survivin gene transfectants. As shown in Figure 6E, the proportion of apoptotic cells induced by survivin siRNA and combination of erlotinib and simvastatin was greater than the theoretical additive effects. However, treatment with erlotinib and simvastatin for 48 h resulted in 37.8% more apoptotic cells than in the untreated cells. Moreover, in the survivin gene transfectants, erlotinib and simvastatin significantly decreased the number of apoptotic cells by only 24.9% (Figure 6F).


A
Erl 10μM
Sim 1μM
p-AKT
AKT
Cleaved PARP
Cleaved caspase-3
Cleaved caspase-8
Cleaved caspase-9
Survivin
GAPDH
B
Control
Erlotinib
Simvastatin
Erl + Sim
Apoptotic cells (%)
Control
Erl
Sim
Erl + Sim
C
LY294002
Erl +Sim
phospho-AKT
AKT
Survivin
GAPDH
D
Erl + Sim
myr-AKT-myc
myr-p-AKT-myc endogenous p-AKT
myr- AKT-myc endogenous AKT
Survivin
GAPDH
E
Control
Erl + Sim
si-SCR + Erl + Sim
si-SUR + Erl + Sim
Apoptotic cells (%)
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Erl + Sim
siSCR + Erl + Sim
siSUR + Erl + Sim
F
Control
SUR
Erl + Sim
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Apoptotic cells (%)
Control
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SUR + Erl + Sim

Figure 6. Effect of combination treatment with erlotinib and simvastatin on apoptosis in HCC827/ER cells. (A) Cells were treated with erlotinib and simvastatin, alone and in combination, for 48 h, after which the cell lysate was subjected to 12% SDS-PAGE. (B) Apoptosis was evaluated by green fluorescent protein-annexin V + propidium iodide. Columns representing the flow cytometry data are presented in the upper part. Bars represent the mean ± S.D. of three independent experiments. $^{*}P < 0.05$, for the combination of erlotinib and simvastatin versus either control or one drug alone. (C) HCC827/ER cells were pretreated with the PI3-kinase inhibitor LY294002, and then further incubated in the presence of erlotinib and/or simvastatin for 48 h. (D) HCC827/ER cells were transiently transfected with constitutively activated AKT constructs, followed by incubation with erlotinib and/or simvastatin for 48 h. (E and F) Apoptosis was evaluated by green fluorescent protein-annexin V + propidium iodide. Columns representing the flow cytometry data are presented in the upper part. The data represent the mean ± S.D. of three independent experiments. $^{*}P < 0.05$ for the indicated comparisons.

Ⅳ. Discussion

Although, based on preliminary results of ongoing clinical trials, BIBW2992 has shown a remarkable ability to control disease progression in NSCLC patients with EGFR mutations, its effect in patients showing T790M-mediated resistance has been disappointing [32], indicating that these irreversible inhibitors may not be suitable for clinical application to manage patients with a secondary T790M mutation. In the present study, we demonstrated that the addition of simvastatin to EGFR-TKIs significantly enhanced apoptotic potential in NSCLC cells with the T790M mutation of EGFR. Simvastatin could overcome EGFR-TKI resistance, which may provide an alternative therapeutic strategy in patients with acquired resistance to EGFR-TKIs through secondary mutation. To date, several studies have shown that the combination of statin with gefitinib induces a potential synergistic cytotoxicity in a variety of tumors without EGFR mutations [14,15]. Han et al. [33] showed that treatment with gefitinib plus simvastatin was not superior to treatment with gefitinib alone in an unselected NSCLC population. To our knowledge, this is the first report to show the potential role of simvastatin as an anticancer drug that may overcome EGFR-TKI resistance in NSCLC cells with the T790M mutation of EGFR.

The AKT signaling pathway plays a crucial role in cell growth and cell survival. It is activated by insulin or growth factors and promotes cell survival by inactivating multiple targets including Bad, caspase-9, and forkhead transcription factors. Increased AKT activity has been noted in lung cancer, and is highly correlated with tumor progression [34]. The effect of simvastatin on the phosphorylation of AKT has also been reported in endothelial cells *in vitro* [35]. There is evidence to suggest that the AKT-mediated survival

pathway may inhibit apoptosis by stimulating survivin synthesis in various cancer cell lines [36,37]. To determine the role of AKT in gefitinib and simvastatin-induced apoptosis of NSCLC cells with the T790M mutation of EGFR, we pharmacologically activated AKT by stimulating cells with insulin or the introducing constitutively activated AKT constructs into cells. In contrast, we inhibited AKT by siRNA-mediated knockdown or use of the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002. In this work, we discovered that gefitinib and simvastatin-caused inhibition of β-catenin was directly regulated by AKT in NSCLC cells with the T790M mutation of EGFR. Moreover, phosphorylation of β-catenin by AKT promotes its nuclear accumulation and transcription of its targets.

Our results showed that the activation of caspase plays an important role in gefitinib- and simvastatin-induced apoptosis. Caspase-3 is the ultimate executioner caspase and is primarily responsible for the cleavage of PARP during apoptosis. Disruption of survivin - microtuble interactions results in the loss of the survivin anti-apoptosis function, an increase in caspase-3 activity, and cleavage of PARP [38]. We demonstrated that down-regulation of survivin by introduction of survivin siRNA into NSCLC cells with the T790M mutation of EGFR resulted in enhanced apoptosis induced by EGFR-TKIs and simvastatin. Therefore, we hypothesized that the up-regulation of the anti-apoptotic protein survivin, which inhibits caspase-3, may be a mechanism by which NSCLC cells with the T790M mutation of EGFR acquired resistance to EGFR-TKIs and simvastatin-induced apoptosis. As we expected, the number of apoptotic cells induced by combination of EGFR-TKIs and simvastatin was clearly decreased by the up-regulation of survivin. This observation indicated that survivin functions as a factor conferring resistance against EGFR-TKIs and simvastatin-induced apoptosis. Based on our experiments, simvastatin

modulated AKT/β-catenin signaling in NSCLC cells with the T790M mutation of EGFR and reduced survivin protein levels, thus implicating the AKT-survivin axis in the activity of simvastatin.

In conclusion, our results indicate that combination treatment with EGFR-TKIs and simvastatin augments their apoptotic potential in NSCLC cells with the T790M mutation of EGFR through AKT/β-catenin signaling-dependent down-regulation of survivin. This combination treatment leads to more complete inhibition of AKT/β-catenin signaling, and may not be achievable by treatment with EGFR-TKIs alone in NSCLC cells harboring the T790M mutation. Taken together, these results indicate that combination treatment with EGFR-TKIs and simvastatin is a clinically promising strategy for treating patients with T790-mediated acquired resistance to EGFR-TKIs.

Ⅴ. Acknowledgments

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