

Implication of the Insulin-like Growth Factor-IR Pathway in the Resistance of Non–small Cell Lung Cancer Cells to Treatment with Gefitinib

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Abstract Purpose: Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors have been found to be effective against lung cancer *in vitro*, but clinical resistance to these agents has developed as their usage has increased. In this study, we determined whether the insulin-like growth factor I (IGF-I) signaling pathway induces resistance of non–small cell lung cancer (NSCLC) cells to the EGFR tyrosine kinase inhibitor gefitinib.

Experimental Design: The effects of gefitinib and cetuximab on NSCLC cells, alone or with an IGF-I receptor (IGF-IR) inhibitor, were assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, the flow cytometry–based terminal nucleotidyl transferase–mediated nick end labeling assay, coimmunoprecipitation, and Western blot analysis. EGFR and IGF-IR expression in NSCLC tissues were examined by Western blot analysis.

Results: Gefitinib inhibited NSCLC cell proliferation by inducing apoptosis when IGF-IR signaling was suppressed. Treatment with gefitinib, but not cetuximab, induced EGFR:IGF-IR heterodimerization and activation of IGF-IR and its downstream signaling mediators, resulting in increased survivin expression in NSCLC cell lines with high levels of IGF-IR expression. Inhibition of IGF-IR activation and knockdown of survivin expression led to increased apoptosis. In contrast, overexpression of survivin protected cells with low IGF-IR expression from gefitinib-induced apoptosis. Most NSCLC tissues with EGFR overexpression had associated high levels of IGF-IR expression.

Conclusions: IGF-IR expression may be useful as a predictive marker for gefitinib treatment of NSCLC. Suppression of IGF-IR signaling pathways may prevent or delay development of gefitinib resistance in patients with NSCLC.

Lung cancer is the leading cause of cancer-related death in both sexes in the United States and throughout the world, and its overall mortality rate has not changed substantially in decades (1). Advances in the understanding of lung tumor biology and oncogenesis have provided several molecular targets for the treatment of non–small cell lung cancer (NSCLC). Of these,

inhibitors of epidermal growth factor receptor (EGFR) tyrosine kinase pathways are the most extensively studied.

EGFR is overexpressed in several solid tumor types, including NSCLC (it has been found in 40-80% of cases; ref. 2). The EGFR signaling pathway activates the phosphoinositide-3-kinase/Akt and mitogen-activated protein kinase (MAPK) pathways, which mediate proliferation, differentiation, and survival in both normal and malignant epithelial cells (3, 4). Activated EGFR also promotes angiogenesis, tumor cell motility, and invasion by regulating the expression and activity of matrix metalloproteinases and by interacting with components of the integrin pathway (5). These findings indicate that EGFR-targeted agents would be effective against cancer.

Small-molecular-weight EGFR tyrosine kinase inhibitors (TKI) and anti-EGFR monoclonal antibodies have been in advanced clinical development (6, 7). Monoclonal antibodies bind to the extracellular domain of EGFR. The EGFR-antibody complex is subsequently internalized, causing decreases in EGFR expression and heterodimerization in a phosphorylation status–independent manner. Treatment with cetuximab, a chimeric human-mouse anti-EGFR monoclonal immunoglobulin G₁ (IgG₁) antibody, has been found to block cell cycle progression by inducing G₁ arrest and to inhibit human cancer xenograft growth in nude mice *in vivo* (8) by inhibiting tumor-induced angiogenesis (9). Treatment with cetuximab has shown therapeutic activities in patients with head and neck

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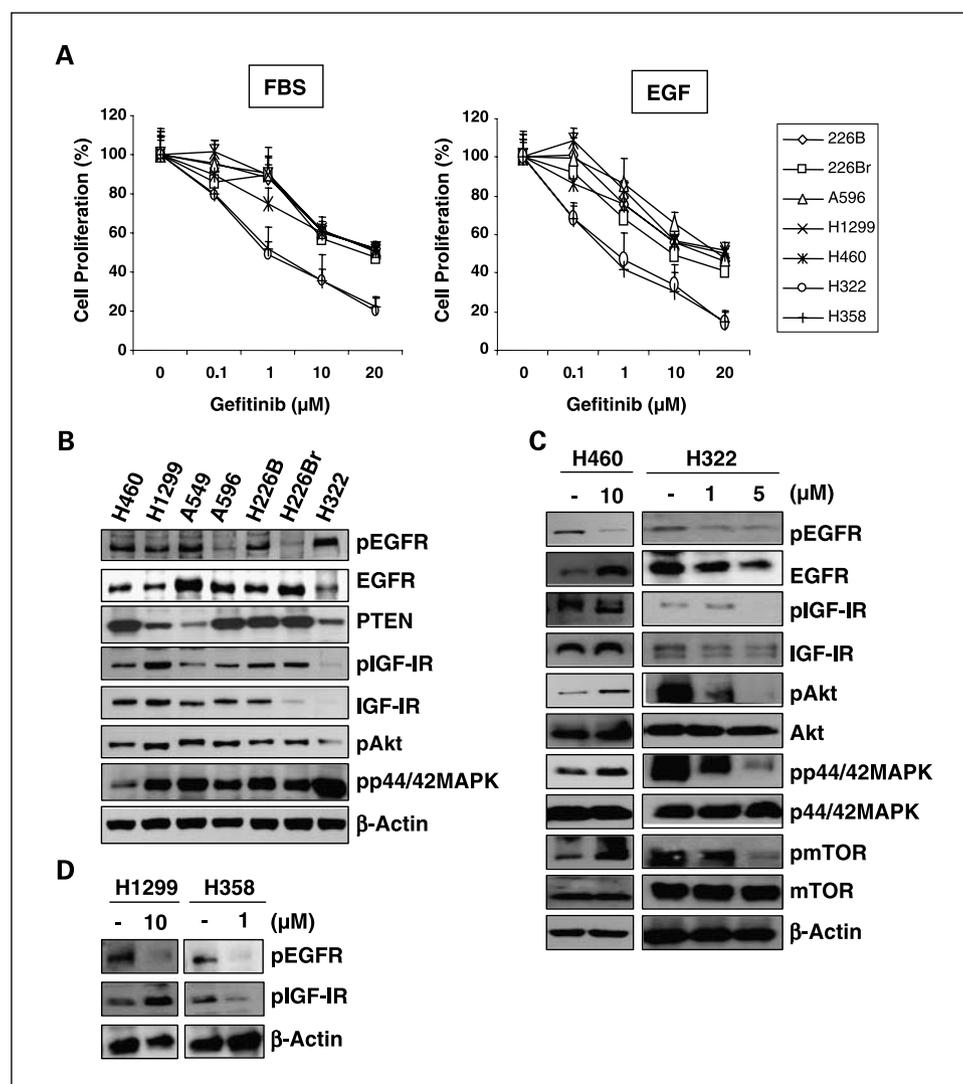


Fig. 1. Stimulation of IGF-IR signaling pathway in gefitinib-resistant NSCLC cell lines. **A**, results of the MTT assay of H460, H1299, A596, H226B, H226Br, H322, and H358 cell lines treated with the indicated concentrations of gefitinib in RPMI 1640 containing 10% FBS or EGF for 3 d. Independent experiments were repeated at least thrice each, and one representative result is shown. Points, mean value of eight identical wells of a single representative experiment; bars, upper 95% CIs. **, $P < 0.01$ and ***, $P < 0.001$ for comparisons between gefitinib-treated and control cells. **B**, basal expression of EGFR, IGF-IR, p44/42 MAPK, Akt, and their phosphorylated forms and PTEN in seven NSCLC cell lines. **C** and **D**, immunoblotting of EGFR, IGF-IR, and their downstream signaling components in H460, H322 (**C**), H1299, and H358 (**D**) cells treated with the indicated concentrations of gefitinib. **B** and **C**, Western blotting on β -actin was included as a loading control.

cancer or colorectal cancer (10, 11). Unlike monoclonal antibodies, TKIs do not affect internalization of the EGF receptor and are often not EGFR specific; thus, they affect the kinase activity of other ErbB family receptors. EGFR TKIs block the ATP pocket of EGFR, thereby inhibiting EGFR phosphorylation and downstream signal transduction (12).

Preclinical studies have shown that the EGFR TKI gefitinib, when used in combination with standard chemotherapeutic agents or radiotherapy, inhibits EGFR activation, causing G_1 arrest and additive-to-synergistic growth inhibition (6). However, negative results from clinical trials (13, 14) have diminished the enthusiasm for gefitinib and indicate that a better understanding is needed of the mechanisms of acquired resistance to this drug. The effectiveness of gefitinib is currently being studied in a population of patients who may have a biomarker that sensitizes their tumors to gefitinib. However, mechanistic studies of gefitinib resistance have not been completed.

Because recent studies have suggested that the insulin-like growth factor I receptor (IGF-IR) pathway is involved in NSCLC cells' resistance to EGFR-targeting agents (15, 16), we studied whether the IGF-IR-mediated signaling pathway influences the NSCLC response to gefitinib and cetuximab. We also sought to

determine the proteins mediating survival of NSCLC cells against gefitinib treatment. In the present studies, we show that treatment with gefitinib but not cetuximab stimulates the IGF-IR pathway and its downstream signaling mediators via the EGFR:IGF-IR heterodimer and induces survivin expression that protects NSCLC cells from apoptosis.

Materials and Methods

Cells and reagents. The human NSCLC cell lines H596, H226B, H226Br, H460, H1299, H358, and H322 were purchased from the American Type Culture Collection and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Life Technologies-BRL) in a humidified atmosphere with 5% CO_2 . EGF was purchased from R&D Systems. Gefitinib (AstraZeneca) was prepared as a 10 mmol/L stock solution in DMSO. Cetuximab was obtained from ImClone Systems and prepared as a 20 mmol/L stock solution. AG1024, an IGF-IR TKI, was purchased from Calbiochem-Novabiochem. Adenoviruses, with and without survivin (Ad-survivin and Ad-EV, respectively), were amplified as described previously (17).

Cell proliferation assay. To determine the effects of gefitinib and AG1024 on NSCLC cell proliferation, we plated 3×10^3 cells per well of the indicated NSCLC cell lines in 96-well plates. The next day, cells were

treated with either 0.1% DMSO as a diluent control or with different concentrations of drugs (0.1-10.0 μmol/L in a final DMSO concentration of 0.1%) in RPMI with 10% FBS or EGF (50 ng/mL). Cell medium was replaced with fresh medium containing gefitinib, AG1024, or both everyday for 3 days. At the end of the treatment period, cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The drug concentrations required to inhibit cell growth by 50% were determined by interpolation from the dose-response curves. Eight replicate wells were used for each analysis, and at least three independent experiments were done. The data from replicate wells are presented as the mean numbers of cells remaining, with 95% confidence intervals (CI). To determine the effect of the combined drug treatments, any potentiation was estimated by multiplying the percentage of cells remaining (% growth) for each agent. The classification index was calculated as described previously (18). Supra-additivity was defined as $\% AB / (\% A \times \% B) > 1.0$; additivity was defined as $\% AB / (\% A \times \% B) = 0.9-1.0$; and subadditivity was defined as $\% AB / (\% A \times \% B) < 0.9$. (In these equations, A and B are the effects of individual agents, and AB is the effect of the combination of the two drugs.)

Cell cycle and apoptosis assays. For cell cycle and apoptosis assays, 2×10^5 NSCLC cells per well were plated in six-well plates. The next day, the cells were treated with various concentrations of gefitinib (5 μmol/L), AG1024 (5 μmol/L), or both. Both adherent and nonadherent cells were harvested, pooled, and fixed with 1% paraformaldehyde and 70% ethanol. To determine the percentages of cells in the phases of the cell cycle (G₁, S, and G₂-M), we stained cells with 50 μg/mL propidium iodide and analyzed them with a flow cytometer (Epics Profile II; Beckman Coulter Inc.) equipped with a 488-nm argon laser. Apoptosis was assessed using a modified flow cytometry-based terminal nucleotidyl transferase-mediated nick end

labeling (TUNEL) assay with an APO-bromodeoxyuridine staining kit (Phoenix Flow Systems) as described previously (19). Data from at least three experiments are presented as means with 95% CIs.

Immunoblotting and coimmunoprecipitation. NSCLC cells were either left uninfected or infected with Ad-EV or Ad-survivin (50 infection-forming units) and then left untreated or treated with gefitinib (1-10 μmol/L), AG1024 (5 μmol/L), and cetuximab (1-10 μmol/L), alone or in combination, in growth medium that was changed daily. For growth factor stimulation, cells were cultured in serum-free medium for 1 day and then incubated with EGF (50 ng/mL). For small interfering RNA (siRNA) transfection, H460 cells in the logarithmic growth phase in six-well plates (5×10^5 cells per well) were transfected with 10 μL of 20 μmol/L survivin siRNA or control scrambled siRNA (Dharmacon) using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol. Cells were incubated for 24 h in growth medium, and gefitinib was added. Cells were harvested after 3 days of incubation.

We did a biochemical analysis of 14 lung tumor and 14 healthy adjacent tissue specimens from patients with NSCLC who had been treated at The University of Texas M.D. Anderson Cancer Center. This study was approved by the M.D. Anderson Cancer Center Institutional Review Board. All tissue specimens were frozen in liquid nitrogen immediately after being resected and rinsed in PBS; they were kept in a -80°C freezer until retrieved for the study. Total protein isolation and Western blot analyses were done as described previously (20).

Immunoprecipitation was done using 3 mg of protein from the total cell lysates and 3 μg of mouse monoclonal anti-EGFR antibody (Santa Cruz Biotechnology), mouse monoclonal anti-IGF-IR antibody (αIR-3; Oncogene Science), or healthy preimmune serum anti-mouse antibody (sc-2025) as the negative control, followed by incubation overnight at 4°C. The immunoprecipitates were resolved by 6% SDS-PAGE and then

Fig. 2. Effects of combined blockade of EGFR and IGF-IR pathways on NSCLC cells. **A**, effects of treatment with 5 μmol/L gefitinib, 5 μmol/L AG1024, or both on the expression of pEGFR and pIGF-IR were determined in H460 cells by Western blot analysis. β-Actin was used as a loading control. **B**, effect of targeting EGFR and IGF-IR on cell proliferation. H460 cells were treated with the indicated concentrations of gefitinib, AG1024, or both in RPMI 1640 containing 10% FBS for 3 d. The results of the MTT assay are shown. Columns, mean value of eight (MTT) identical wells in a single representative experiment; *n* = 3. Bars, upper 95% CIs. ***, *P* < 0.001 for comparisons between cells treated with a single drug or both drugs. **C**, effects of treatment with the indicated concentrations of gefitinib, AG1024, or both on the cell cycle distribution of H460 cells. DNA content was evaluated by propidium iodide uptake, and the percentages of cells in specific phases of the cell cycle were determined using flow cytometry. Columns, mean value of three identical experiments. **, *P* < 0.01; ***, *P* < 0.001. **D**, apoptosis in H460 cells assessed using a modified TUNEL assay. Two independent experiments were done; the data shown are from a single representative experiment.

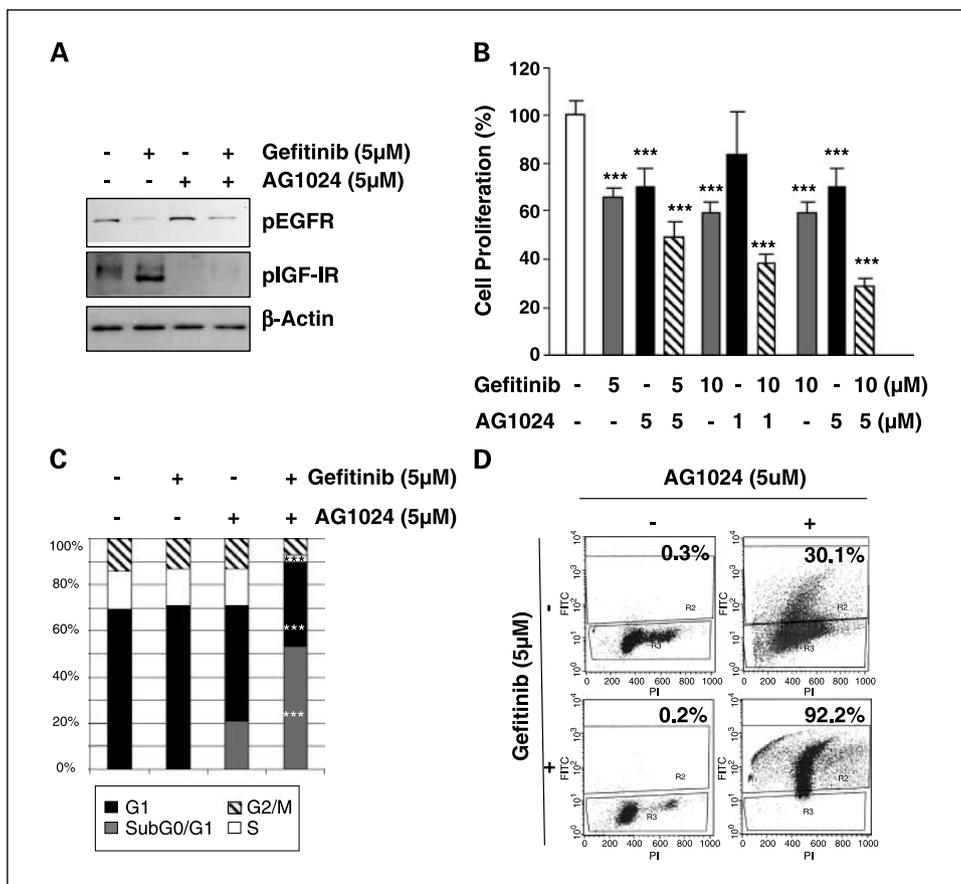


Table 1. Synergistic indices of combination treatment with gefitinib and AG1024

Treatment A				Treatment B				Combination treatment			Index*
Drug	Concentration ($\mu\text{mol/L}$)	MGI	<i>P</i> value [†]	Drug	Concentration ($\mu\text{mol/L}$)	MGI	<i>P</i> value [†]	Expected [‡]	Observed [§]	<i>P</i> value [†]	
Gefitinib	1	0.85	5.3E-05	AG1024	1	0.87	0.0299	0.73	0.69	0.000124	1.05
	5	0.65	1.9E-09		5	0.70	7.5E-07	0.59	0.57	1.3E-09	1.03
	10	0.59	4.3E-10		1	0.87	0.0299	0.56	0.50	8.3E-08	1.12
					5	0.70	7.5E-07	0.45	0.44	1.9E-10	1.022
					1	0.87	0.0299	0.51	0.32	1.2E-12	1.59
					5	0.70	7.5E-07	0.41	0.28	9.9E-14	1.46

NOTE: H460 cell proliferation treated with indicated concentrations of gefitinib, AG1024, or their combinations was calculated by the MTT assay. Abbreviation: MGI, mean growth inhibition rate = growth rate of treated group/growth rate of untreated group.

*Calculated by dividing the expected growth inhibition rate by the observed growth inhibition rate. An index more than 1 indicates synergistic effect and <1 indicates less than additive effect.

[†]*P* value (two-sided) was calculated by *t* test compared with no treatment.

[‡]Growth inhibition rate of treatment A \times growth inhibition rate of treatment B.

[§]Growth inhibition rate of combined treatment on treatments A and B.

analyzed by Western blot using rabbit polyclonal antibodies against human phosphorylated EGFR (pEGFR; Tyr¹⁰⁶⁸), EGFR, and phosphorylated IGF1R (pIGF-IR and Tyr¹¹³¹/Tyr¹¹⁴⁶; 1:1,000; Cell Signaling Technology); rabbit polyclonal antibodies against human IGF1R (1:500; Santa Cruz Biotechnology); rabbit polyclonal antibodies against human phosphorylated Akt (pAkt; Ser⁴⁷³; 1:1,000), and Akt (1:1,000) and a mouse monoclonal antibody against human anti-phosphorylated p44/42 MAPK (pp44/42MAPK and Thr²⁰²/Tyr²⁰⁴; 1:500; Cell Signaling Technology); goat polyclonal antibodies against p44/42 MAPK (1:1,000; Cell Signaling Technology); and a rabbit polyclonal anti-poly(ADP-ribose) polymerase antibody (1:1,000; VIC 5; Roche Molecular Biochemicals). Other products used in the Western blot analysis included rabbit polyclonal caspase-3 (1:1,000), rabbit polyclonal antibodies against X inhibitor of apoptosis protein (XIAP; 1:1,000; Cell Signaling Technology), rabbit polyclonal antibodies against mammalian target of rapamycin (mTOR; 1:1,000; Cell Signaling Technology), pmTOR (1:1,000; Cell Signaling Technology), mouse monoclonal survivin (1:1,000; Santa Cruz Biotechnology), goat polyclonal antibody against β -actin (1:4,000, Santa Cruz Biotechnology), and rabbit anti-mouse IgG-horseradish peroxidase conjugate (1:2,000; DakoCytomation).

Statistical analysis. The MTT assay data were analyzed using the Student's *t* test. Eight replicate wells were used for each analysis, and data from replicate wells are presented as mean values with 95% CIs. At least three independent experiments were done to obtain each result, and cell survival among groups was compared using ANOVA for a 2 \times 2 factorial design. The mean values from three experiments with the eight replicates and 95% CIs were calculated using the SAS software (version 8.02; SAS Institute). In all statistical analyses, two-sided *P* values of <0.05 were considered statistically significant.

Results

Stimulation of the IGF-IR signaling pathway in gefitinib-resistant NSCLC cell lines. We determined the effects of gefitinib on cell proliferation in H596, H226B, H226Br, H460, H1299, H358, and H322 cell lines. We treated cells with gefitinib in the presence of 10% FBS or 50 ng/mL EGF. The MTT assay revealed that gefitinib decreased NSCLC cell proliferation in a dose-dependent manner (Fig. 1A). H322 and H358 cells were more sensitive to gefitinib than were the other cell lines [H322 in 10% FBS, 47.5% (95% CI, 41.5-53.5%; *P* < 0.001); H322 in EGF, 46.8% (95% CI, 40.8-52.8%; *P* < 0.01); H358 in 10% FBS, 52.1% (95% CI, 50.0-54.2%; *P* < 0.001); and H358 in EGF, 41.6% (95% CI, 37.3-45.9%; *P* < 0.01) after 72 h]. The gefitinib concentrations required to inhibit H596, H226B, H226Br, H460, and H1299 cell growth by 50.0% were 10 to 20 times higher than were those for H322 and H358 cells.

We next determined the mechanisms responsible for the sensitivity of NSCLC cells to gefitinib. We first measured the basal levels of EGFR and pEGFR in the cell lines. As shown in Fig. 1B, all cell lines except H322 had high levels of EGFR expression. Four of these six cell lines also had high levels of pEGFR expression, indicating that no relationship exists between gefitinib response and EGFR activation. Because of the role of phosphatase and tensin homologue (PTEN) in NSCLC cells' resistance to EGFR TKIs (21), we next determined

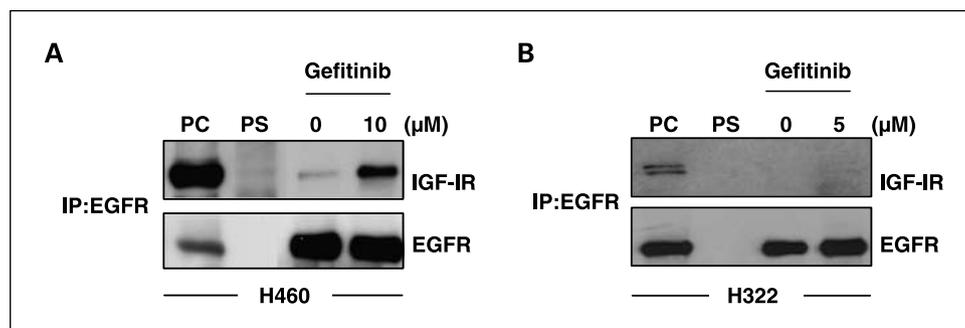
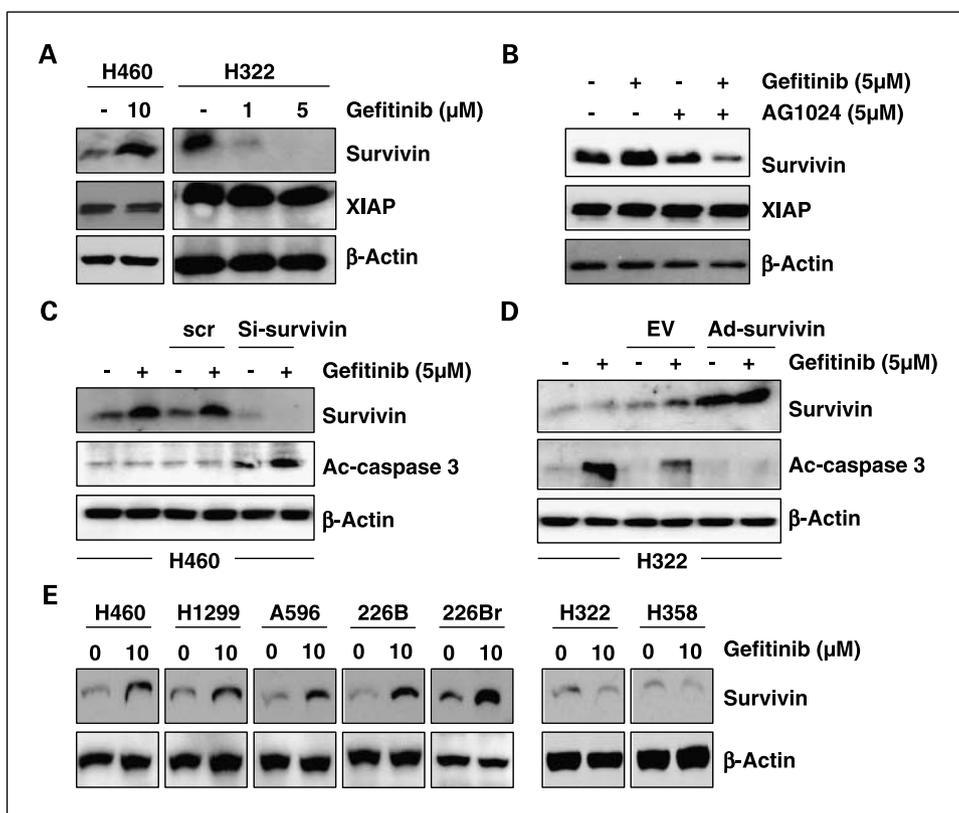


Fig. 3. Effect of gefitinib on EGFR:IGF-IR heterodimerization and activation of IGF-IR signaling pathway. Whole-cell extracts (3 mg) from (A) H460 and (B) H322 cells, left untreated or treated with gefitinib (10 and 5 $\mu\text{mol/L}$, respectively) for 3 d, were immunoprecipitated (IP) with anti-EGFR antibodies. The immunoprecipitates were subjected to Western blot analysis with the indicated antibodies. Input (PC) represents cell lysates (30 μg) that were not subjected to immunoprecipitation. Control immunoprecipitation was done using mouse preimmune serum.

Fig. 4. Role of survivin in the resistance of NSCLC cells to gefitinib. *A*, immunoblotting of survivin and XIAP in H460 and H322 cells treated with the indicated concentrations of gefitinib. *B*, effects of treatment with 5 $\mu\text{mol/L}$ gefitinib, 5 $\mu\text{mol/L}$ AG1024, or both on the expression of survivin and XIAP in H460 cells. *C* and *D*, effect of knockdown of expression or overexpression of survivin on H460 and H322 cells treated with gefitinib. H460 cells were transfected with scrambled (*scr*) or survivin siRNA and left untreated or treated with gefitinib for 48 h. H322 cells were infected with a control virus (Ad-EV or Ad-survivin) and incubated for 3 d in the presence of gefitinib. The protein extracts were subjected to Western blotting for evaluation of survivin and active caspase-3. *E*, Western blot analysis of survivin expression in NSCLC cell lines treated with 10 $\mu\text{mol/L}$ gefitinib. β -Actin was used as a loading control.



the level of PTEN protein expression. PTEN was expressed in all cell lines, suggesting that gefitinib resistance is not caused by a PTEN deficiency. We then determined the expression of IGF-IR, pIGF-IR, Akt, and pAkt and found that most cell lines had high levels of IGF-IR expression, which were associated with high levels of pIGF-IR expression. The levels of pIGF-IR, IGF-IR, and pAkt expression were higher in H460, H1299, A549, H226B, and H226Br cells than in H322 cells, and the levels of pEGFR, EGFR, and pp44/42MAPK expression did not substantially differ among these cell lines. H358 cells also had lower levels of IGF-IR expression than did H460, H1299, A549, H226B, and H226Br cells (data not shown). The two cell lines most sensitive to gefitinib treatment (H322 and H358) had the lowest level of IGF-IR expression, suggesting that IGF-IR is involved in NSCLC cells' sensitivity to gefitinib.

We selected four representative cell lines, two resistant (H1299 and H460) and two sensitive (H322 and H358) to gefitinib, to determine whether IGF-IR and its downstream signaling components confer resistance to gefitinib. We first determined the phosphorylated and unphosphorylated levels of IGF-IR, EGFR, Akt, and mammalian target of rapamycin (mTOR) in H460 and H322 cells after 72 h of treatment with gefitinib. The treatment (10 $\mu\text{mol/L}$ for H460 cells and 1 or 5 $\mu\text{mol/L}$ for H322 cells) resulted in the complete inhibition of pEGFR expression, verifying gefitinib's effects on EGFR tyrosine kinase activity (Fig. 1C). It was surprising that the levels of pIGF-IR, pAkt, pp44/42MAPK, and pmTOR expression increased in H460 cells after treatment with 10 $\mu\text{mol/L}$ gefitinib, the drug concentration that had completely suppressed pEGFR expression. However, these proteins remained suppressed in gefitinib-treated H322 cells. A gefitinib-induced increase in

pIGF-IR expression was observed in H1299 cells that had high levels of IGF-IR expression but not in H358 cells with low levels of IGF-IR expression (Fig. 1D). Taken together, these findings suggest that gefitinib treatment induces activation of the IGF-IR pathway and its downstream signaling components.

Effects of the combined blockade of the EGFR and IGF-IR pathways on the proliferation of NSCLC cells. To determine the roles of the IGF-IR signaling pathway in the development of gefitinib resistance, we evaluated the effects of gefitinib and AG1024, an IGF-IR TKI (22), on the proliferation and apoptosis of H460 cells.

Treatment with gefitinib and AG1024 efficiently blocked the gefitinib-induced increase in pIGF-IR expression in H460 cells (Fig. 2A). We observed more sensitivity to gefitinib when we simultaneously treated H460 cells with gefitinib and AG1024 than with either agent alone (Fig. 2B). In fact, the combined treatment resulted in synergistically enhanced antiproliferative effects on H460 cells [10 $\mu\text{mol/L}$ gefitinib, 59% (95% CI, 55.9-62.1%; $P < 0.001$); 5 $\mu\text{mol/L}$ AG1024, 70.1% (95% CI, 64.7-75.5%; $P < 0.001$); 10 $\mu\text{mol/L}$ gefitinib plus 5 $\mu\text{mol/L}$ AG1024, 28.5% (95% CI, 26-31%; $P < 0.001$; Table 1].

No obvious changes in cell cycle progression were found in H460 cells that had been treated with gefitinib for 3 days; however, a decrease in the G_1 phase population and an increase in the sub G_0/G_1 phase population were found in those treated with AG1024. Combined treatment with gefitinib (5 $\mu\text{mol/L}$) and AG1024 (5 $\mu\text{mol/L}$) for the same duration led to a decrease in the S population, a further decrease in the G_1 population, and an increase in the sub G_0/G_1 population ($P < 0.001$ for all; Fig. 2C). Moreover, TUNEL staining of cells treated with gefitinib and AG1024 showed a marked increase in apoptosis

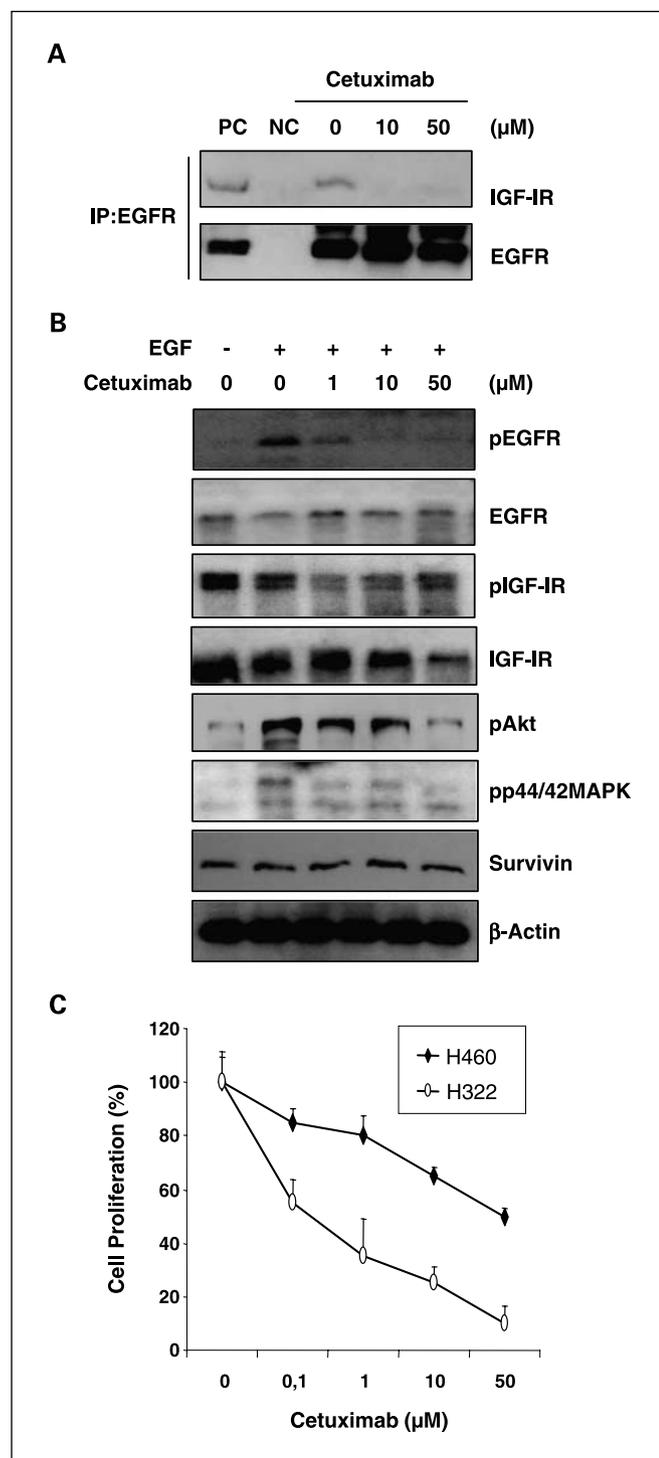


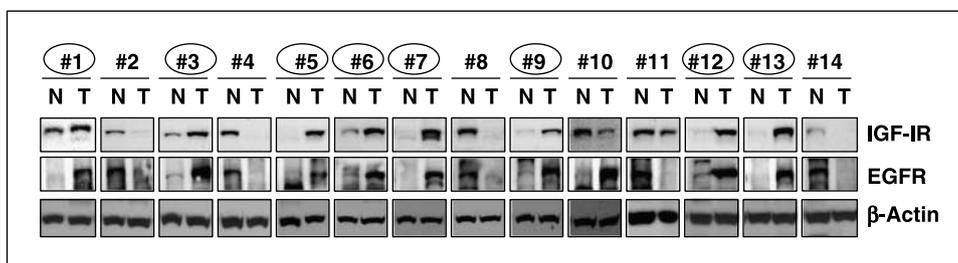
Fig. 5. Effects of cetuximab on EGFR:IGF-IR interaction in NSCLC cells. *A*, effect of cetuximab treatment on EGFR:IGF-IR heterodimerization and activation of the IGF-IR and EGFR signaling pathways. Whole-cell extracts (3 mg) from H460 cells were left untreated or treated with cetuximab (10 and 50 μmol/L) for 3 d. They were then immunoprecipitated with anti-EGFR antibodies, and the immunoprecipitates were subjected to Western blot analysis with the indicated antibodies. Input represents cell lysates (30 μg) that were not subjected to immunoprecipitation. Control immunoprecipitation was done using mouse preimmune serum (PS) as a negative control. *B*, Western blot analysis of survivin, pIGF-IR, IGF-IR, pEGFR, EGFR, pAkt, and pp44/42MAPK expression in H460 cells treated with cetuximab (1, 10, and 50 μmol/L) for 3 d in the absence or presence of EGF (50 ng/mL). *C*, results of the MTT assay of H460 and H322 cell lines after treatment with cetuximab in RPMI 1640 containing 10% FBS for 3 d. Points, mean value of eight identical wells of a single representative experiment; bars, upper 95% CIs.

(Fig. 2D); administration of 5 μmol/L of both gefitinib and AG1024 as single agents induced apoptosis in 0.2% (95% CI, 0.0-2.8%; $P > 0.05$) and 30.1% (95% CI, 14.3-37.6%; $P < 0.05$) of cells, respectively, whereas combined treatment with gefitinib and AG1024 induced apoptosis in more than 90% (95% CI, 64.4-89.8%; $P < 0.001$). These findings suggest that the IGF-IR pathway provides an alternative proliferation or survival mechanism for NSCLC cells in which the EGFR pathway is blocked by gefitinib treatment. Thus, cotargeting the IGF-IR and EGFR pathways may be an effective therapeutic strategy for NSCLC.

Gefitinib induces heterodimerization of IGF-IR and EGFR in H460 cells. EGFR signaling can be modulated by several mechanisms, including heterodimerization with other members of the EGFR family, such as HER-2, and other growth factor receptors, such as IGF-IR (15, 16, 23, 24). We recently observed increased heterodimerization between EGFR and IGF-IR in NSCLC cells after treating them with erlotinib, an EGFR TKI (25). In the current study, we determined the effects of gefitinib on the interaction between EGFR and IGF-IR in H460 and H322 cells. EGFR immunoprecipitates from gefitinib-treated H460 cells showed an obvious increase in IGF-IR binding, whereas control immunoprecipitates that had been treated with preimmune serum had no immunoreactive bands (Fig. 3A). In contrast, both EGFR and control immunoprecipitates from H322 cells lacked immunoreactive bands (Fig. 3B). These results suggest that EGFR and IGF-IR interact through physical contact, and that treatment with gefitinib increases the heterodimerization of EGFR and IGF-IR in H460 cells.

Role of survivin in resistance of NSCLC cells to gefitinib. We next evaluated the signaling mediator that connects gefitinib-induced IGF-IR pathway activation and H460 cell survival. Because inhibitors of apoptosis protein (IAP) such as survivin and XIAP play a role in tumor cells' resistance to chemotherapeutic drugs (26), we determined the effects of gefitinib on the expression of these proteins in H460 and H322 cells. As shown in Fig. 4A, the expression level of survivin but not XIAP markedly increased in H460 cells after treatment with gefitinib. In contrast, no changes were detected in the expression levels of survivin or XIAP in these cells. Moreover, treatment with gefitinib (5 μmol/L) and AG1024 (5 μmol/L) efficiently suppressed the gefitinib-induced increase in survivin expression (Fig. 4B). We then evaluated the influence of survivin on gefitinib-induced apoptosis in NSCLC cells by knockdown of expression or overexpression of survivin. H460 cells transfected with control scrambled or survivin siRNA before treatment with 10 μmol/L gefitinib (a concentration that did not induce apoptosis) had an obvious increase in the rate of apoptosis as determined by a Western blot analysis of an increase in activated caspase-3 expression (Fig. 4C). Furthermore, among H322 cells infected with Ad-survivin, the level of gefitinib-induced apoptosis was substantially reduced (Fig. 4D). These findings suggest that NSCLC cells can escape apoptosis by inducing survivin expression. To determine whether the induction of survivin by gefitinib is related to the resistance of cell lines to the drug, we compared the expression of survivin in a subset of NSCLC cell lines that had been treated with TKI. The five nonsensitive or resistant NSCLC cell lines (H460, H1299, A596, 226B, and 226Br cells) showed an increase in survivin expression after treatment with gefitinib; no increase

Fig. 6. Expression of EGFR and IGF-IR in paired lung tumor and normal tissue specimens from patients with NSCLC. Proteins were extracted from lung tumor and healthy lung tissue and subjected to Western blot analysis to determine the expression of EGFR and IGF-IR. β -Actin was used as a loading control. The samples marked with a circle had increased IGF-IR and EGFR expression.



was found in the two most sensitive NSCLC cell lines (H322 and H358; Fig. 4E). These results suggest that NSCLC cells' resistance to gefitinib can be mediated by survivin induction.

Effects of cetuximab on EGFR:IGF-IR interaction in NSCLC cells. We evaluated the effects of cetuximab on the interaction between IGF-IR and EGFR. EGFR precipitates from cetuximab-treated H460 cells showed no detectable binding to IGF-IR, suggesting that cetuximab suppressed the interaction between EGFR and IGF-IR (Fig. 5A). Moreover, treatment with cetuximab decreased EGF-stimulated pEGFR expression in H460 cells in a dose-dependent manner, without inducing pIGF-IR, pAkt, pp44/42MAPK, or survivin expression (Fig. 5B). A cell proliferation assay showed that H460 cells were more resistant to the antibody than were H358 cells (Fig. 5C). Overall, these findings suggest that heterodimerization between IGF-IR and EGFR, activation of IGF-IR, and induction of survivin expression are at least partly responsible for the induction of acquired resistance to EGFR TKIs, but not to the monoclonal antibody against EGFR in NSCLC cells.

Expression of EGFR and IGF-IR in human lung tissue. Our data suggested that IGF-IR expression has an important role in determining NSCLC cells' sensitivity to EGFR TKIs. Therefore, we determined the levels of EGFR and IGF-IR expression in NSCLC tissue. Nine of the fourteen tumor specimens had higher levels of EGFR expression than did normal tissue specimens from the same patients. Eight of those specimens also had higher levels of IGF-IR expression than those did in normal tissue (Fig. 6).

Discussion

We have previously shown that erlotinib, an EGFR TKI, induces heterodimerization of EGFR/IGF-IR, resulting in the activation of IGF-IR and induction of Akt/mTOR-mediated synthesis of survivin protein, which protects NSCLC cells from the drug-induced apoptosis (25). In the present study, we show that the activation of the IGF-IR via heterodimerization of EGFR/IGF-IR and consequent induction of survivin expression also mediate gefitinib resistance in NSCLC cells, confirming and extending our previous findings (25). Our studies also show that IGF-IR pathway did not affect antiproliferative activities of cetuximab, a monoclonal antibody blocking EGFR. Overall, these findings suggest that heterodimerization between IGF-IR and EGFR, activation of IGF-IR, and induction of survivin expression contribute to the acquired resistance to EGFR TKIs, but not to the monoclonal antibody against EGFR in NSCLC cells.

In clinical trials, the EGFR TKIs, such as erlotinib and gefitinib, were found to be effective in the treatment of patients with NSCLC (27, 28). These agents were approved for NSCLC

treatment after resulting in response rates of 10% and survival advantages in patients previously treated with chemotherapy (29). However, large-scale phase III clinical trials in advanced NSCLC have had contrasting results (13, 14, 29, 30). In the current study, we found that gefitinib treatment did not inhibit NSCLC cell proliferation at doses sufficient to suppress EGFR activation, suggesting that the development of resistance to EGFR TKIs is caused by the activation of alternative cell survival signaling mechanisms.

To develop better anticancer therapeutic strategies using gefitinib, we sought to identify the pathways whereby NSCLC cell trigger alternative survival signaling. PTEN expression and EGFR and Kirsten ras (*KRAS*) somatic mutation have been involved in the cellular response to EGFR-targeted therapy (31–33). Bianco et al. (34) found that the loss of PTEN expression in the MDA-468 breast cancer cell line contributed to gefitinib resistance. These findings suggest that PTEN-deficient cell lines maintain their Akt activity and survive when EGFR is inactivated. However, deletion of PTEN does not frequently occur in NSCLC cells (31, 35). In fact, all NSCLC cell lines used in our study expressed PTEN, suggesting that gefitinib resistance in NSCLC cells is not caused by a PTEN deficiency. Similarly, the NSCLC cell lines without somatic mutations of *EGFR* responded to gefitinib, which is consistent with previously reported findings (36, 37). Moreover, in our study, gefitinib showed antiproliferative effects on H358 cells that have somatic mutation in *KRAS* (38). Together, these findings indicate that expression of PTEN and mutational status of *EGFR* and *KRAS* are not entirely responsible for NSCLC cells' resistance to gefitinib.

Our *in vitro* data suggest that the cross-talk between the IGF-IR and EGFR signaling pathways is involved in the development of gefitinib resistance in NSCLC cells. First, we found that the levels of IGF-IR and pIGF-IR expression in NSCLC cells were inversely associated with the antiproliferative effects of gefitinib. Second, gefitinib induced phosphorylation of IGF-IR and its downstream mediators in NSCLC cell lines with high levels of IGF-IR expression. Third, gefitinib induced heterodimerization between IGF-IR and EGFR and survivin expression in the high IGF-IR-expressing H460 cells but not in the low IGF-IR-expressing H322 cells. Finally, gefitinib exhibited apoptotic activity in H460 cells, in which IGF-IR activation was blocked. These NSCLC cell lines have shown a similar response to the erlotinib treatment (25). EGFR and IGF-IR have similar extracellular domain structures (39); therefore, it is plausible that EGFR TKIs induce a direct interaction between EGFR and IGF-IR, leading to the activation of IGF-IR pathways and induction of survivin expression and, thus, maintain NSCLC cell proliferation. Recent reports showing a direct interaction between EGFR and IGF-IR (40, 41) also support our hypothesis.

The induced expression of survivin found in our study seemed to protect H460 cell from apoptosis in the presence of gefitinib: survivin overexpression protected H322 cells from gefitinib-induced apoptosis. In addition, gefitinib induced apoptosis in H460 cells, in which transfection with survivin-specific siRNA silenced survivin expression. Survivin is a member of the IAP family and is associated with both cancer progression and drug resistance (42, 43). Therefore, the resistance and sensitivity of NSCLC cells to gefitinib may be determined, at least in part, by their ability to activate the IGF-IR-mediated pathway and induce survivin expression. Most strikingly, we found no evidence of EGFR and IGF-IR heterodimerization, IGF-IR activation, and induction of survivin expression in H460 cells after cetuximab treatment. These findings suggest that activation of the IGF-IR pathway and the subsequent expression of survivin are at least partly responsible for the sensitivity of NSCLC cells to EGFR TKIs but not to anti-EGFR monoclonal antibodies.

In summary, we showed that the cross-talk between IGF-IR and EGFR has a specific role in inducing gefitinib resistance in NSCLC cells. Gefitinib induced heterodimerization of EGFR and IGF-IR; stimulated IGF-IR and downstream pathways, including phosphoinositide-3-kinase/Akt/mTOR and p44/42 MAPK; and increased survivin expression in NSCLC cell lines. Overexpression of the EGFR family and its ligands have been found in 30% to 80% of NSCLC (30, 44, 45). However, we found no

interaction between EGFR and IGF-IR in NSCLC cells with low levels of IGF-IR expression, suggesting that the basal level of IGF-IR expression is important for initiating the formation of the EGFR:IGF-IR complex. Nine of the fourteen (64%) tumor specimens in our study exhibited higher levels of EGFR expression, and eight of those had related higher IGF-IR expression compared with paired normal tissue. The number of IGF-IRs may determine the response to IGF-I; activated IGF-IRs in sufficient numbers change the mode of IGF's effect from nonmitogenic to mitogenic, resulting in cell cycle progression, translation, and DNA synthesis (46). Overexpression of IGF-IR has been associated with survival of NSCLC patients treated with gefitinib (47). Therefore, our newly identified mechanism of EGFR TKI resistance could provide new therapeutic avenues for NSCLC. Treatment with the anti-EGFR monoclonal antibody cetuximab may be effective once resistance to EGFR TKIs has been established, and the use of combination regimens with EGFR TKIs and IGF-IR inhibitors may be effective at treating NSCLC in patients with high levels of EGFR and IGF-IR expression. However, the results of an *in vitro* study with a limited number of cell lines are not sufficient to generalize the roles of the IGF-IR signaling pathway and survivin expression in gefitinib resistance in NSCLC cells. Further clinical trials are needed to determine whether combined treatment with EGFR TKIs and IGF-IR pathway inhibitors enhances objective responses and survival in patients with NSCLC.

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