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ORIGINAL ARTICLE

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Activated Src and Ras induce gefitinib resistance by activation of signaling pathways downstream of epidermal growth factor receptor in human gallbladder adenocarcinoma cells

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Abstract *Purpose*: Although gefitinib, a selective inhibitor of epidermal growth factor receptor (EGFR) tyrosine kinase, has been demonstrated to exhibit its antitumor activity by the blockade of EGF receptor, the role of signaling pathways downstream of EGFR in gefitinib sensitivity remains unknown. In this study, we investigated the mechanistic role of Src and Ras, major oncogene products implicated in the pathogenesis of many human cancers in gefitinib sensitivity. *Methods*: Using parental and v-*src*- or c-H-*ras*-transfected HAG-1 human gallbladder adenocarcinoma cell lines, effects of gefitinib on cytotoxicity, cell cycle purtubation and apoptosis, and tyrosine phosphorylation of EGFR, Akt, and Erk were determined by WST-1 assay, flow cytometry, and Western blots, respectively. *Results*: Activated Ras and Src conferred a strong resistance to gefitinib by nearly 30-fold and 200-fold, respectively. Gefitinib induced accumulation of cells in the G0/G1 phase of the cell cycle at 24-h, with progressive expansion of apoptotic cell population in parental HAG-1 cells, but these effects were completely abolished in v-*src*- or c-H-*ras*-transfected cell line. Upon gefitinib treatment, EGFR activation and subsequent downstream activation through Erk and Akt were significantly inhibited in HAG-1 cells. By contrast, gefinitib failed to inhibit the activation of both Akt and Erk in v-src-transfected cells and Erk, but not Akt in c-H-ras-transfected cells, despite the blockade of EGFR activation in these respective cell lines. Treatment of v-src-transfected cells with herbimycin A, a Src tyrosine kinase inhibitor, partially reversed the gefitinib resistance, with concomitant inhibition of Akt and Erk. *Conclusion*: Our results suggest that activated Ras and Src could induce gefitinib resistance by activating either or both of Akt and Erk signaling pathways, thus providing a strategic rationale for assessment of these specific signaling molecules downstream of EGFR to customize treatment.

Keywords Gefitinib · EGFR · Akt · Erk · Apoptosis · Src · Ras

Abreviatons EGFR: Epidermal growth factor receptor · EGF: Epidermal growth factor · RTK: Receptor tyrosine kinase · MAPK: Mitogen activated protein kinase · Erk: Extracellular signal-regulated kinase · PI-3K: Phosphatidylinositol 3'-kinase

Introduction

Over the past decade, a variety of receptor tyrosine kinases have been identified to play a central role in the pathogenesis of various human cancers. Among these, epidermal growth factor receptor (EGFR) is overexpressed in a wide variety of epithelial malignancies including non-small cell lung, head, neck, colon, and breast cancers [1–4]. The overexpression of this receptor has been also detected in gallbladder cancer [5–7], a highly lethal disease with no known curative modality. Upon ligand binding, EGFR is activated through autophosphorylation by forming homodimerization or heterodimerization with other members of the HER family [8, 9], and transduces a variety of signals to downstream signal transduction cascades that lead to cellular proliferation and survival [10, 11]. Therefore, the inhibition of the EGFR signaling cascades may provide a rational therapeutic target of these chemotherapy-refractory cancers.

Gefitinib, a quinazoline derivative that inhibits EGFR tyrosine kinase activity, has been shown to be effective in

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preclinical studies and in late stages of clinical trials for non-small cell lung cancer [12, 13], although its activity appears not to be associated with the expression level of EGFR, but with the certain background of population, specific types of histology, and activating somatic mutations in the tyrosine kinase domain of EGFR [14–16]. This drug has been shown to inhibit major cell survival and growth signaling pathways such as Ras-Raf-Erk kinase pathway and phosphatidylinositol-3 kinase (PI-3K)-AKT pathway, as a consequence of inactivation of EGFR [17–20]. Conversely, persistent activity of the Ras/Erk and PI3K/Akt kinase pathways contributes to resistance of NSCLC cells to EGFR inhibitors [21–23]. Therefore, signaling molecules that activate these pathways might have the possibility to induce gefitinib resistance. The representative signaling molecules that share downstream signaling pathways with EGFR are Ras and Src, crucial cellular oncogene products implicated in the pathogenesis of many human cancers. Activation of Ras through gene amplification or point mutation was most frequently identified in a variety of human cancers, including adenocarcinoma of the pancreas, colon, and lung [24]. Ras transmits a signal to the serine/threonine kinase Raf, which subsequently activates mitogen-activated protein (MAP) kinase, resulting in cell proliferation through the transcriptional activation of a variety of targets, such as c-fos [25]. Activation of Src as detected by the elevation of tyrosine kinase activity was also identified in a variety of human cancers, such as breast, colon, skin, bladder, and pancreas [26]. Specifically, c-Src has been found to be highly activated in colon cancer metastasized to the liver [27]. Src phosphorylates a number of intracellular substrates on tyrosine residue [28], resulting in a generation of mitogenic and tumorigenic signals from Src to downstream signaling such as PI 3K-Akt and Ras-Raf-Erk kinase pathways.

Since the role of such oncogenic signalings in the gefitinib sensitivity remains to be clarified, we have investigated here the mechanistic role of Src and Ras, in gefitinib sensitivity, specifically through Akt and Erk pathways using parental and v-*src*- or H-*ras*-transfected HAG-1 human gallbladder carcinoma cell lines. In those cell lines showing EGFR-independent activity of the PI3K/Akt or Ras/Erk pathways, the relationship between the activity of these pathways and the ability of gefitinib to induce apoptosis was assessed.

Materials and methods

Cells and cultures and chemicals

HAG-1 is a human epithelial cell line derived from a moderately differentiated adenocarcinoma of the gallbladder [29]. No mutations and amplifications of H-, K-, or N-*ras* genes have been detected in this cell line. The HAG/ras5-1 cells were obtained by transfecting HAG-1 parental cells with activated c-H-*ras*, while HAG/src3-1 cells that express $p60^{v\text{-}src}$ protein were obtained by transfection of the pSV2/v-*src* into HAG-1 cells [30]. HAG/neo3-5 cells were obtained by transfection of HAG-1 cells with pSV2neo alone, which carries the gene for neomycin resistance. v-Src has a constitutively activated tyrosine kinase activity by the lack of negative regulatory domain. HAG-1 cells were cultured in Dulbecco's minimum essential medium (DMEM, Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 100 UI/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. HAG/ras5-1 and HAG/src3-1 cells were grown in the same conditions, except that G418 (200 μg/ml) was added to the culture medium.

Gefitinib was kindly provided by AstraZeneca (Macclesfield, UK). Stock solutions were prepared in dimethyl sulfoxide (DMSO, Wako, Osaka, Japan) and stored at −20°C. The final concentration of DMSO for all experiments and treatments (including controls where no drug was added) was maintained at less than 0.02%. Herbimycin A was purchased from Wako Chimicals (Osaka, Japan). These conditions were found to be non-cytotoxic. Anti-EGF receptor, anti-MAPK, anti-Akt antibodies, and Protein A agarose were purchased from BD Biosciences (San Jose, CA USA).

Cytotoxicity, cell cycle analysis, and apoptosis measurement

The cytotoxic effect of gefitinib on HAG-1 cells was assessed by WST assay using manufacturer's instructions (DOJIN, Kumamoto, Japan). The WST assay is a colorimetric method in which the intensity of the dye is proportional to the number of the viable cells. Briefly, 100 μl cell suspension of HAG-1 cells was seeded into a 96-well plate at a density of 1,000 cells /well. After overnight incubation, 100 μl of gefitinib solutions at various concentrations were added. The effect of herbimycin A, a Src tyrosine kinase inhibitor, on the resistance to gefitinib was assessed in HAG/sec3-1 cells by co-treatment of gefitinib and herbimycin A. After incubation for 69 h at 37°C, 10 μl of solution A and solution B mixture was added to each well and the plates were incubated for a further 3 h at 37°C. Then the optical density was measured at 450 and 620 nm using an IMMUNO-MINI NJ-2300 spectrophotometer (Nalge Nunc International, Chester, NY, USA). Each experiment was performed using six replicate wells for each drug concentration and was carried out independently for three times. The IC_{50} value was defined as the concentration needed for a 50% reduction in the absorbance.

Control or gefitinib-treated cells were harvested by trypsinization, washed with PBS, and then fixed in 100% ethanol and stored at 4°C for up to 3 days prior to cell cycle analysis. After the removal of ethanol by centrifugation, cells were then washed with PBS and stained with a solution containing PI and RNase A on ice for 30 min. Cell cycle analysis was performed on a Becton Dickinson FACS/Calibur Flow Cytometer using the CELLQuest or

ModFit 3.0 software packages (Becton Dickinson, San Jose, CA USA), and the extent of apoptosis was determined by measuring the sub-G1 population.

Immunoprecipitation and Western blot analysis

The cells were washed twice with ice-cold PBS and scraped into 1 ml of radioimmunoprecipitation assay lysis buffer (50 mM Tris–HCl (pH 7.6), 300 mM NaCl, 0.4% (v/v) TritonX-100, 400 μM EDTA•2Na, 400 μM Na_3VO_4, 10 mM NaF, 10 mM $Na_4P_2O_7$•$10H_2O$, 1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin). After removal of cell debris by centrifugation, protein concentrations of the supernatants were determined by using a BCA protein assay kit (Pierce, Rockford, IL). For Western blot, equal amounts of proteins or immunoprecipitated target proteins were resolved by 10% SDS-PAGE (polyacrylamide gel electrophoresis) and electrotransferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA). Non-specific binding sites were blocked by incubating the membranes in blocking buffer (5% non-fat milk in 1× TBS with 0.1% Tween-20) at room temperature for 1 h. The membranes were then incubated with primary antibodies against either phospho-EGFR (Tyr1068, Cell Signaling Technology), phospho-p44/42 MAPK (Thr202/Tyr204, Cell Signaling Technology), or phospho-Akt (Ser473, Cell Signaling Technology). The membranes were hybridized with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology). Immunoblots were developed with the enhanced chemiluminescence (ECL) system from Amersham Biosciences (Buckinghamshire, UK) and were then exposed to ECL hyperfilm according to the manufacture's instructions (Amersham Biosciences, Buckinghamshire, UK). The blots were striped and reprobed with primary antibodies against EGFR (2232, Cell Signaling Technology), MAPK (9102, Cell Signaling Technology), and Akt (9272, Cell Signaling Technology). For reblotting, membranes were incubated in stripping buffer (62.5 mM Tris/HCl, pH 6.8/2% (w/v) SDS/100 mM 2-mercaptoethanol) for 30 min at 50°C before washing, blocking, and incubating with antibody. Triplicate determinations were made in separate experiments.

Statistical analysis

The data were analyzed by the Mann-Whitney *U* test for statistical significance of the difference between groups. A P value of <0.01 was considered to indicate statistical significance.

Results

Effect of gefitinib on cytotoxicity of HAG-1, HAG/src3-1, and HAG/ras5-1 cells

To determine whether activated Src and Ras affect the gefinitib sensitivity, we examined the drug sensitivity in H-ras-transfected HAG/ras5-1 and v-src-transfected HAG/src3-1cells, and compared their IC_{50} values with those of parental HAG-1 cell line. The IC_{50} values of 72-h exposure of gefitinib were 0.12±0.05 μM for HAG-1 cells, 3.6±0.52 μM for HAG/ras5-1 cells, and 22±6.7 μM for HAG/src3-1 cells, indicating approximately 30-fold and 200-fold increases in resistance to gefitinib in HAG/ras5-1 and HAG/src3-1 cells, respectively, as compared with that of parental HAG-1 cells (Fig. 1).

Time course analysis of the effect of gefitinib on cell cycle progression and apoptosis

To examine whether the inhibitory effect observed in cytotoxicity assays reflect the arrest of cell cycle or apoptotic cell death, cells were treated with gefitinib for indicated times and the cell cycle progression and apoptosis was evaluated after PI staining by fluorescence-activated cell sorting analysis. When HAG-1 parental cells were treated with gefitinib at a dose of 1 μM, the proportion of cells in a G0/G1 phase increased from 60 to 87 % at 24 h from the beginning of the treatment, with corresponding decrease in cells in S and G2-M phase and reached a plateau afterward. The percentage of sub-G0/G1 cell population became evident after (72 h, 20%) 72 h post-treatment and progressively increased upon further treatment (96 h, 34%; 120 h, 50%) (Fig. 2a). Because cells in the sub-G0/G1 population represent apoptotic cells, the cytotoxicity by the treatment of gefitinib appeared to be due to progressive expansion of apoptotic cell population. By contrast, when HAG/ras5-1 or HAG/src3-1cells was treated with the same concentration of gefitinib, neither arrest of cells in the G0/G1 phase nor the sub-G0/G1 cell population became evident with incubation times in both cell lines (Fig. 2b, c).

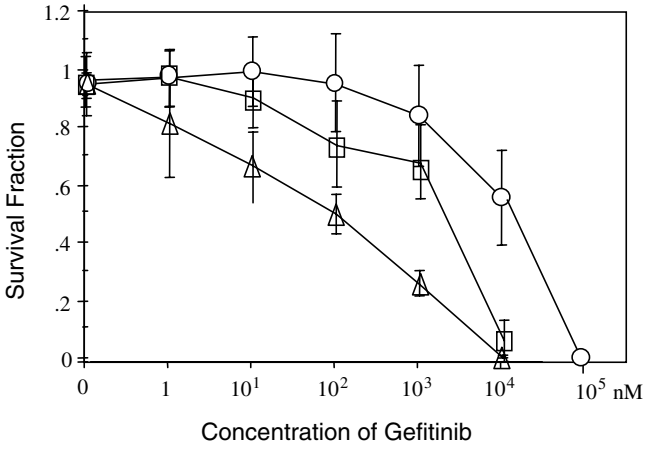

Fig. 1 Cytotoxicity of gefitinib against HAG-1 (*open triangle*), HAG/ras5-1 (*open square*), and HAG/src3-1 (*open circle*) cells. Cells were treated with various concentration of gefitinib for 72-h and assessed for cytotoxicity by WST-1 assay as described in Materials and methods. The data represent the means from three independent experiments. Bars, SD

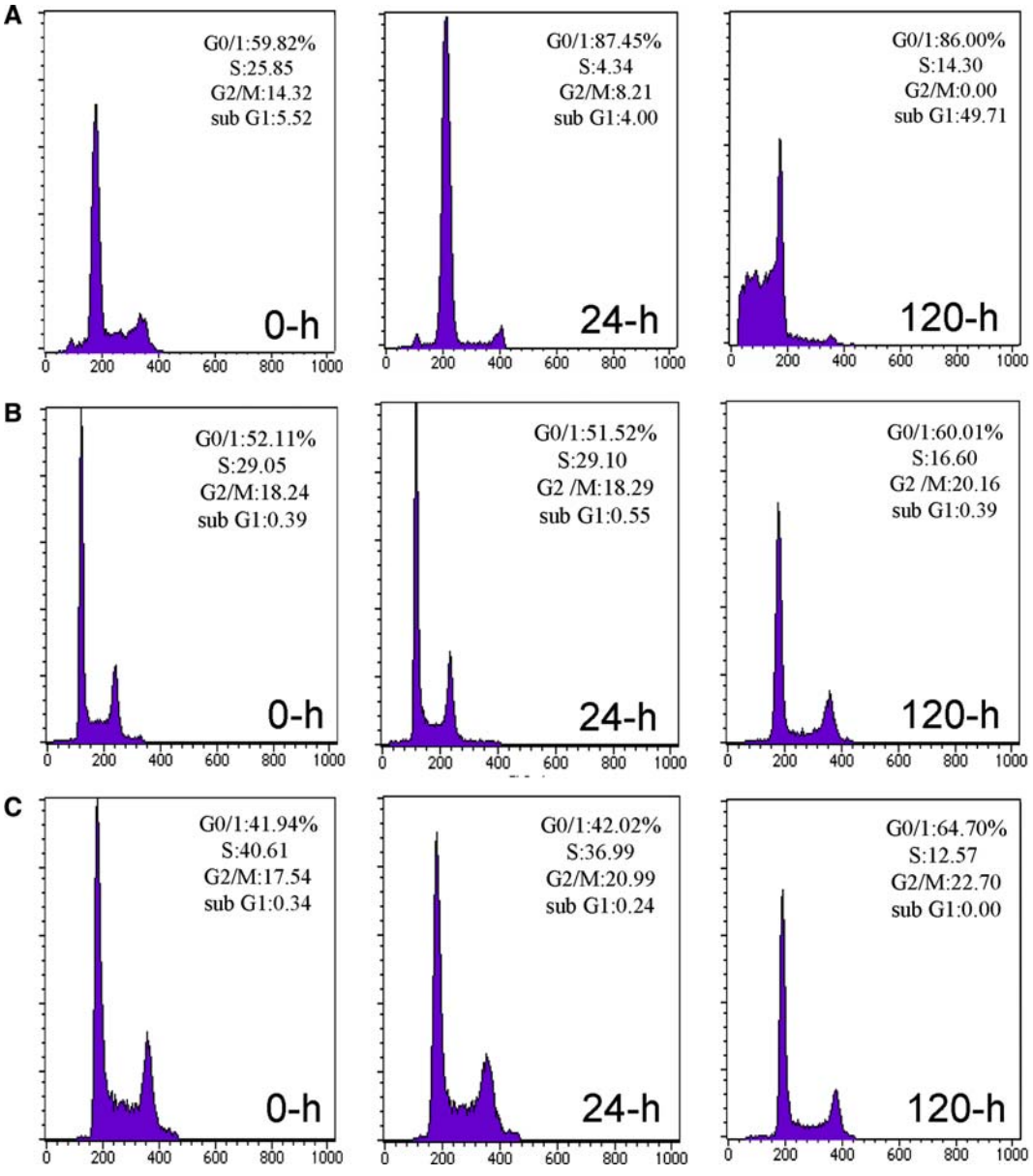

Fig. 2 Time course analysis of the effect of gefitinib on Cell cycle progression and apoptosis. HAG-1 (**a**), HAG/ras5-1 (**b**), and HAG/src3-1 (**c**) cells were stained with propidium iodide after exposure to gefitinib (1.0 μM) for 0, 24, and 120 h and analyzed by flow cytometry. Percentages of the total cell population in the different phases of cell cycle were determined with curve fitting using the ModFit 3.0 software. The mean values for each phase of the cell cycle are shown on the top right of each panel. Representative results of at least three experiments are shown

Inhibition of tyrosine phosphorylation of the EGFR by gefitinib in parental HAG-1, HAG/ras5-1, and HAG/src3-1 cells

To demonstrate the effect of gefitinib on the EGFR activation, we examined the expression and activation of EGFR in these three cell lines. As shown in Fig. 3, phosphorylated EGFR at tyrosine was detected without EGF stimulation in all three cell lines. When parental HAG-1 cells were treated with 1 μM gefitinib, constitutive phosphorylation of EGFR was significantly inhibited at 2-h post-treatment and remained to be suppressed over 24 h, without changing the relative amount of EGFR. Similarly, in HAG/ras5-1 cells, the same concentration of gefitinib completely suppressed the constitutive phosphorylation of EGFR at 2-h post-treatment and remained to be suppressed over 24 h. In HAG/src3-1 cells, however, inhibition of gefitinib on the constitutive phosphorylation of EGFR appears to be modest, declining gradually over 12-h post-treatment, with subsequent recovery to the initial level at 24 h.

Effects of gefitinib on autophosphorylation of Akt and Erk in parental HAG-1, HAG/ras5-1, and HAG/src3-1 cells

To demonstrate the effect of gefitinib on signaling pathways downstream of EGFR, we examined the expression

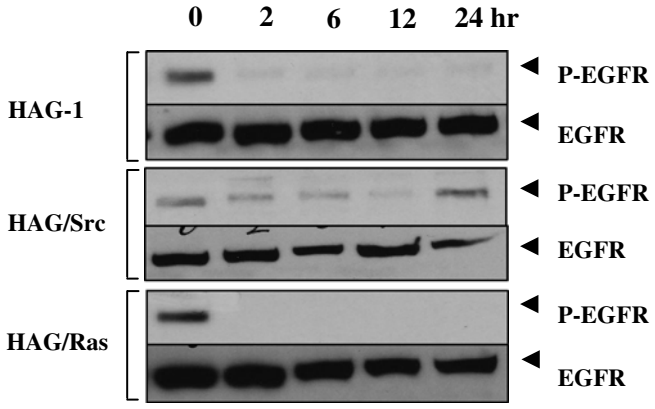

Fig. 3 Effect of gefitinib on the phosphorylation of EGFR in HAG-1, HAG/ras5-1, and HAG/src3-1 cells. Cells were exposed to 1 μM of gefitinib and incubated for indicated times. Western blots are shown for phospho- and total EGFR

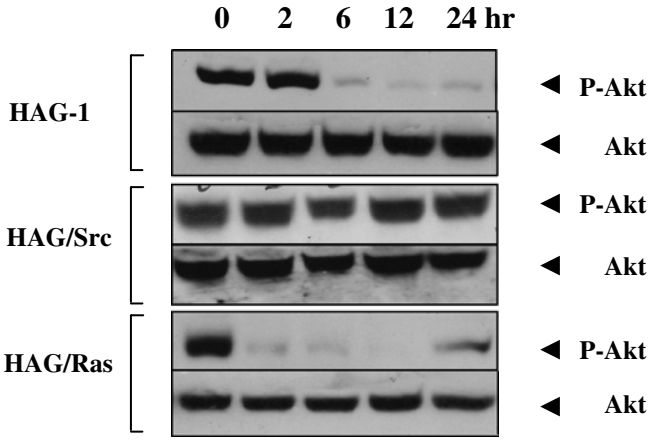

Fig. 5 Effect of gefitinib on the phosphorylation of AKT in HAG-1, HAG/ras5-1, and HAG/src3-1 cells. Cells were exposed to 1 μM of gefitinib and incubated for indicated times. Western blots are shown for phospho- and total Akt

and activation of Erk and Akt in these three cell lines. As shown in Fig. 4, tyrosine phosphorylation of Erk was seen in all the cell lines. Upon treatment with gefitinib, the phosphorylation of Erk was significantly suppressed only in parental HAG-1 cells, but was never suppressed in HAG/src3-1 and HAG/ras5-1 during the incubation periods. As shown in Fig. 5, tyrosine phosphorylation of Akt exhibited by HAG/src3-1 cells was never inhibited by gefitinib treatment during the incubation period. By contrast, these constitutive activations of Akt were significantly inhibited by gefitinib in parental HAG-1 cells. Of note, the activation of Akt is similarly inhibited in H-ras-transfected HAG/ras5-1 cells, indicating that activated Ras could not drive Akt activation in these cells.

Effects of Src kinase inhibitor on gefitinib resistance and Src-induced Akt/Erk tyrosine phosphorylation

To determine whether Src kinase activity is responsible for resistance to gefitinib in v-src-transfected cells, we studied the effect of herbimycin A on the gefitinib resistance in mock-transfected (HAG/neo3-5) and v-src-transfected (HAG/src3-1) cell lines. Combined treatment with gefitinib and 50 ng/ml of herbimycin A did not alter the sensitivity of gefitinib in HAG/neo3-5 cells, but significantly reduced gefitinib resistance in v-src-transfected HAG/src3-1 cells (Fig. 6). In v-src-transfected cells, gefitinib did not affect the phosphorylation status of both Akt and Erk (Fig. 7a), but exhibited its inhibitory activity against Akt and Erk phosphorylation when co-treated with herbimycin A (Fig. 7b). These data suggest that gefitinib resistance observed in HAG/src3-1 cells might be induced by Src kinase activity through activation of Akt and Erk.

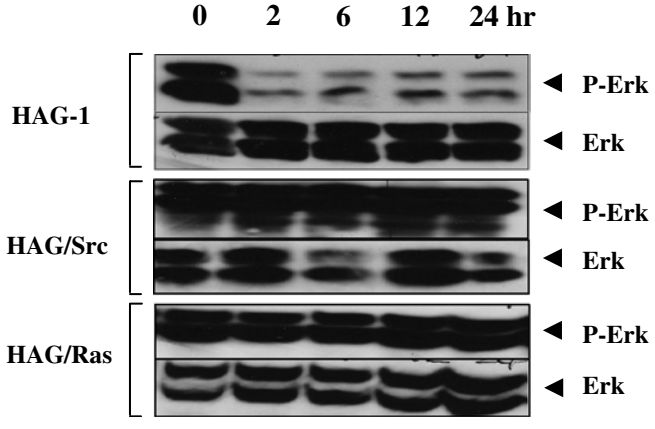

Fig. 4 Effect of gefitinibb on the phosphorylation of ERK in HAG-1, HAG/ras5-1, and HAG/src3-1 cells. Cells were exposed to 1 μM of gefitinib and incubated for indicated times. Western blots are shown for phospho- and total Erk

Discussion

In the present study, we found that the IC_{50} of gefitinib against HAG-1 cells was 0.12 μM for 72 h exposure, a comparable IC_{50} concentration exhibited by highly sensitive A431 squamous carcinoma cell line [21]. Using this gefitinib-sensitive cell line, we examined the role of activated Ras and Src in the gefitinib resistance after transfection with activated c-H-*ras* or v-*src*, since these oncogenes are major signaling molecules that share downstream signaling pathways with EGFR and closely associated with the pathogenesis of many human cancers. We found that activation of Ras and Src conferred a strong resistance to gefitinib by nearly 30-fold and 200-fold, respectively, and abolished completely its apoptosis-inducing activity. Moreover, v-Src-induced gefitinib resistance was partially reversed by the Src kinase inhibitor, indicating a potential role of Src tyrosine kinase activity in inducing gefitinib resistance. Recently, activating K-ras mutations has been shown to be specifically detected in gefitinib-resistant cells, suggesting that the occurrence of K-Ras mutations is correlated with

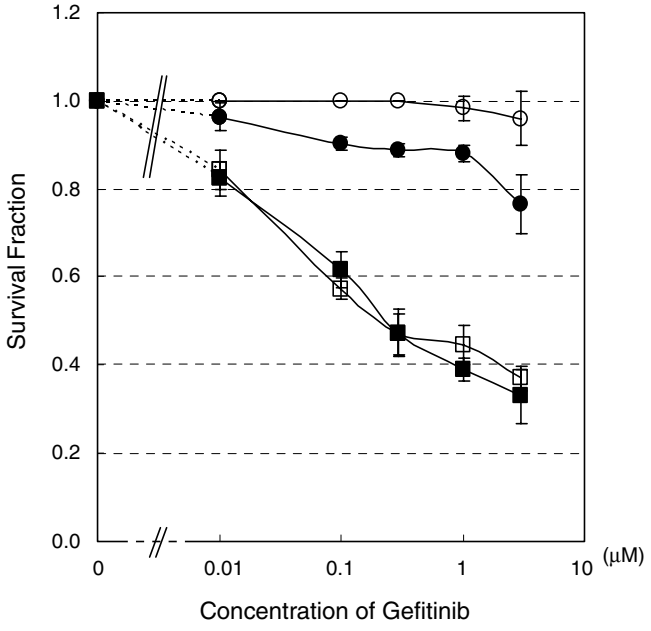

Fig. 6 Effects of herbimycin A on the cytotoxicity of gefitinib in HAG/neo3-5 and HAG/src3-1 cells. Cells were treated with various concentration of gefitinib with or without 50 ng/ml of herbimycin A and assessed for cytotoxicity by WST-1 assay as described in Materials and methods. HAG/neo3-5 cells with (*filled square*) or without (*open square*) herbimycin A. HAG/src3-1 cells with (*filled circle*) or without (*open circle*) herbimycin A. The data represent the means from three independent experiments. Bars, SD

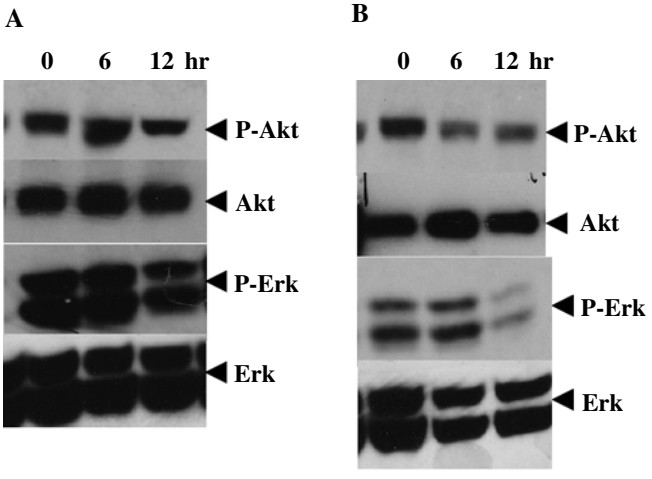

Fig. 7 Effects of gefitinib on the phosphorylation of Akt and Erk in HAG/src3-1 cells. The cells were exposed to 1 μM of gefitinib alone (**a**) or in combination with 100 ng/ml of herbimycin A (**b**) and incubated for indicated times. Western blots are shown for phospho- and total Akt and Erk

resistance to EGFR antagonists [31]. Taken together, these data suggest that concomitant presence of either of these activated oncogenes could induce a strong resistance to gefitinib.

In the downstream of EGFR, there are two major cell survival and growth signaling pathways, i.e., Ras/Raf-1/Erk pathway and PI-3K-AKT pathway. Recently, it has been reported that simultaneous inhibition of both Ras/Raf-1/Erk and PI-3K/AKT pathways are important for the execution of gefitinib-induced antiproliferative effect and apoptosis, and that persistent activity of either of these signaling pathways is involved in the decreased or lack of sensitivity to EGFR inhibitor [21, 32]. Therefore, we examined the activity of these signaling pathways by measuring the activity of EGFR, Akt, and Erk following treatment with gefitinib. Upon gefitinib treatment, tyrosine phosphorylation of EGFR was significantly inhibited in all three cell lines, although the inhibition was modest in v-src-transfected cell line. Lower inhibition of phosphorylated EGFR in v-src transfected cells might be explained by the direct phosphorylation of EGFR by Src kinase because the physical association between Src and EGFR [33] and direct phosphorylation of EGFR on tyrosine 845 [34] have been reported. Activation of ERK and AKT was significantly inhibited in HAG-1 parental cells, followed by accumulation of cells in the G0/G1 phase of the cell cycle, with progressive expansion of apoptotic cell population. However, neither arrest of cell cycle nor apoptosis was evident in both v-*src*- and c-H-*ras*-transfected cell lines. Moreover, gefitinib failed to inhibit the phosphorylation of both Akt and Erk in v-*src*-transfected cells and Erk, but not Akt in c-H-*ras*-transfected cells. These data suggest that activated Src can induce gefitinib resistance by activating both PI-3K-AKT and Ras-Raf-Erk pathways and that activated Ras induce gefitinib resistance by activating Ras-Raf-Erk pathway alone. Accordingly, herbimycin A partially reversed the resistance to gefitinib, with concomitant inhibition of Akt and Erk in v-src-transfected cells. These data suggest that gefitinib resistance might be induced by activating either or both of these Akt and Erk signalings. Similar observations have been reported in non-small cell lung cancer cell lines, indicating that simultaneous inhibition of PI-3K/Akt and MEK/Erk reduces tumor cell survival more effectively than inhibition of each pathway alone [21]. Recent immunohistochemical study in patients with chemotherapy-refractory non-small cell lung cancer showed that positive expression of phosphorylated Erk is significantly associated with poor response to gefitinib [35]. In our experiments, however, a strong resistance was observed with concomitant activation of Akt and Erk rather than a single activation of Erk, indicating that Akt activation would also be crucial for gefitinib resistance.

Of note, in this study, the activated c-H-Ras could not activate the Akt, suggesting that the signaling pathway from c-H-Ras to PI-3K-Akt might not operate in the HAG-1 cell line. This is in contrast to the previous studies showing that mutant K-Ras and H-Ras preferentially activate the Ras/Erk pathway and the PI3K/Akt pathway, respectively [36–38]. Therefore, it is suggested that preferential signaling downstream of Ras may vary from cell to cell, controlling elegantly the cell growth and survival depending on cell types. Likewise, the mechanism of gefitinib resistance cannot be explained by the

activation of PI-3K/Akt and/or Ras/Erk pathways. Src phosphorylates a number of intracellular substrates associated with cell growth and survival. The signaling pathway downstream of EGFR other than Ras/Raf-1/Erk and PI-3K/Akt is Janus tyrosine kinase (Jak)/signal transducers and activators of transcription (STAT) pathways. In our previous study, the Jak2/Stat3 has been shown to be activated in v-Src-transfected HAG-1 cells [39]. Therefore, activation of Stat3 might be involved in gefitinib resistance in these cells. We are currently investigating this possibility.

In recent study, strong correlations have been reported between EGFR mutations and improved response and survival in patients with non-small cell lung cancer, who have been treated with gefitinib [40]. Therefore, assessment of EGFR mutations is currently recommended to customize treatment. Likewise, the search for activated oncogenes such as Src and Ras as well as identification of signaling molecules downstream of EGFR would be beneficial for prediction of clinical response to gefitinib, and combination with specific inhibitors against Src, Ras, MEK, or PI-3K might become useful to overcome gefitinib-resistance.

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