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Original Article

Gefitinib, an Epidermal Growth Factor Receptor Blockade Agent, Shows Additional or Synergistic Effects on the Radiosensitivity of Esophageal Cancer Cells *in Vitro*

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Human esophageal cancers have been shown to express high levels of epidermal growth factor receptor (EGFR) and a relationship between high EGFR expression and local advance, the number of lymph node metastases, life expectancy, and sensitivity to chemo-radiotherapy has been demonstrated. We examined the use of gefitinib, an orally active EGFR-selective tyrosine kinase inhibitor, as a new strategy for treatment of esophageal carcinoma. The effects of gefitinib were evaluated in monotherapy and in combination with radiotherapy in human esophageal carcinoma cell lines. Gefitinib produced a dose-dependent inhibition of cellular proliferation in all of the 8 esophageal carcinoma cell lines examined, with IC_{50} values ranging from 5.7 μM to 36.9 μM. In combination, gefitinib and radiotherapy showed a synergistic effect in 2 human esophageal carcinoma cell lines and an additive effect in 5 cell lines. Western blotting demonstrated that gefitinib blocked activation of the EGFR-extracellular signal-regulated kinase (Erk) pathway and the EGFR-phosphoinositide-3 kinase (PI3K)-Akt pathway after irradiation. These results suggest that further evaluation of EGFR blockade as a treatment for esophageal cancer should be performed, and that radiotherapy combined with EGFR blockade may enhance the response of esophageal carcinoma to therapy.

Key words: gefitinib, esophageal cancer, radiosensitivity, epidermal growth factor receptor

In the development of tolerance to irradiation, an intracellular phosphorylation cascade starting from auto-phosphorylation of transmembrane tyrosine kinase receptors, and of epidermal growth factor receptor (EGFR) or HER-1 in particular, has been recognized as a signaling pathway associated with cell survival. An *in vivo* study using 9 cancer cell lines has demonstrated a negative relationship between the expression levels of EGFR and sensitivity to radiation [1]. Low-dose irradiation in A431 human vulvar squamous carcinoma cells has been shown to lead to autophosphorylation of EGFR and activated mitogen-activated protein kinase (MAPK), resulting in an induced proliferation response [2]. In addition, apoptosis is increased when MAPK activation is inhibited by PD98059, a MAPK kinase (MEK)

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inhibitor, following low-dose irradiation in A431 cells [3], and PD98059 also shows a radiosensitizing effect in leukemia cells and mammary cancer cell lines [4, 5]. A study in prostatic cancer cell lines has demonstrated that following irradiation, the activated transforming growth factor-alpha (TGF-alpha)-EGFR-MAPK pathway is inhibited by a neutralizing antibody of TGF-alpha, indicating a radiosensitizing effect [6]. These results suggest that the radiosensitizing effect on cancers caused by inhibition of the EGFR-MAPK pathway may have potential in clinical practice. The phosphoinositide-3 kinase (PI3K)-Akt is an important anti-apoptosis signaling pathway downstream of EGFR. An immunohistochemical study in head and neck cancers has showed a correlation of the phosphorylated Akt level in tissues to the efficacy of radiotherapy [7]. Irradiation activates the PI3K-Akt pathway and produces an anti-apoptotic effect, and the radiosensitizing effects of Wortmannin and LY294002, which are PI3K inhibitors, have been demonstrated in human melanoma cells, non-small cell lung cancer cell lines and human glioblastoma cell lines [8–10]. Given that the MAPK and PI3K-Akt pathways led to radiation resistance through activation in an EGFR-dependent manner, EGFR inhibitors may be promising radiosensitizing agents, and C225, an anti-EGFR monoclonal antibody, has shown encouraging results regarding the treatment of head and neck cancers in combination with radiotherapy in a clinical trial [11].

EGFR has been found to be highly expressed in 40–70% of esophageal carcinomas [12, 13], and a relationship between high EGFR expression and local advance, the number of lymph node metastases, life expectancy, and sensitivity to chemo-radiotherapy has been demonstrated [14–17]. Based on these observations, we hypothesized that clinical application of gefitinib, an orally active EGFR-selective tyrosine kinase inhibitor, may provide a new strategy for treatment of esophageal carcinoma. The present study is the first to investigate the effects of gefitinib alone and in combination with radiotherapy in esophageal carcinomas, and its purpose was to evaluate these effects in several esophageal carcinoma cell lines.

Materials and Methods

Drugs and reagents. Gefitinib was provided by AstraZeneca Pharmaceuticals (London, United Kingdom). The MEK inhibitor PD98059 and the PI3K inhibitor LY294002 were obtained from Alexis Corporation (San Diego, CA, USA). All inhibitors were dissolved in dimethylsulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO, USA) and stored at −20 °C as stock solutions for use in all the experiments. Control cells were treated with medium containing an equal volume of DMSO. HEPES, EDTA, Igepal, DTT, phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, sodium fluoride (NaF), sodium orthovanadate (Na_3VO_4), β-glycerophosphate, sodium phosphate (Na_2HPO_4), citric acid, propidium iodide, and RNase A were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Goat polyclonal anti-EGFR (1005), goat polyclonal anti-p-EGFR (Tyr1173), anti-HER2, anti-HER3, rabbit polyclonal anti-ERK1 (K-23), mouse monoclonal anti-p-ERK (E-4) [which specifically reacts with ERK1 and ERK2 phosphorylated at Tyr-204], rabbit polyclonal anti-cyclin D1 (M-20), mouse monoclonal anti-p-Tyr (PY20), rabbit polyclonal anti-Akt1/2 (H-136), and rabbit polyclonal anti-Bad (C-20) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). We also used rabbit polyclonal anti-AKT/PKB (pS^{473}) phosphospecific antibody, anti-human BAD (pS^{112}) phosphospecific antibody (BioSource International, Camarillo, CA, USA), and monoclonal anti-β-actin (clone AC-74) mouse ascites fluid (Sigma). Peroxidase-labeled antibodies against mouse, rabbit, and goat were purchased from Amersham (Arlington Heights, IL, USA).

Cell lines and culture conditions. The TE series of human esophageal cancer cell lines was obtained from the Cancer Cell Repository, Research Institute for Tuberculosis and Cancer, Tohoku University. These cells were cultured in RPMI1640 medium with 10% fetal bovine serum (Sigma) at 37 °C in a 5% CO_2 incubator. All cell lines carried p53 mutations [18, 19].

Cellular proliferation assay. Cancer cells were seeded on 96-well plates at a concentration of 5×10^3 cells/well with complete culture medium, and were allowed to adhere to the plate overnight. The adherent cells were incubated in the presence of vari-

ous amounts of gefitinib (0.01–100 μM) for a further 72 h at 37 °C in 5% CO_2. After the treatment, an MTS assay was performed using a CellTiter 96^R AQueous One Solution Cell Proliferation Assay (Promega, WI, USA). The absorbance of the treated samples against the blank control was measured using an Immuno Mini NJ-2300 (Nalge Nunc International K.K., NY, USA). The wavelength for measuring the absorbance of the formazan product was 490 nm, and the reference wavelength was 700 nm. The antiproliferative activities of gefitinib are shown in terms of IC_{50} values. For determination of the cytotoxic effects of radiation alone, cancer cells were seeded on 96-well plates at a concentration of 5×10^3 cells/well and allowed to adhere to the plate overnight. Cancer cells were then irradiated with a Hitachi X-ray machine (MBR-1520R) operating at 150 kV and 15 mA, with 1.0-mm Al filtration, a focus-source distance of 30 cm, and a dose rate of 0.9 Gy/min. A total of 10 doses (3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 Gy) were used, and irradiation was performed in air at room temperature. Following irradiation, cancer cells were incubated for a further 72 h at 37 °C in 5% CO_2, and an MTS assay was then performed. Dose-response curves were obtained for 8 esophageal cancer cell lines and the cytotoxic effects of the ionizing radiation are shown in terms of ED_{50} values. Cell proliferation assays were performed in 3 wells, and each experiment was repeated three times. The effects of gefitinib and ionizing radiation in combination were tested at three different ratios of the gefitinib concentration to the radiation dose. Cancer cells were irradiated as described above in the presence of gefitinib (gefitinib was added to each well 1 h prior to irradiation), and an MTS assay was performed, as also described above. The timing of exposure to gefitinib (before or after irradiation) is important in the investigation of the combination effect. Autophosphorylation of EGFR after irradiation has been reported to occur very early (only 2 min after irradiation), and we also obtained a similar result in a preliminary study [20]. Since the present study was performed to analyze the combined effects of irradiation and inhibition of EGFR signaling by an EGFR inhibitor, for this purpose cells were exposed to gefitinib from 1 h before irradiation. The ratio of the gefitinib concentration to the radiation dose is also important in the investigation of the combination effect. Since sensitivities to gefitinib and irradiation vary among the 7 esophageal cancer cell lines in which the combination effects were investigated, use of a single combination ratio in all the cell lines may not be appropriate. Thus, the effects of 3 combination ratios of the gefitinib concentration (μM) and radiation dose (Gy), 3:1, 1:1 and 1:3, were evaluated.

Combination index (CI) calculations. The cytotoxic effects obtained with the different gefitinib / radiation combinations were analyzed according to the Chou and Talalay method, using Calcusyn software (Biosoft, Cambridge, UK) [21]. Interactions between gefitinib and radiation were assessed by means of an automatically computed combination index (CI) [22]. CI was determined at 50% and 75 % cell death, and was defined as follows:

$$CI_{A+B} = [(D_{A/A+B})/D_A] + [(D_{B/A+B})/D_B] + \\ + [\chi(D_{A/A+AB} \times D_{B/A+B})/D_A D_B]$$

where

CI_{A+B} = CI for a fixed effect (F) of the combination of cytotoxic A and cytotoxic B.

$D_{A/A+B}$ = concentration of cytotoxic A in the combination A + B giving an effect F.

$D_{B/A+B}$ = concentration of cytotoxic B in the combination A + B giving an effect F.

D_A = concentration of cytotoxic A alone giving an effect F.

D_B = concentration of cytotoxic B alone giving an effect F.

χ = parameter with a value of 0 when A and B are mutually exclusive and 1 when A and B are mutually nonexclusive.

The combination index indicates the following behavior: synergism, < 0.8; additivity, > 0.8 and < 1.2; antagonism, > 1.2; slight synergistic and additive cytotoxic activity for values of 0.8 and 1.2, respectively.

Western blotting. Generally, cells plated in a 60-mm dish were used for each sample. After the treatment, the cells were washed once with ice-cold PBS without calcium and lysed by the addition of 50 μl per dish of ice-cold lysis buffer (50 mM HEPES-NaOH (pH 7.4), 250 mM NaCl, 1 mM EDTA, 1% Igepal, 1 mM DTT, 1 mM PMSF, 5 μg/ml aprotinin and leupeptin, 1 mM NaF, 2 mM

Na_3VO_4, 10 mM β-glycerophosphate). The cells were lysed for 20 min on ice and clarified by centrifugation. Whole cell lysate was heated in 5 × SDS sample buffer (312.5 mM Tris-HCl (pH 6.8), 50% Glycerol, 10% SDS, 25% 2-ME, 0.01% BPB) for 5 min at 95 °C and then subjected to SDS-PAGE. After SDS-PAGE, the protein was transferred to polyvinylidene difluoride membranes (Hybond-P, Amersham Pharmacia Biotech, Buckinghamshire, England) and immunoblotted with appropriate primary antibodies. For detection, the blots were incubated with the appropriate secondary antibodies conjugated with horseradish peroxidase and were developed using the enhanced chemiluminescence plus Western blotting detection system (Amersham), according to the manufacturer's instruction.

Flow cytometry and TUNEL assay. Trypsinized cells were pelleted by centrifugation at 500 g for 5 min at 4 °C and fixed in 70% ethanol for 48–72 h at −20 °C. After fixation, cells were suspended in 100 μl of phosphate-citrate buffer (0.19 M Na_2HPO_4, 4 mM citric acid) and were incubated for 30 min at room temperature, before being resuspended in 1 ml of PBS containing 10 μg/ml of propidium iodide and 10 μg/ml of RNase A. The propidium iodide-stained cell samples were assayed at FL2 on a FACSCalibur (Beckton Dickinson, San Jose, CA, USA), and data analysis was performed with CELLQuest software (Beckton Dickinson). Instrument quality control was conducted daily to monitor fluorescence intensity, alignment, and voltage standardizations. A minimum of 10,000 cells was collected in each run. The cells were selected by pulse-area and pulse-width analysis, and only integrated signals were collected to reject doublets. Apoptotic cells were detected by fluorescence microscopy (TUNEL assay) using an In Situ Cell Death Detection Kit with Fluorescein (Roche Diagnostics), according to the manufacturer's protocol.

Results

Association between HER family expression levels in esophageal cancer cell lines and antiproliferative effects of gefitinib and radiation. HER family (EGFR, HER2, HER3) expression levels in 8 esophageal carcinoma cell lines were analyzed by Western blotting. TE-4 cells exhibited the highest EGFR expression levels, followed by TE-8, TE-3, and TE-5 cells (Fig. 1). Gefitinib showed growth-inhibitory activity in all the esophageal carcinoma cell lines examined, with IC_{50} values ranging from 5.7 μM in TE-4 cells to 36.9 μM in TE-13 cells (Table 1). A statistically significant linear inverse correlation was found between EGFR expression levels and IC_{50} values in the cell lines ($p = 0.033$, $r = -0.706$), but there was no correlation between the expression levels of other HER family members and IC_{50} values. TE-4 cells were the most radioresistant (ED_{50}: 32.1 Gy) and TE-10 cells the most radiosensitive (ED_{50}: 11.7 Gy). A statistically significant linear correlation was found between EGFR expression levels and ED_{50} values for radiation ($p = 0.005$, $r = 0.866$), but there was no correlation between the expression levels of other HER family members and ED_{50} values.

Combination effects of gefitinib and radia-

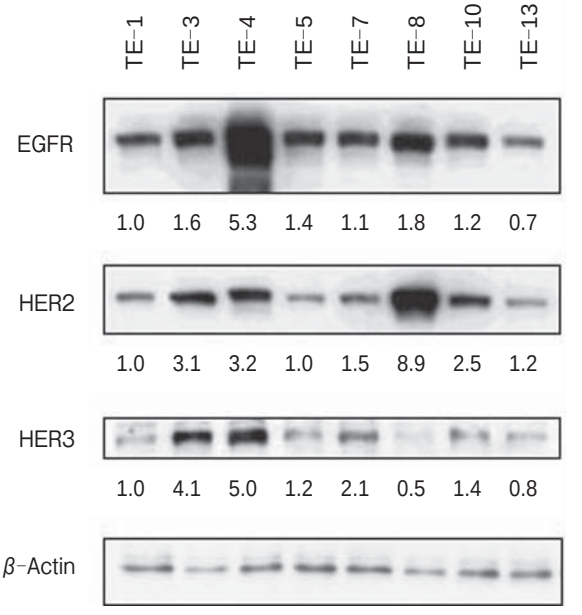

Fig. 1 Expression levels of HER family members (EGFR, HER2, and HER3) in each esophageal carcinoma cell line were analyzed by Western blotting. Each band was analyzed using NIH image. Comparison of protein expression levels among the cell lines is based on the ratio of expression of a protein in each cell line to that in TE-1 (set equal to 1). Different levels of EGFR expression occurred in the TE series, with TE-4 cells exhibiting the highest EGFR expression levels, followed by TE-8, TE-3, and TE-5 cells. β-Actin levels in each cell line are shown as a control.

tion. The effects of gefitinib and radiotherapy in combination were evaluated in 7 esophageal carcinoma cell lines (TE-1, -3, -4, -5, -7, -8, and 10). A typical dose-response curve is shown in Fig. 2, and examples of the experimental data and CI values at 50% and 75% cell death are shown in Table 2. The linear correlation coefficient, *r*, of the median-effect plot for the data in Table 2 was reasonably good, since an r value above 0.9 is considered acceptable for cell culture systems [21]. In this experiment, the CI value was accepted only when the *r* value was 0.95 or higher at all combination ratios of gefitinib and RT (3:1, 1:1, and 1:3). The combination effects were judged using the mean CI value at ED_{50} for 2 independent experiments. Among the 7 esophageal cancer cell lines investigated, an obvious synergistic effect was observed in TE-3 and TE-4 cells, while the combination effect was additive in the other cell lines (Table 3).

Effects of gefitinib combination treatment on intracellular signaling mechanisms follow-

Table 1 IC_{50} values of gefitinib and ED_{50} values of radiation in the cell lines

Cell lines	IC50s mean (s.d.)	ED50s mean (s.d.)
	Gefitinib (μM)	Radiation (Gy)
TE-1	34.7 (1.0)	21.9 (3.2)
TE-3	8.3 (0.1)	15.5 (3.2)
TE-4	5.7 (0.2)	32.1 (7.9)
TE-5	20.8 (3.5)	17.0 (1.8)
TE-7	28.6 (1.5)	17.5 (2.8)
TE-8	9.6 (1.2)	18.4 (2.0)
TE-10	27.1 (0.9)	11.7 (0.7)
TE-13	36.9 (2.2)	13.2 (2.1)

The values shown are the means of 3 independent experiments, with the S.D. given in parentheses.

Table 2 An example of the results: A Combination Index experiment in TE-4 cells

	Ratio Gefinitib (μM): Radiation (Gy)	Combination Index Values at		*r*
		ED50	ED75	
Experiment 1	3:1	0.22	0.95	0.97
	1:1	0.85	0.76	0.97
	1:3	1.23	1.11	0.98
Experiment 2	3:1	0.43	0.83	0.98
	1:1	0.22	0.43	0.99
	1:3	0.83	1.05	0.99
Average		**0.63**	**0.85**	

Gefitinib and radiation were concomitantly administered at ratios of gefitinib concentration (μM) to radiation dose (Gy) of 3:1, 1:1 and 1:3 in each experiment, and the Combination Index was calculated using Calcusyn software. The CI values at ED_{50} and ED_{75} are shown. The CI value was considered acceptable only when the *r* value was 0.95 or higher for all 3 combination ratios. The mean CI values for 2 experiments are shown in bold letters.

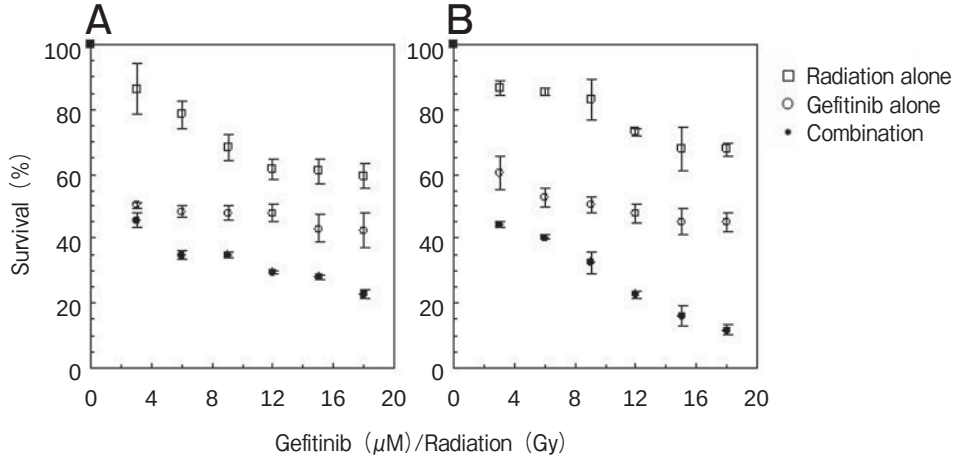

Fig. 2 Typical dose-response curves in TE-3 (**A**) and TE-4 (**B**) cells. The ratios of the gefitinib concentration (μM) and radiation dose (Gy) were 1:1 in both cell lines. Bars represent means with S.D. (error bars).

Table 3 Combination Index and effects of gefitinib / radiation

Cell lines	Combination Index Values at ED50	Combination Index Values at ED75	Effect
TE-1	1.18 (0.21, 0.95–1.48)	1.78 (1.04, 0.93–2.95)	Additive
TE-3	0.35 (0.25, 0.12–0.71)	0.95 (0.11, 0.79–1.01)	Synergistic
TE-4	0.63 (0.41, 0.22–1.23)	0.85 (0.25, 0.43–1.11)	Synergistic
TE-5	1.19 (0.26, 0.70–1.39)	1.13 (0.14, 1.04–1.37)	Additive
TE-7	1.14 (0.30, 0.79–1.58)	0.91 (0.58, 0.41–1.75)	Additive
TE-8	1.06 (0.25, 0.62–1.25)	1.16 (0.41, 0.70–1.74)	Additive
TE-10	0.92 (0.17, 0.72–1.19)	1.40 (0.97, 0.66–3.26)	Additive

The CI values at ED_{50} and ED_{75} in the cell lines. The effects were judged based on the CI at ED_{50}, as described in the Methods (S.D., range).

ing irradiation. Using TE-3 cells, in which a synergistic effect of gefitinib and radiation was observed, time-course changes in the intracellular EGFR phosphorylation pathway after irradiation and the effects of the EGFR inhibitor were investigated by Western blotting. Exposure to 1 μM gefitinib alone for 1 h inhibited the levels of phosphorylation of EGFR and Akt (Fig. 3A). The EGFR phosphorylation level in TE-3 cells was initially elevated 5 min after irradiation at 3 Gy, and then gradually increased over 24 h (Fig. 3B). Phosphorylation of Erk, a representative MAPK, was observed between 5 min and 1 h after irradiation, with this effect followed by rapid diminution of phosphorylation, with only a slightly elevated phosphorylation level remaining after 24 h. In accordance with the initial activation of Erk, TE-3 cells showed the highest Cyclin D1 protein level 12 h after irradiation. In combination with gefitinib, phosphorylation of EGFR and

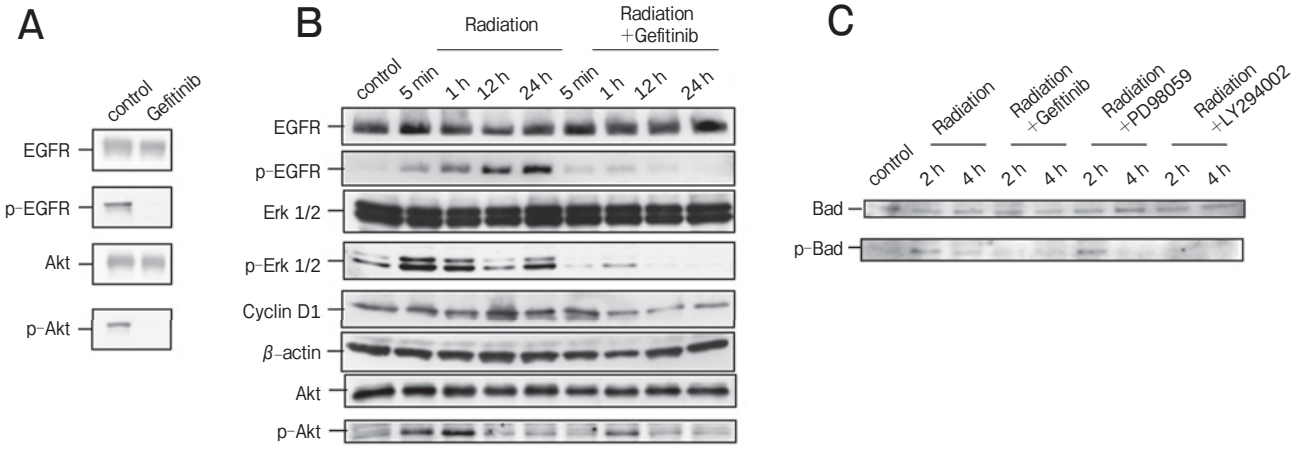

Fig. 3 **A**, Exposure of TE-3 cells to 1 μM gefitinib alone for 1 h resulted in inhibition of phosphorylation of EGFR and Akt; **B**, TE-3 cells harvested 5 min and 1, 12, and 24 h after irradiation at a dose of 3 Gy, with or without treatment with 1 μM gefitinib, were assayed by Western blotting. In the combination treatment, the cells were exposed to gefitinib from 1 h before irradiation until harvesting. Irradiation resulted in limited phosphorylation of EGFR, spike-like phosphorylation of Erk1/2, and an increase in Cyclin D1 after 12 h (Lanes 2–5). Gefitinib inhibited the activation of EGFR-Erk and the increase in Cyclin D1 (Lanes 6–9). Irradiation also induced Akt phosphorylation with a peak 5 min to 1 h after irradiation (Lanes 2–5). Gefitinib markedly suppressed the Akt phosphorylation (Lanes 6–9). Lane 1 shows the results for untreated controls; **C**, The phosphorylation levels of Bad, a target of Akt, were determined 2 and 4 h after irradiation. TE-3 cells showed Bad phosphorylation with a peak 2 h after irradiation (Lanes 2 and 3). Combination use of gefitinib suppressed Bad phosphorylation (Lanes 4 and 5). Phosphorylation was not suppressed by 10 μM PD98059 (Lanes 6 and 7), but was suppressed by 10 μM LY294002 (Lanes 8 and 9). Lane 1 shows the results for untreated controls. Independent experiments performed in triplicate gave the same results.

Erk in TE-3 cells was continuously suppressed, and the Cyclin D1 level was lowest after 12 h, in contrast to the cells exposed to radiation alone. The PI3K-Akt pathway in TE-3 cells was also examined. Phosphorylation of Akt started 5 min after irradiation and peaked after 1 h, but combination treatment with gefitinib suppressed phosphorylation of Akt. The phosphorylation level of Bad, which is considered to be a target factor for Akt, was elevated from 2–4 h after irradiation (Fig. 3 C). Exposure to gefitinib after irradiation suppressed Bad phosphorylation. In order to determine whether the PI3K-Akt pathway is able to control Bad phosphorylation, TE-3 cells were also treated with LY294002 and PD98059, a PI3K inhibitor and a MEK inhibitor, respectively. LY294002 suppressed Bad phosphorylation from 2–4 h after irradiation, similarly to gefitinib, whereas PD98059 showed no such suppression.

Inhibition of initial mitosis and increased apoptosis induced by gefitinib after irradiation. Analysis of the cell cycle following irradiation at 3 Gy showed that TE-3 cells underwent a pronounced increase in DNA levels 24 h after irradiation, indicating a G2/M transition, and then returned to a control level of DNA after 48 h (Fig. 4). This mitosis after irradiation was inhibited by treatment with gefitinib (1 μM) 3 h after irradiation and by

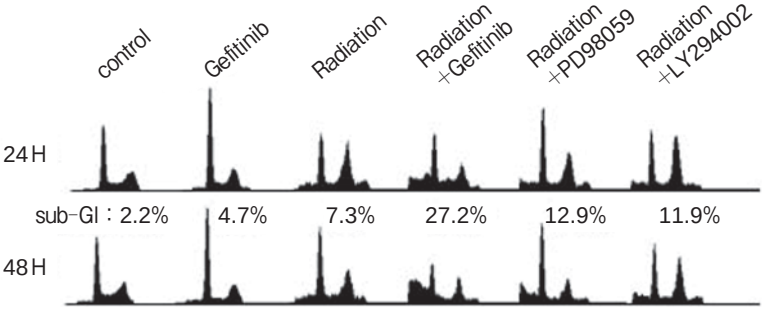

Fig. 4 TE-3 cells were irradiated at a dose of 3 Gy, with or without exposure to a drug (gefitinib 1 μM, PD98059 10 μM, or LY294002 10 μM) 3 h after irradiation. They were then treated with trypsin 24 or 48 h after irradiation to prepare samples for FACS analysis, as described in the Methods. Inhibition of the G2/M transition after irradiation and a marked increase in the sub-G1 population were observed in the gefitinib combination group. The experiments were performed in triplicate, and consistent results were obtained.

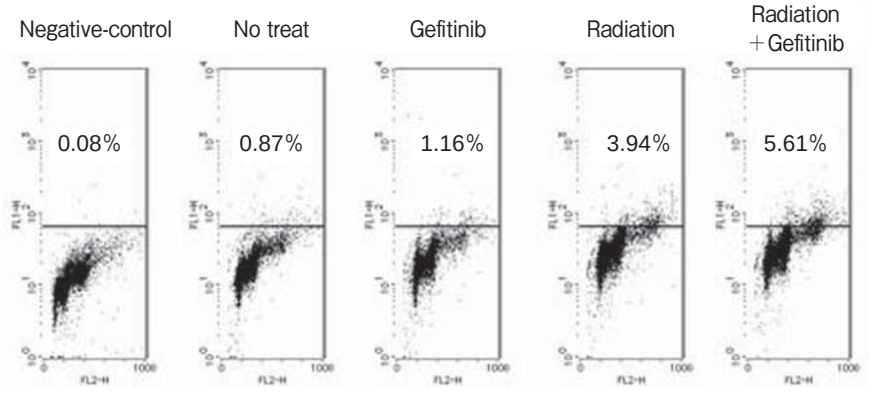

Fig. 5 TE-3 cells were irradiated at a dose of 3 Gy, with or without exposure to gefitinib (1 μM) 3 h after irradiation. They were then treated with trypsin 48 h after irradiation for use in a TUNEL assay. Propidium iodide-stained cell samples were assayed at FL2 and TUNEL-positive cell samples at FL1. Treatment with irradiation combined with gefitinib resulted in a significant increase in the number of TUNEL-positive cells.

the MEK inhibitor PD98059 (10 μM), but not by the PI3K inhibitor LY294002 (10 μM). Based on the proportion of apoptotic cells counted as sub-G1 48 h after irradiation, PD98059 and LY294002 slightly enhanced the sub-G1 population, but this effect was less than that of gefitinib. In a TUNEL assay, irradiation combined with gefitinib produced a significant increase in the number of apoptotic cells (Fig. 5).

Discussion

The results of the MTS-1 assays showed that gefitinib had an antiproliferative effect on all the esophageal carcinoma cell lines examined, with IC_{50} values ranging from 5.7 μM to 36.9 μM. The antitumor effects of gefitinib appear to occur through inhibition of signal transduction pathways associated with cell growth or apoptosis, and the existence of abnormal signaling that is independent of the EGFR cascade could therefore be a possible mechanism through which resistance to gefitinib may develop. Ras mutations occur commonly in tumors and lead to abnormal signaling, but the incidence of a Ras mutation is very low in esophageal carcinoma [23, 24]. In light of the high expression of EGFR in esophageal carcinoma [25], which implies enhanced signaling activity, gefitinib appears to be appropriate for the clinical treatment of esophageal carcinoma. The relationship between the EGFR expression levels with sensitivity to gefitinib is an interesting issue. Our results indicate a significant correlation between EGFR expression levels and the IC_{50} of gefitinib, suggesting that the effects of gefitinib may be lessened at lower EGFR expression levels. This finding is consistent with *in vivo* studies demonstrating a similar correlation between EGFR gene expression levels with the antitumor effects of gefitinib in epidermoid carcinoma, lung cancer, and prostatic cancer cell lines [26], and with a study demonstrating a linear inverse correlation of EGFR expression levels with IC_{50} values of gefitinib in head and neck cancer and colonic cancer cell lines [27]. However, clinical data obtained to date for gefitinib have not demonstrated any clear relationship between EGFR expression and gefitinib efficacy [28], although it has recently been reported that a mutation in EGFR is related to the therapeutic effects of gefitinib in lung cancer, which is of interest [29].

A relationship between the EGFR signal transduction system and radiosensitivity has been suggested previously. This signal transduction pathway is initiated by autophosphorylation of EGFR, which occurs after irradiation. Repeated irradiation of mammary cancer MCF-7 cells has been shown to result in increased TGF-alpha mRNA levels [30], and cleavage and extracellular release of pro-TGF-alpha could act as a trigger for EGFR activation after irradiation, with activation of factors downstream of EGFR [20]. Based on these reports, it is of interest to determine if the combination effects of an EGFR inhibitor and radiation are effective for esophageal cancer, for which radiotherapy is an important therapeutic approach. The present study shows that an EGFR inhibitor and irradiation can have a synergistic effect in esophageal cancer cells, although this synergistic effect occurred in only 2 of the 7 cell lines investigated, while the effect was additive in the other 5 cell lines. Differences in the expression of HER family members were investigated in TE-3 and TE-4 cells, in which a synergistic effect was noted, and cell lines in which an additive effect occurred, but no significant differences were apparent between the 2 groups. Thus, the cellular characteristics that lead to synergism in the effects of irradiation and EGFR inhibition remain unknown. However, the marked synergistic effect noted in TE-4 cells, which strongly express EGFR and are resistant to irradiation, is noteworthy. Western blotting showed that the EGFR phosphorylation pathway is dramatically activated after irradiation. The MAPK and PI3K-Akt cascades are the pathways that are primarily affected, and these cascades were activated soon after irradiation. Radiosensitivity enhancement by MAPK inhibitors has previously been demonstrated [3–6], and it can therefore be concluded that blockage of MAPK signaling activation may be a mechanism through which the radiosensitizing effects of gefitinib occur.

The PI3K-Akt pathway is considered to be important as an anti-apoptotic pathway, in which Caspase-9 and Bad are reported to be targets of Akt; however, much remains to be clarified regarding this pathway. In esophageal carcinoma cell lines, we found that the EGFR-PI3K-Akt pathway was activated after irradiation and that gefitinib inhibited its activation. Irradiation induced Bad phosphorylation in TE-3

cells, and Bad phosphorylation was inhibited by gefitinib. Phosphorylation of Bad diminishes its binding to Bcl-xl or Bcl-2, which is an apoptosis-induction factor [31], and the PI3K-Akt pathway controls Bad phosphorylation and suppresses mitochondrial cytochrome C release, thereby reducing apoptosis [32, 33].

In summary, the following key points emerged from the present study. The combination effects of gefitinib and radiotherapy in esophageal cancer cell lines were found to be synergistic-additive. Inhibition of EGFR activation after irradiation and inhibition of activation of downstream death signals in the EGFR-MAPK and PI3K-Akt cascades by gefitinib were considered to be the mechanisms underlying the synergistic effect. Hence, radiotherapy combined with EGFR blockade may enhance the treatment response of esophageal carcinoma, particularly in a case that is resistant to radiotherapy.

Gefitinib has been clinically applied to lung cancer, and good efficacy of gefitinib for lung cancer associated with EGFR mutation has recently been reported [29]. To facilitate the clinical application of gefitinib in esophageal cancer, clarification of the basic relationship between the incidence and effects of EGFR mutations in esophageal cancer cell lines and the effects of gefitinib will be of importance.

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