the certain background of population, specific types of histology, and activating somatic mutations in the tyrosine kinase domain of EGFR [Lynch et al., 2004; Paez et al., 2004; Sordella et al., 2004]. This drug has been shown to inhibit major cell survival and growth signaling pathways such as Ras-Raf-MAP kinase pathway and phosphatidylinositol-3 kinase (PI-3K)-Akt pathway, as a consequence of inactivation of EGFR [Anderson et al., 2001; Moasser et al., 2001; Moulder et al., 2001; Janmaat et al., 2003]. Although induction of apoptosis has been considered as a major mechanism for gefitinib-mediated anti-cancer effects [Gilmore et al., 2002; Janmaat et al., 2003], the molecular mechanism for gefitinib-induced apoptosis has not been fully elucidated. Pro-apoptotic Bad, a BH3 only member of the Bcl-2 family, and anti-apoptotic Bcl-2 have been shown to be respectively involved in sensitivity and resistance to gefitinib-induced apoptosis [Gilmore et al., 2002; Janmaat et al., 2003], while the role of Bax, a multi-BH domain pro-apoptotic protein which appears to act downstream of Bad, in the gefitinib-induced apoptosis has yet to be clarified. Bax appears to have a more direct role than Bad in the regulation of pore formation in the outer membrane of the mitochondrion [Epand et al., 2002]. Bax protein undergoes conformational changes that expose membrane-targeting domains, resulting in its translocation from cytosol to mitochondrial membranes where Bax inserts and causes release of cytochrome C, followed by caspase activation and DNA degradation [Wolter et al., 1997; Pastorino et al., 1998]. Bax has been shown to undergo post-translational modification during apoptosis. For example, p18 Bax generation through wild type Bax cleavage has been observed in response to various stimuli such as Interferon-alfa [Yanase et al., 1998] and chemotherapeutic agents [Wood et al., 1998]. This p18 Bax fragment has been shown to be as efficient as full-length Bax in promoting cytochrome C release [Wood et al., 1998; Gao and Dou, 2000] or more potent than full-length Bax in inducing apoptotic cell death [Toyota et al., 2003].

In this report, we have investigated the molecular mechanism of gefitinib-induced growth inhibition and apoptosis using EGFR-expressing HAG-1 human gallbladder adenocarcinoma cells. We present evidence that blockade of the EGFR activity with gefitinib causes suppression of downstream signaling pathway through Erk and Akt, and induces apoptosis through activation of p18 Bax.

MATERIALS AND METHODS

Cell Culture and Chemicals

HAG-1 is a human cell line derived from a moderately differentiated adenocarcimona of the gallbladder and its cellular and molecular features were well characterized [Nakano et al., 1994]. The cells was cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Gefitinib was kindly provided by AstraZeneca (Macclesfield, United Kingdom). 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%. These conditions were found to be non-cytotoxic. Anti-EGF receptor, anti-Bax, anti-Bad, anti-Bcl-2, anti-p27, anti-p53 antibodies, and Protein A agarose were purchased from BD Biosciences (San Jose, CA). Anti-phospho-p53 (Ser15) antibody was purchased from Cell Signaling Technology Inc. (MA).

The siRNA (sense and anti-sense strands) against Bax gene was purchased from Qiagen (Germantown, MD). The sense and anti-sense strands sequences of Bax were 5′-GATGATTGCCGCCGTGGACA-TT and 5′-AAAGTAGGAGAGGAGGCCGT-TT, respectively. In vitro transfections were performed using the Transit-TKO polymer/lipid from Mirus (Madison, WI) as recommended. For 6×10^6 cells in 10 ml of medium, 2 μg of siRNA were used. Cells were washed 24 h after transfection.

Determination of Growth and Growth Inhibition

To determine the effect of gefitinib on cellular growth, replicate dishes (Falcon 3001) inoculated with $1\sim2 \times 10^4$ HAG-1 cells were incubated with or without gefitinib. Cell number was determined every day by Coulter counter after removal of the cells from plates with 0.05% trypsin and 0.02% EDTA in Ca- and Mg-free phosphate-buffered saline. The anti-proliferative 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 drug solution at various concentrations were added. 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). Each experiment was performed using six replicate wells for each drug concentration and was carried out independently three times. The IC_{50} value was defined as the concentration needed for a 50% reduction in the absorbance.

Detection of EGFR by Flow Cytometry

Cells were harvested using trypsin and incubated for 1 h at 4°C with 1 μg of the anti-EGFR antibody (Santa Cruz Biotechnology, Santa Cruz, CA). As a control for non-specific binding, 1 μg of protein of human IgG1lamda (Sigma) was used as isotype-matched non-binding antibody for the EGFR. Subsequently, cells were washed twice with ice-cold PBS containing 0.5% BSA and incubated at 4°C in the dark for 1 h with FITC-conjugated goat anti-human IgG antibody, diluted 1:50 in PBS/BSA. After two washing steps with ice-cold PBS/BSA, cells were resuspended in 0.5 ml of ice-cold PBS/BSA and analyzed on a FACS/Calibur Flow Cytometer using CELLQuest software. Relative expression levels were calculated as the ratio between the mean fluorescence intensity of cells stained with the specific antibody and the mean fluorescence intensity of cells stained with the control antibody.

Cell Cycle Analysis and Apoptosis Measurement

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), and the extent of apoptosis was determined by measuring the sub-G1 population.

Reverse Transcriptase Polymerase Chain Reaction

mRNA was extracted from HAG-1 cells using the Trizol Reagent (Life Technologies, Grand Island, NY). cDNA first-strand synthesis was performed by incubating 250 ng RNA in 20 μl RT reaction buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 15 mM $MgCl_2$, 10 mM dithiothreitol, and 500 μM dNTP containing 20 pmol of random primers) with 200 U avian myeloblastosis virus-reverse transcriptase (Promega, Madison, WI) at 42°C for 1 h. The cDNA was amplified in 50 μl PCR buffer containing 50 pmols if each primer, 200 μM dNTP, and one unit of Taq polymerase (Promega). The primer pairs for cyclin D1, p21, p27, and Bax were: cyclin D1: forward, 5′-TGCATCTACACCGACAACTC-3′, reverse, 5′-CAATGAAATCGTGCGGGGTC-3′, p21: forward, 5′-GAAGTAAACAGATGGCACTT-3′, reverse, 5′-TATCAAGAGCCAGGAGGGTA-3′, p27: forward, 5′-TCTGAGGACACGCATTTGGT-3′, reverse, 5′-TGAGTAGAAGAATCGTCGGT-3′, Bax: forward, 5′-TGGTTGCCCTTTTCTACTTTG-3′, reverse, 5′-GAAGTAGGAAAGGAGGCCATC-3′. After a first denaturation step (5 min at 97°C), samples were subjected to 30 cycles consisting of 30 sec at 95°C, 30 sec at 60°C, and 30 sec at 72°C, with a final extension step of 10 min. PCR products were resolved by a 1.2% agarose gel electrophoresis and bands were visualized by ethidium bromide staining.

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 $Na_4P_2O_7 \cdot 10H_2O$, 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 Bradford method or a BCA protein assay kit (Pierce, Rockford, IL). For immunoprecipitation, equal amounts of protein were incubated for 1 h at 4°C with specific antibodies against p53, phosphorylated p53 (pS^{15}). Immune complexes were precipitated with protein A agarose beads, washed with radioimmunoprecipitation assay lysis buffer and then boiled in electrophoresis sample buffer

(250 mM Tris pH6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% β-mercaptoethanol). For Western blot, equal amounts of proteins or immunoprecipitated target proteins were resolved by 5–20% 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% nonfat 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), phospho-Akt (Ser473, Cell Signaling Technology), p27 (Transduction Laboratories), Bax, Bad, or Bcl-2 (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 then were exposed to ECL hyperfilm according to the manufacture's instructions (Amersham Biosciences). The blots were striped and reprobed with primary antibodies against EGFR (2232; Cell Signaling Technology) and 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.

Isolation of Mitochondrial Fraction

Cells were lysed in 1 ml of 20 mM HEPES–KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 250 mM sucrose. The cells were broken open with 6 passages through a 26-gauge needle applied to a 1 ml syringe. The homogenate was centrifuged at 800*g* for 10 min at 4°C to remove nuclei and unbroken cells. The supernatant was transferred to a 1.5 ml centrifuge tube. Centrifugation was conducted at 10,000*g* for 15 min at 4°C. The supernatant contained the cytosolic fraction. The resulting mitochondrial pellet was lysed in 50 μl of 20 mM Tris (pH 7.4), 100 mM NaCl, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1% Triton X-100. Then the lysate was centrifuged at 15,000*g* for 5 min at 4°C, and the resultant supernatant was kept as the solubilized enriched mitochondria fraction. Cell fractions were assayed for protein concentration using the Bio-Rad Dye Binding protein assay (Bio-Rad Laboratories), then equivalent amounts of protein were analyzed for Bax expression by Western/ECL analysis.

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 Proliferation and Survival in HAG-1 Cells

The EGFR expression was examined in HAG-1 cells by flow cytometry. As shown in Figure 1A, EGFR was detected in HAG-1 cells, with approximately 10-fold relative EGFR expression. The IC_{50} of the gefitinib against HAG-1 cells was 0.12 μM for 72 h exposure (Fig. 1B). The population doubling times of HAG-1 cells was 26.4 h (Fig. 1C), but was prolonged to 104 h when the cells were treated with 1 μM gefitinib, indicating that gefitinib depressed the growth of HAG-1 cells by approximately fourfold (Fig. 1D). When the treatment exceeded 72 h, the cell number abruptly decreased, and the decline of the growth appeared to be irreversible, because the cell number still decreased upon removal of gefitinib at 72 h (Fig. 1D). These data indicate that gefitinib delays the growth of the cells initially, but leads to cell death when treated over 72 h.

Time-Course Analysis of the Effect of Gefitinib on Cell Cycle Progression and Apoptosis

To examine whether the inhibitory effect observed in growth assays reflects a delay or arrest of cells in the G0/G1 phase, cells were treated with gefitinib for indicated times, and the cell cycle progression was evaluated after PI staining by fluorescence-activated cell sorting analysis (Fig. 2). Upon treatment 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

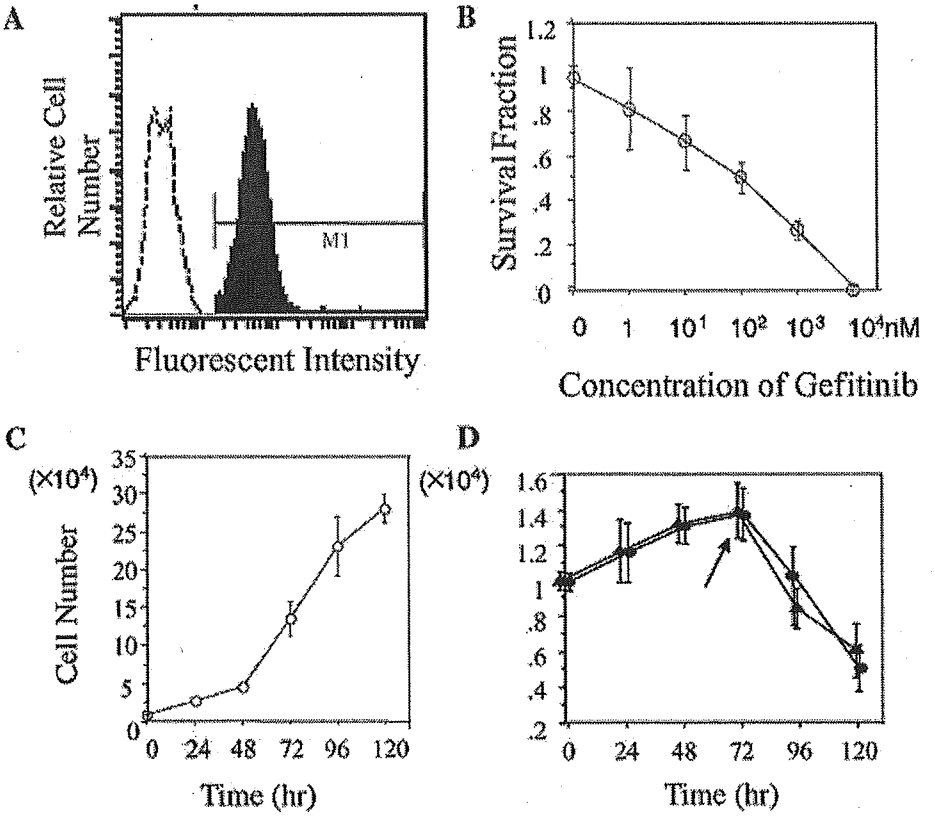

Fig. 1. Expression of EGFR, cytotoxicity of gefitinib, and effects of gefitinib on the growth of HAG-1 cells. **A:** Expression of EGFR was analyzed by FACS after treatment of cells for 1 h at 4°C with 1 μg of the anti-EGFR antibody. **B:** Cytotoxicity was determined by using WST-1 assay. Cells were seeded into a 96-well microplate, and treated with gefitinib at various concentrations of gefitinib for 72 h. **C:** The proliferation of HAG-1 cells without gefitinib treatment (○). **D:** Effect of gefitinib on cell growth. Cells were seeded and treated with gefitinib at 1.0 μM for 120 h (●), or treated with gefitinib at 1.0 μM for 72 h, followed by incubation with normal medium (▲). Arrow indicates removal of gefitinib. Values represent the means of three experiments; *bars*, SE. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

corresponding decrease in cells in S and G2–M phase, and reached almost a plateau afterwards. By contrast, the sub-G0/G1 cell population became evident (72 h, 20%) 72 h post-treatment, and progressively increased upon further treatment (96 h, 34% and 120 h, 50%). Because cells in the sub-G0/G1 population represent apoptotic cells [Janmaat et al., 2003], the irreversible growth decline appeared to be due to progressive expansion of apoptotic cell population.

Effects of Gefitinib on Autophosphorylation of EGFR, Akt, and Erk

To assess the effect of gefitinib on the EGFR activation and subsequent downstream activation, we examined the expression and activation of EGFR, Akt, and Erk. As shown in Figure 3, phosphorylated EGFR was detected without EGF stimulation. Upon treatment with 1 μM gefitinib, tyrosine phosphorylation of EGFR was significantly inhibited with incubation for 2 h, and continued to be suppressed over 24 h, without changing the relative amount of EGFR. In parallel, Erk was also phosphorylated without EGF, and significantly suppressed upon treatment with gefitinib. Unlike EGFR and Erk, autophosphorylation of Akt was modest, but subsequent suppression of Akt was also observed.

Gefitinib Induces Growth Inhibition and Apoptosis Through G1 Arrest and p18 Bax Expression

To identify the molecular basis for gefitinib-induced G0/G1 arrest, we examined the effects of gefitinib on the mRNA expression level of the cyclin D1 and p21 by using a

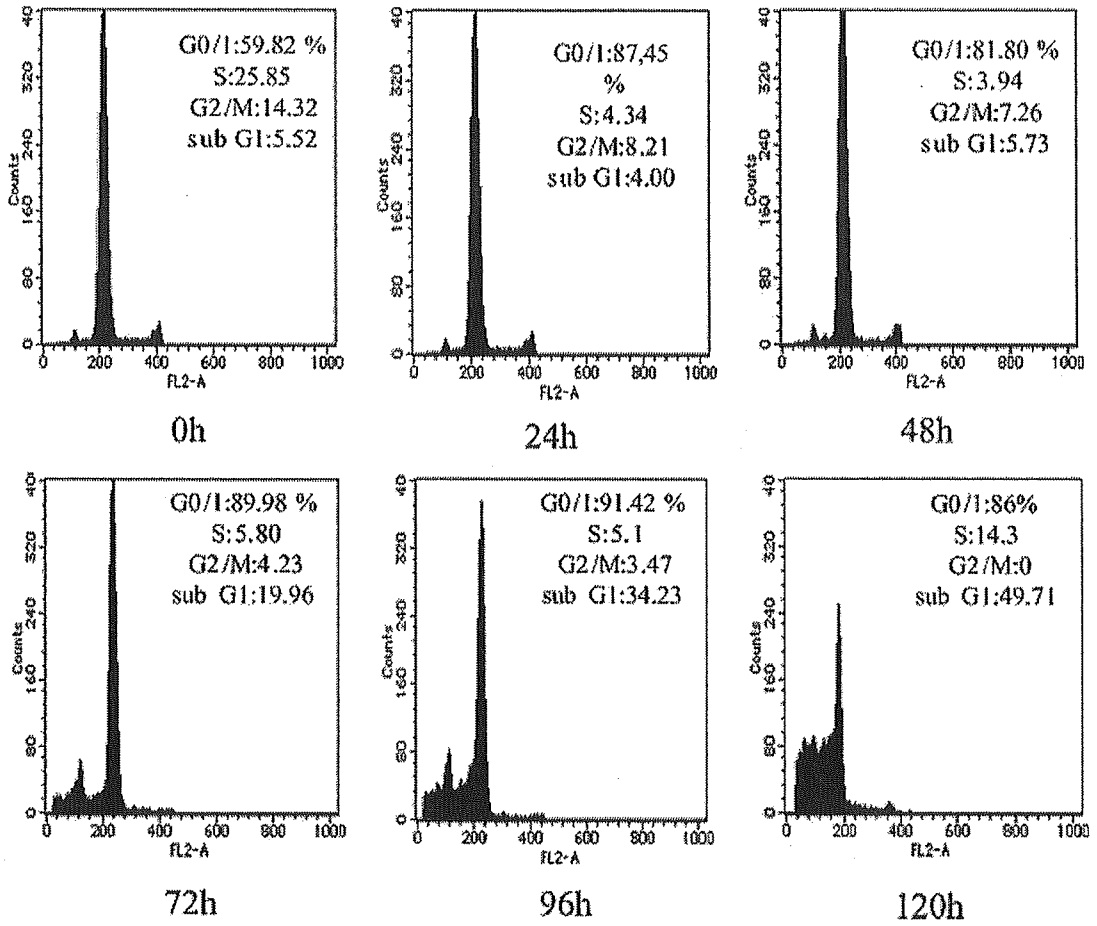

Fig. 2. Time course analysis of the effect of gefitinib on cell cycle progression and apoptosis. HAG-1 cells were stained with propidium iodide after exposure to gefitinib (1.0 μM) for 0, 24, 48, 72, 96, and 120 h, and analyzed by flow cytometry. Percentages of the total cell population in the different 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. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

semi-quantitative RT-PCR method. As shown in Figure 4A, mRNA expression level of cyclin D1 is decreased significantly at 24 h from the beginning of the treatment, and remained low during the entire period of experiments. In contrast, mRNA expression of p21 was upregulated. It has been demonstrated that a blockade of the EGFR-mediated pathway induced upregulation of p27 [Busse et al., 2000], we next examined the effect of gefitinib on the expression of p27. Although mRNA expression of p27 was not affected by the treatment of gefitinib throughout the experiments (Fig. 4A), gefitinib increased p27 protein by fivefold at 24 h from the beginning of the treatment, and levels remained high up to 120 h (Fig. 4B). These results, together with the cell cycle analysis, indicate that accumulation of p27 might be responsible for gefitinib-induced growth arrest at the G0/G1 phase. To investigate the apoptotic mechanism, pro-apoptotic p53, Bad, and Bax and anti-apoptotic Bcl-2 were evaluated following gefitinib treatment. As shown in Figure 4B, total p53 protein level, which acts upstream of p27, and phosphorylated p53 at serine 15, which stabilizes and enhances accumulation of p53, both were not altered after treatment with gefitinib (Fig. 4B). As shown in Figure 4C, gefitinib substantially increased the expression of p18 Bax, an active subtype of Bax protein, 72 h post-treatment, with maximal expression at 120 h. By contrast, Bcl-2 and Bad expressions were unchanged during the incubation period. Since gefitinib did not affect Bax mRNA levels, an

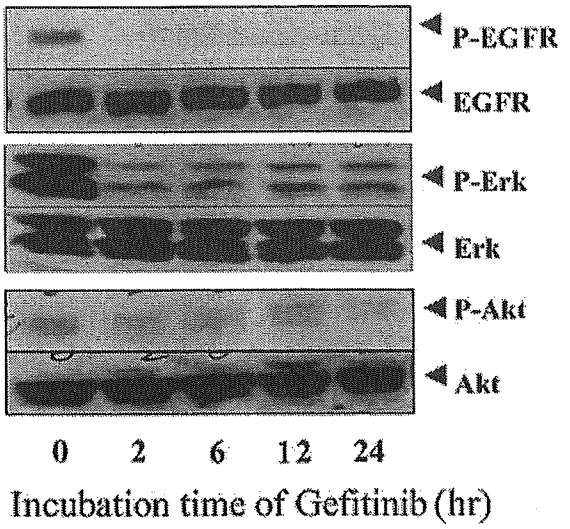

Fig. 3. Effects of gefinitib on the phosphorylation of EGFR and downstream Akt and Erk 1/2. Western blots are shown for phospho- and total EGFR, Erk 1/2, and Akt.

increase in gefitinib-induced expression of total Bax (p21 Bax and p18 Bax) could not be explained on a transcription level.

Attenuation of Apoptosis by Blocking Bax Activity With RNA Interference and Translocation of Bax to Mitochondria

To investigate the direct role of Bax in gefitinib-induced apoptosis, HAG-1 cells were transfected with anti-Bax siRNA, and gefitinib-induced apoptosis was evaluated. Anti-Bax siRNA significantly prevented the cells from gefitinib-induced apoptosis from 45 to 25% (45% reduction in apoptosis) (Fig. 5A,B) after incubation with gefitinib for 120 h. In parallel with the inhibition of apoptosis, anti-Bax siRNA was shown to significantly inhibit the amount of gefitinib-induced p18 Bax and p21 Bax protein, as compared to control siRNA that was constructed based on no significant homology with Bax RNA. Densitometric analyses showed approximately 70% reduction in Bax protein level (Fig. 5C). Western immunoblot analysis of mitochondrial-enriched fractions, obtained after cells were treated with 1 μM of gefitinib for indicated times, showed a time-dependent increase of p18 Bax, accompanied by time-dependent decrease of p21 Bax (Fig. 5D). Since wild-type p21 Bax has been shown to be cleaved into p18 Bax in the mitochondria [Wood et al., 1998], these data indicate that gefitinib

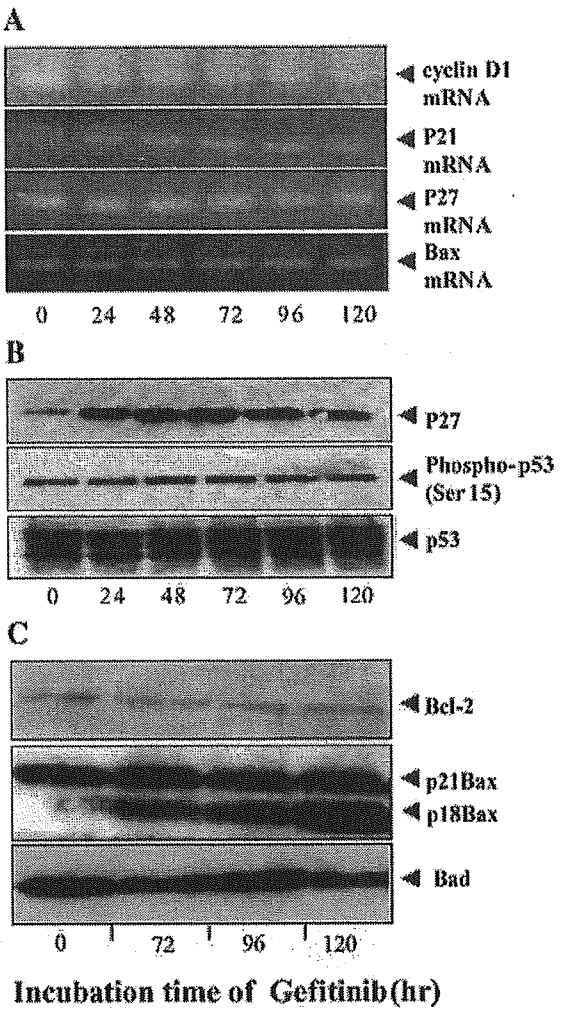

Fig. 4. Quantitative evaluation of apoptosis-associated proteins and RNA transcript in HAG-1 cells treated with gefitinib. The cells were exposed to 1.0 μM gefitinib for indicated times, and processed for RT-PCR and immunoblot analyses as described in Materials and Methods. **A:** Quantitative analysis of transcripts by RT-PCR of cyclin D1, p21, p27 and Bax. **B:** Western blot analyses of p27, p53, and phosphorylated p53 at serine 15. Equivalent amounts of immunoprecipitates were subjected to 12% SDS–PAGE, followed by transfer to nitrocellulose, and then blotting by respective antibodies. **C:** Western blot analyses of Bcl-2, Bax, and Bad.

activates Bax through translocation of Bax from the cytosol to the mitochondria, thereby inducing apoptosis.

DISCUSSION

The most frequent molecular abnormalities associated with pathogenesis of gallbladder cancer are overexpression of EGFR [Yukawa

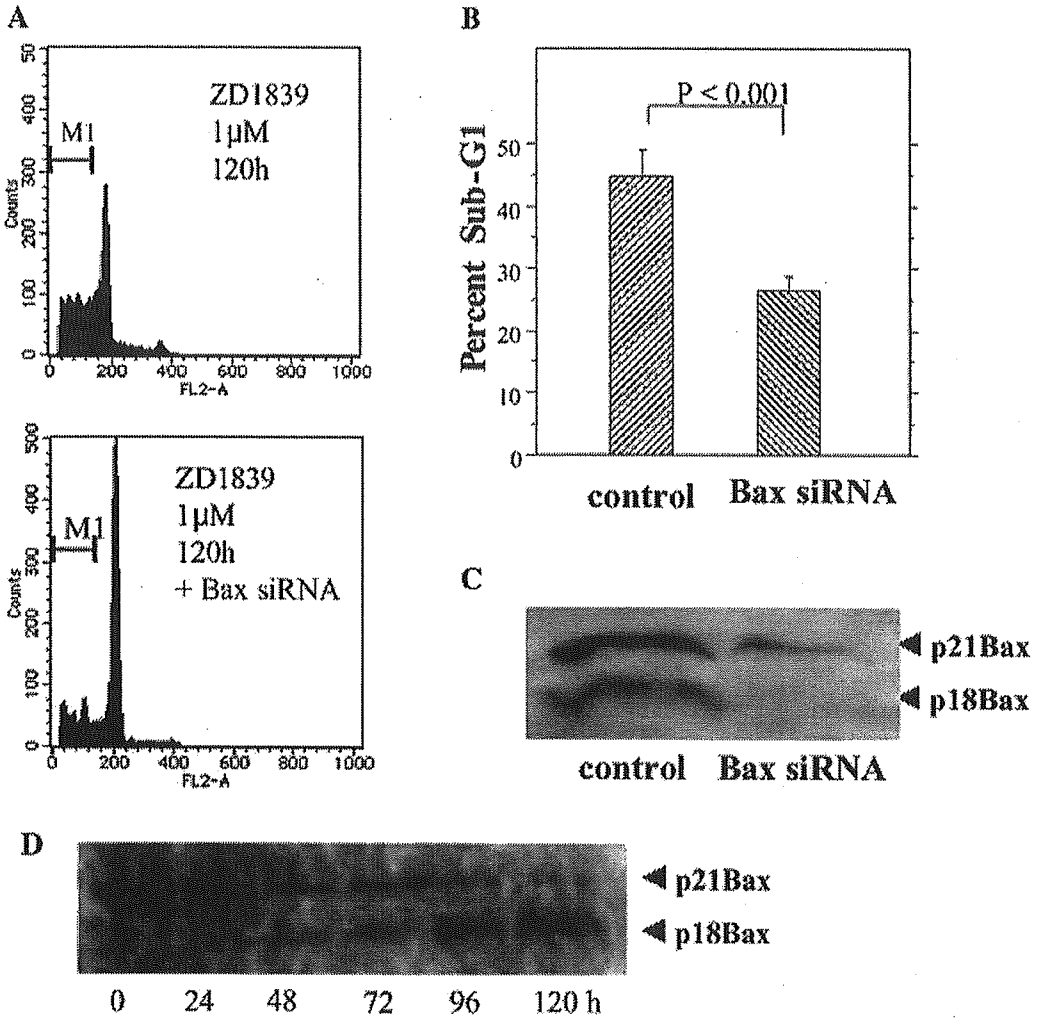

Fig. 5. Attenuation of apoptosis by Bax siRNA and translocation of Bax to mitochondria. HAG-1 cells were transfected with anti-Bax siRNA and processed for FACS analyses as described in Materials and Methods. **A:** Flow cytometric analysis of cell cycle progression at 120 h following treatment with gefitinib. **B:** Levels of gefitinib-induced apoptosis as measured by the percentage of sub-G1 phase cell population at 120 h post-treatment. **C:** Amount of Bax protein at 120 h post treatment, measured by Western blot. **D:** Amount of Bax protein in mitochondrial-enriched fraction.

et al., 1993; Valerdiz-Casasola, 1994; Lee and Pirdas, 1995]. Thus, we investigated here the possibility of EGFR signaling as a potential therapeutic target for gallbladder cancer by studying in vitro effects of the orally active EGFR inhibitor, gefitinib, against an EGFR-expressing HAG-1 gallbladder adenocarcinoma cell line. We have 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 [Janmaat et al., 2003]. Using this cell line, we showed that gefitinib inhibited the cell growth by arresting the cells in G0/G1 phase, followed by the increase in apoptotic cell population (sub-G0/G1 phase). The arrest of the cell cycle at the G0/G1 phase was accompanied by depression of cyclin D1 mRNA as well as accumulation of p27 protein, a critical negative regulator of the cell cycle, that inhibits the activity of cyclin/cdk complexes during G0 and G1 [Slingerland and Pagano, 2000], being consistent with a previous report showing a critical role of p27 in the anti-proliferating activity of gefitinib on tumor cells using p27 anti-sense construct [Di Gennaro et al., 2003]. Moreover, gefitinib upregulated p27 protein levels without affecting p27 mRNA expression.

Degradation of p27 has been shown to be a critical event for the G1/S transition occurring through ubiquitination and subsequent degradation by the 26S-proteasome [Slingerland and Pagano, 2000; Masuda et al., 2002]. Therefore, it is suggested that gefitinib may affect the ubiquitin–proteasome pathway of p27 degradation.

When the treatment of HAG-1 cells with gefitinib exceeded 72 h, cell death became evident with a progressive expansion of apoptotic population with incubation time until 120 h. Correspondingly, gefitinib upregulated the expression of total Bax, with subsequent increase in p18 Bax that has been shown to be generated through cleavage of full-length Bax during apoptosis [Wood et al., 1998] and regarded as a more potent inducer of apoptotic cell death than full-length Bax [Toyota et al., 2003]. The observed expression of p18 Bax appears to be a cause of gefitinib-induced apoptosis, not only because the amount of p18 Bax increased in the mitochondria, a characteristic feature of Bax activation toward apoptosis [Gross et al., 1999], but also because the blockade of Bax using anti-Bax siRNA significantly reduced gefitinib-induced apoptosis. This is the first report demonstrating the direct role of Bax in gefitinib-induced apoptosis. With regard to the mechanism of gefitinib-induced Bax upregulation, it has been reported in colorectal cancer that inhibition of EGFR by anti-EGFR monoclonal antibody C225 induces apoptosis by enhanced expression of newly synthesized Bax protein [Mandal et al., 1998]. However, in the present study, an increase in the gefitinib-induced Bax protein might be due to the decreased degradation of Bax, because levels of Bax mRNA expression and levels of total and phosphorylated p53 that regulates Bax [Zhan et al., 1994] were not altered following treatment with gefitinib. Recently, it has been demonstrated that Bax is degraded by the ubiquitin-proteasome pathway [Chang et al., 1998; Li and Dou, 2000]. Moreover, inhibition of proteasome function has been shown to increase levels of ubiquitinated forms of Bax protein, without any effects on Bax mRNA expression, thereby inducing apoptosis as a consequence of upregulation of Bax [Fan et al., 2001; Nam et al., 2001]. We are currently investigating the mechanism of gefitinib-induced accumulation and activation of Bax through the ubiquitin-proteasome pathway as well as cleavage pathway of wild-type Bax into p18 Bax.

There are two major cell survival and growth signaling pathways downstream of EGFR, i.e., the Ras-Raf-MAPK and PI-3K-Akt pathways. Recently, it has been reported that simultaneous inhibition of both the MAP kinase and PI-3K Akt pathways is important for the execution of gefitinib-induced anti-proliferative effect and apoptosis, and that persistent activity of either of these signaling pathways is involved in the decreased or lack of sensitivity to EGFR inhibitors [Janmaat et al., 2003; Li et al., 2003]. The inactivation of Bad through activation of these pathways has been demonstrated to be involved in gefitinib-induced apoptosis, since activation of either of MAP kinase or Akt pathway has been shown to abrogate the pro-apoptotic function of Bad by phosphorylating its specific serine residues [Datta et al., 1997; Fang et al., 1999; Shimamura et al., 2000; Zhou et al., 2000]. In the present study, however, Bad appears not to be involved in the gefitinib-induced apoptotic events, because Bad is unchanged during the treatment despite inactivation of Akt and Erk. With regard to Bax, there is only a report that inhibition of Akt led to an increased protein level of Bax in a pancreas cancer cell line [Fahy et al., 2003]. In this study, we have found that activation of MAP kinase and Akt is significantly inhibited by gefitinib, suggesting that simultaneous inhibition of these pathways by gefitinib may lead to Bax accumulation and subsequent apoptosis.

Although the observations were obtained on a single human gallbladder cancer cell line, the present data suggest the possibility of EGFR signaling as a potential therapeutic target for gallbladder carcinoma and may serve as a rational basis for a therapeutic approach to this incurable disease with EGFR tyrosine kinase inhibitors.

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Clinical Application of Immunoreactivity of Dihydropyrimidine Dehydrogenase (DPD) in Gastric Scirrhous Carcinoma Treated with S-1, a New DPD Inhibitory Fluoropyrimidine

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Abstract. *Background: A highly specific antibody against recombinant human dihydropyrimidine dehydrogenase (DPD) has been developed to immunohistochemically assess DPD expression in tumors. A new oral DPD inhibitory fluoropyrimidine (DIF), S-1, is reportedly effective against gastric scirrhous carcinoma. Patients and Methods: In this study, the relationship between immunoreactivity to DPD in biopsy specimens and the effects of chemotherapy were investigated in 61 patients treated with first-line fluoropyrimidine-based chemotherapy (S-1:DIF, 5-FU:non-DIF) for gastric scirrhous carcinoma. Results: The response rate was significantly higher in patients with DPD-positive tumors than in those with DPD-negative tumors in the S-1 group (45.5%, 10.0% : $p<0.05$), as compared to the 5-FU group (0%, 5.6%: $p=0.398$). According to the median survival time, there was no significant difference between patients with DPD-positive tumors (364 days) and those with DPD-negative tumors (406 days; $p=0.626$) in either the S-1 group or the 5-FU group (181 days and 256 days, respectively; $p=0.543$). Conclusion: This study indicates that S-1 may be effective even in gastric scirrhous carcinoma with a high level of DPD activity.*

Borrmann-type-4 gastric cancer, clinically synonymous with gastric scirrhous carcinoma, is generally resistant to systemic chemotherapy. This type of gastric cancer is characterized by diffuse malignant lesions with indistinct borders, and is usually diagnosed at a very advanced stage. High rates of lymph node metastasis, invasion of neighboring structures and peritoneal dissemination pose a great challenge for medical care. The outcome is usually poor, with 5-year survival rates ranging from 0% to 20% (1). Although most gastric scirrhous carcinomas are resistant to conventional 5-fluorouracil (5-FU)-based regimens, several recent case studies have reported a good response to S-1 (2, 3) Many studies have demonstrated that dihydropyrimidine dehydrogenase (DPD) is a biomarker for response in patients treated with 5-FU-based chemotherapy (4-7). DPD is a rate-limiting enzyme in the metabolism of 5-FU, and its expression by tumors is thought to attenuate the response to 5-FU (8-10). Since more than 80% of the administered dose of 5-FU is degraded in the liver by DPD to fluorinated β-alanine, the level of DPD activity is also a major determinant of 5-FU toxicity (11).

Recently, encouraging clinical results have led to the development of a new generation of oral fluoropyrimidines, commonly referred to as DPD inhibitory fluoropyrimidines (DIF) (12, 13). S-1 is a combined preparation consisting of 1 M tegafur, 0.4 M 5-chloro-2,4-dihydroxypyridine (CDHP), and 1 M potassium oxonate (Oxo). CDHP is a potent inhibitor of DPD, approximately 180 times more active than uracil in inhibiting DPD *in vitro*, and maintains prolonged 5-FU concentrations in plasma and tumors (14-16) Oxo protects against 5-FU-induced gastrointestinal toxicity. Two phase II studies of S-1 monotherapy in patients with metastatic gastric cancer yielded response rates of about 50%, with minimal toxicity (17-19). S-1 is now used to treat advanced gastric cancer as a single agent or in combination with other anticancer agents, including cisplatin, CPT-11, paclitaxel and docetaxel (20).

A technique using highly specific antibodies against recombinant human DPD (rhDPD) has been developed to immunohistochemically assess DPD expression in tumors (21-23) and thereby predict the clinical response to 5-FU-based chemotherapy. Several studies have examined the relationship between the DPD immunoreactivity of tumors

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and the response to oral fluoropyrimidines, but the clinical impact of DPD activity on response remains unclear for new drugs such as S-1, and there are no reports on the treatment of gastric scirrhous carcinoma. In this study, intra-tumoral levels of DPD were assessed immunohistochemically using anti-DPD polyclonal antibodies, and the relationship between the immunoreactivity of DPD and the antitumor effects of S-1 were investigated. We propose that S-1 might circumvent the resistance to 5-FU in gastric scirrhous carcinoma with a high level of DPD activity. Our aim was to clarify the differences between the antitumor activities and mechanisms of action of S-1 as a DIF and 5-FU as a non-DIF.

Patients and Methods

Patients. Sixty-one patients with Borrmann-type-4 gastric scirrhous carcinoma, who received S-1 or 5-FU as first-line chemotherapy at the National Cancer Center Hospital (Tokyo, Japan) between February 2000 and January 2003, were studied retrospectively. Thirty-one patients were given S-1 and 30 were given 5-FU. Tumor biopsy specimens were obtained from all patients before chemotherapy.

Treatment schedule and evaluation of response. S-1 was administered at a dose of 40 mg/m^2 of body surface area (BSA) twice daily in one of the following doses: 40 mg (BSA<1.25 m^2), 50 mg (1.25 m^2≤BSA <1.50 m^2), or 60 mg (BSA ≥1.50 m^2). S-1 was given for 28 consecutive days, followed by a 14-day rest period. This period was defined as one course of treatment. S-1 was purchased from Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan) in the form of 20 and 25 mg capsules. 5-FU (800 mg/m^2/day) was administered as a 5-day (120 h) intravenous continuous infusion, repeated every 28 days, comprising one course of treatment.

Both treatments were continued until tumor progression, unacceptable toxicity, or refusal by the patient to continue further therapy. The response of measurable target lesions to chemotherapy was objectively evaluated according to the WHO criteria after each course of treatment. The response of primary lesions was also evaluated according to the roentgenographic and endoscopic criteria proposed by the Japanese Research Society of Gastric Cancer for "c-lesions" (24). Complete response (CR) was defined as the disappearance of all invasive findings. Partial response (PR) was defined as a decrease of 50% or greater in the affected area on X-ray films after barium administration, obtained in the same position as that before treatment. Progressive disease (PD) was defined as a 25% or greater increase in lesions or the appearance of new lesions. Responses not falling into any of these categories were classified as stable disease (SD). The survival time was calculated from the start date of the first course of treatment to the date of death or to the final date of confirmed survival.

Immunohistochemical examination. DPD immunoreactivity in the tumor biopsy specimens was examined with the use of an anti-recombinant human DPD polyclonal antibody (diluted at 1:1000, The Second Cancer Laboratory, Taiho Pharmaceutical Co., Ltd., Saitama, Japan). The tissues were routinely fixed in 10% formalin and embedded in paraffin wax. Sections 3 μm thick were cut and mounted on aminopropyltriethoxysilane-coated slides, and were deparaffinized with xylene and rehydrated in graded ethanol. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxidase in methanol for 30 min at room temperature. Representative specimens were evaluated by the following antigen retrieval procedure. Three types of soaking solutions were employed: 10 mM citrate buffer, pH 6.0, 10 mM citrate buffer, pH 7.0 and 1mM EDTA solution, pH 8.0. After pressure cooking, the sections were left at room temperature for 30 min. The sections were incubated with polyclonal antibody against DPD overnight at room temperature. The specificity of this antibody has been reported previously (21). After rinsing in phosphate-buffered saline (PBS), pH 7.2, the sections were incubated with universal immunoperoxidase polymer, anti-mouse and rabbit (Histofine Simple Stain MAX PO, Nichirei, Tokyo, Japan), at room temperature for 30 min. The reaction products were visualized in 50 mM Tris-HCl buffer, pH 7.6, containing 50 mg/dl diaminobenzidine tetrahydrochloride and 0.006% hydrogen peroxidase. The nuclei were lightly counterstained with Mayer's hematoxylin, and the specificity of immunostaining with the polyclonal antibody was checked by preabsorption experiments using representative samples. Before immunostaining, the diluted antibody was combined with recombinant human DPD (Taiho Pharmaceutical Co., Ltd.) at final concentrations of 0.01, 0.1, 1.0 and 10 μg/ml, at 37°C for 1 h. As a positive control, we employed tumor tissue obtained from a xenograft of the human pancreatic cancer cell line MIAPaCa-2 in nude mice, established to have high DPD expression. Negative

Table I. *Patient characteristics in both regimen (S-1 : DIF, 5-FU : non-DIF) groups.*

Characteristics	S-1	5-FU	*p*-value
Total noumber of patients	31	30	
Age, years, median (range)	53.7 (30-73)	58.2 (39-70)	0.387
Gender (men/women)	16/15	18/12	0.592
ECOG performance status			
0	10	4	0.214
1	20	22	
2	1	4	
Histological type			
Intestinal type	2	1	0.978
Diffuse type	29	29	
Number of organs involved			
1	8	12	0.151
2	17	11	
3	6	7	
Site of metastatic disease			
Peritoneum	29	16	0.117
Distant lymph nodes	17	20	0.672
Liver	9	12	0.867
Lung	2	2	0.978
Others	3	5	0.330
Surgery (total gastrectomy)			
yes	17	11	0.126
no	14	19	
Treatment duration, days, median (range)	217 (27-767)	76 (25-258)	0.006
Number of chemotherapy cycles, mean (range)	5.0 (1-16)	2.4 (1-5)	0.045

controls were prepared by substituting PBS for the primary antibody (Rabbit Immunoglobulin Fraction: DAKO ENVISION). The slides were counterstained with hematoxylin.

Evaluation of immunostaining. Immunohistochemical staining intensity was semiquantitatively graded (– to 3+) on the basis of the proportion of positively-stained cancer cells in the lesions: –, negative; 1+, less than 1/3 of cancer cells positive; 2+, from 1/3 to less than 2/3 of cancer cells positive; 3+, 2/3 or more of cancer cells positive. A staining intensity of – to 1+ was considered negative, and that of 2+ to 3+ was considered positive. Immunohistochemical staining was evaluated independently by four investigators blinded to clinical outcomes. Any disagreement was resolved by consensus.

Statistical analysis. The statistical significance of the relationships of DPD immunoreactivity and TS immunoreactivity to the patients' responses to chemotherapy was evaluated with χ^2-tests. Survival curves were calculated with the Kaplan-Meier method and analyzed with the use of log-rank tests.

Results

Patients' characteristics. The patients' characteristics are provided in Table I. Thirty-four men and 27 women, with a median age of 55 years (range, 30-73 years) were included. Fifty-six patients (91.8 %) had a performance status of 0 or 1 on the Eastern Cooperative Oncology Group scale, and all patients received S-1 or 5-FU chemotherapy as first-line treatment, including preoperative neoadjuvant chemotherapy.

DPD immunoreactivity. DPD immunoreactivity was diffusely distributed in the cytoplasm of tumor cells, with some differences in staining intensity within a given tumor. All grading patterns of DPD immunoreactivity using anti-recombinant human DPD polyclonal antibody are shown in Figure 1 .

Immunoreactivity and response to chemotherapy. The overall response rate was 22.6% (7/31) in the S-1 group and 3.3% (1/30) in the 5-FU group. Positive rates for DPD were, respectively, 35.5% (11/31) in the S-1 group and 40.0% (12/30) in the 5-FU group. Response rates were 45.5% (5/11) in patients with DPD-positive tumors and 10% (2/20) in those with DPD-negative tumors ($p=0.044$) in the S-1 group, as compared with 0% (0/12) and 5.6 % (1/18) ($p=0.398$), respectively, in the 5-FU group.

Relationship between survival and DPD activity. The median survival time of all patients was 340 days (S-1: 393 days, 5-FU: 226 days). The median survival times were 364 days in patients with DPD-positive tumors and 406 days in those with DPD-negative tumors in the S-1 group ($p=0.626$), as compared with 181 days and 256 days, respectively, in the 5-FU group ($p=0.543$). The median survival time did not differ significantly between patients with DPD-positive tumors and those with DPD-negative tumors in either treatment group.

Discussion

Our study indicates that S-1 may be effective in the treatment of gastric scirrhous carcinoma with higher DPD activity. Several studies focusing on human cancer cell lines have suggested that intratumoral DPD levels, assessed on the basis of either enzymatic activity or mRNA expression, are good predictors of the response to 5-FU-based chemotherapy (25-27). Previous studies have also shown that inhibition of intratumoral DPD increases sensitivity to 5-FU, and that thymidylate synthase (TS) overexpression plays a major role in the resistance. Here, we focused on the antitumor effect of S-1 as a newly-developed DIF, and examined the correlation with a DIF antitumor effect and a biomarker (DPD). Immunohistochemical analysis has several important advantages over measuring protein and mRNA levels, since it is labor-saving, low-cost and can be used for tissue specimens fixed in formalin. We believe that it would be valuable to establish a simple and reliable method to assess DPD expression in biopsy specimens, since this is the only available material capable of providing information on the biological properties of tumors before chemotherapy. Antibodies against DPD have recently become available for immunohistochemical analysis, and studies have shown that DPD immunoreactivity correlates with DPD activity and the level of mRNA expression in cancer tissue. Cancer cells that express higher levels of DPD are considered more resistant to 5-FU and may be unresponsive to chemotherapy. However, our findings suggest that S-1 may be effective against gastric scirrhous carcinoma with higher DPD activity. Although there was no significant difference in median survival time between DPD-positive patients and -negative patients in the S-1 group ($p=0.626$) as compared with those of the 5-FU group ($p=0.528$), S-1 showed a higher response rate in tumors with a high DPD activity ($p<0.05$). These results indicates that S-1 could be more effective in gastric scirrhous carcinoma patients resistant to 5-FU only and with high DPD activity. One remarkable point was that all patients who responded to S-1 in the DPD-positive group showed shrinkage of primary lesions. Although DPD has been documented as an important determinant of chemosensitivity to 5-FU, most previous studies have found that the levels of DPD mRNA, protein and activity in tumors are unrelated to outcome. Our results, which showed no correlation of the DPD score in biopsy specimens with survival or time to progression, are in agreement with these findings.

In tumors with low DPD activity, inhibition of DPD by CDHP did not enhance cytotoxicity, even if tumor DPD activity was further reduced. In contrast, maximum

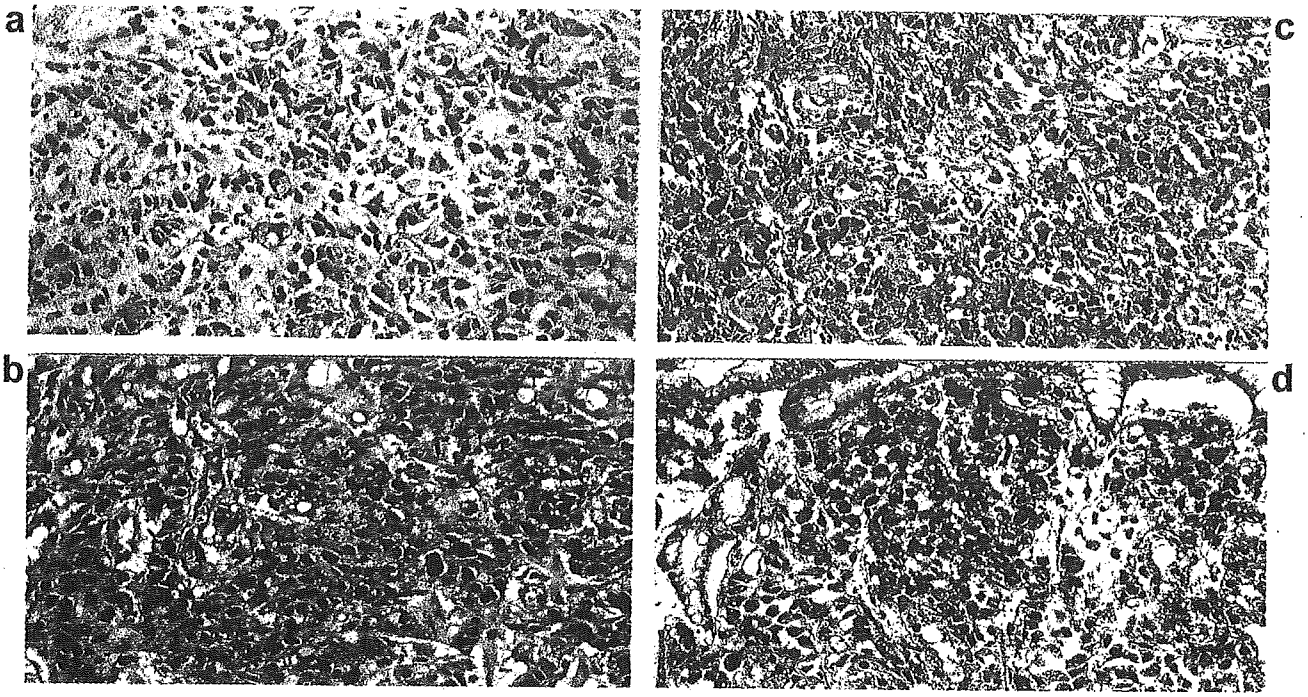

Figure 1. *All grading pattern of immunohistochemical staining for DPD with polyclonal antibody (x400 magnification). Positive staining for DPD is observed in the cytoplasm of cancer cells, with some differences in staining intensity within a given tumor. (a. DPD -, b. DPD 1+, c. DPD 2+, d. DPD 3+). A staining intensity of – to 1+ was considered negative, and that of 2+ to 3+ was considered positive.*

enhancement of the antitumor effect of S-1 would be expected in patients whose tumors have high DPD activity (28). Although the proportion of intratumoral DPD activity inhibited by CDHP is not clinically known, S-1 is expected to show antitumor effects, regardless of the status of intratumoral DPD. Similar to our results, several recent case studies have reported that S-1 is associated with shrinkage of primary lesions of Borrmann-type-4 gastric scirrhous carcinoma (2, 3). Although the mechanism of the response of primary lesions to S-1 remains unclear, strong inhibition of DPD, resulting in prolonged active concentrations of 5-FU in plasma and tumors, may be responsible for the shrinkage of these lesions.

In conclusion, our results suggest that S-1 may be effective against gastric scirrhous carcinoma, even in tumors with high levels of DPD activity. The relationship between DPD and the clinical response to other chemotherapeutic regimens should be investigated to determine whether intra-tumoral DPD activity is useful for selecting the best suited chemotherapeutic regimen. Further immunohistochemical studies on DPD with larger numbers of patients will hopefully contribute to the development of tailor-made DIF-based regimens designed to optimize response.

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ORIGINAL ARTICLE

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A phase I/II study of nedaplatin and 5-fluorouracil with concurrent radiotherapy in patients with T4 esophageal cancer: Japan Clinical Oncology Group trial (JCOG 9908)

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Abstract
Background. Nedaplatin is an analogue of cisplatin with less nonhematologic toxicity. The combination of nedaplatin and 5-fluorouracil showed a promising response rate in a previous phase II study for metastatic esophageal cancer. The purpose of this study was to determine a recommended dose and to evaluate the efficacy of nedaplatin and 5-fluorouracil combined with concurrent radiotherapy.
Methods. Eligibility criteria included squamous cell carcinoma of the thoracic esophagus; T4 disease without distant organ metastasis; age 20–70 years; performance status 0–2; and adequate organ functions. Patients received two cycles of nedaplatin (80 mg/m^2 or 90 mg/m^2) on day 1 and continuous infusion of 5-fluorouracil 800 mg/m^2/day on days 1–5, every 5 weeks with concurrent radiotherapy 60 Gy in 30 fractions.
Results. Between December 1999 and April 2002, 26 patients were accrued. The recommended dose of nedaplatin was 90 mg/m^2. Common grade ≥3 toxicities included leukopenia 9, neutropenia 5, thrombocytopenia 4, esophagitis 4, and esophageal fistula 3. Three of 26 patients achieved complete response (12%; 95% confidence interval, 2%–30%). With a minimum follow-up of 26 months for surviving patients, the median survival time was 12 months (95% confidence interval, 9–22 months), and the 2-year overall survival was 31% (95% confidence interval, 13%–49%).
Conclusions. This combined therapy is active with acceptable toxicity, however, the survival figure remains poor. Further investigation into more effective treatment is needed.

Key words Esophageal neoplasms · Drug therapy · Radiotherapy · Clinical trial

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Introduction

Esophageal cancer has become an important disease in the fight against cancer. In recent years, the number of patients with stage I disease has been increasing, but most patients are diagnosed with advanced disease and their prognoses are still daunting.

Over the last decade, chemoradiotherapy (CRT) for esophageal cancer has revealed promising results [1,2]. After the report of an intergroup randomized controlled trial (Radiation Therapy Oncology Group 85-01) that compared CRT with radiotherapy alone, the combined-modality treatment became a standard for patients with esophageal cancer who received nonsurgical treatment [3,4]. Most reports of CRT used cisplatin (CDDP) and fluorouracil (FU) with concurrent radiotherapy, and this combination is thought to be standard [1–6].

Nedaplatin (NDP; *cis*-diammine-glycolatoplatinum), a novel second-generation platinum compound, has shown promising antitumor activity with less nephrotoxicity, gastrointestinal toxicity, and neurotoxicity than CDDP in some preclinical and clinical studies [7–11]. The combination of NDP and FU also showed promising results in a phase II study for metastatic esophageal cancer [12]. Following the results of this phase II study, we decided to investigate this combination with concurrent radiotherapy in locally advanced disease. To determine a recommended dose and to evaluate the efficacy of NDP and FU combined with concurrent radiotherapy, we conducted a phase I/II study in patients with T4 (according to the International

Union Against Cancer tumor-node-metastasis system, 1997) esophageal cancer.

Patients and methods

Eligibility criteria

Eligibility criteria included previously untreated patients with pathologically proven squamous cell carcinoma of the thoracic esophagus; clinical tumor-node-metastasis system T4 disease without distant organ metastasis but supraclavicular and celiac nodes metastases were allowed; age, 20–70 years; performance status (PS; based on the Eastern Cooperative Oncology Group scale), 0–2; adequate hematologic [white blood cell count (WBC) count ≥ 4000/mm^3, platelet count ≥ 100000/mm^3, and hemoglobin ≥ 9.5 g/dl], hepatic [aspartate amino transferase (AST) and alanine amino transferase (ALT) level ≤ 2.5 times the upper limit of normal, and total bilirubin ≤ 1.5 mg/dl], and renal (creatinine ≤ 1.2 mg/dl and creatinine clearance ≥ 50 ml/min) functions; PaO_2 ≥ 70 torr; no esophageal fistula; no pleural and pericardial effusion; and no serious comorbidity. All patients gave written informed consent in accordance with institutional review boards.

Pretreatment evaluation

Pretreatment evaluation included history and physical examination; complete blood cell count; serum chemistries; chest radiograph; barium swallow; endoscopy of the esophagus; computed tomography (CT) scan of the neck, the chest, and the abdomen; and electrocardiogram. Endoscopic ultrasonography of the esophagus was optional. Bronchoscopy was performed if tracheobronchial involvement was suspected and surgical resection was under consideration. The tracheobronchial tree was judged to be involved if the tumors extended into the lumen or caused deformity of the lumen. The descending aorta was judged to be involved if the contact angle of the tumor was 90° or greater on the CT scan. Metastatic lymph nodes were defined if they were ≥1 cm in longest diameter on any imaging.

Treatment details

The treatment consisted of two cycles of NDP (level 1, 80 mg/m^2; level 2, 90 mg/m^2) on day 1 and continuous infusion of FU 800 mg/m^2/day on days 1–5, every 5 weeks, with concurrent radiotherapy at 60 Gy in 30 fractions over 6 weeks. The dose level of NDP was set referring to the results of a preceding phase I/II study in patients with metastatic esophageal cancer (data not shown). The second cycle of chemotherapy was set in the 6th week referring to a CDDP/5-FU chemoradiation regimen used in our institutions [5]. Radiotherapy was delivered with megavoltage equipment using anterior/posterior opposed fields up to 40 Gy including the primary tumor and the metastatic lymph nodes. An additional dose of 20 Gy was given to the primary tumor and the metastatic lymph nodes for a total dose of 60 Gy using bilateral oblique or multiple fields. The clinical target volume for the primary tumor was defined as the gross tumor volume plus 3 cm craniocaudally, and the clinical target volume for the metastatic nodes was the same as the gross tumor volume. The planning target volumes for the primary tumor and the metastatic lymph nodes were determined with 0.5- to 2-cm margins, taking account of setup variations and internal organ motion. Elective nodal irradiation was not intended in this study. Lung heterogeneity corrections were not used.

Toxicity assessment

Patients were observed weekly during treatment to monitor toxicity. Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria (version 2.0) [13]. Late toxicity was graded according to the Radiation Therapy Oncology Group (RTOG)/European Organisation for Research and Treatment of Cancer (EORTC) late radiation morbidity scoring scheme. Late toxicity was defined as that occurring more than 90 days after treatment initiation.

Follow-up evaluation

The following evaluations were performed until disease progression every 3 months for the first years and every 6 months thereafter: physical examination, toxicity assessment, complete blood cell count, serum chemistry profile, endoscopy of the esophagus, and CT scan of the neck, the chest, and the abdomen. Biopsy of the primary tumor site was routinely performed at each follow-up examination. Pulmonary function test, electrocardiogram, and cardiac ultrasound were performed when indicated.

Response assessment

Complete response (CR) for the primary tumor was defined by endoscopy when all visible tumors, including ulcerations, disappeared with negative biopsy and lasted for ≥4 weeks.

Responses of the metastatic lymph nodes were assessed using the World Health Organization response criteria for measurable diseases. Briefly, CR was defined as the complete disappearance of all measurable and assessable disease for ≥4 weeks. Uncertain CR (uCR) was defined when small nodes (≤1 cm) persisted with no evidence of progression for ≥3 months after completion of treatment, and it was also included in CR.

Patterns of failure

Patterns of failure were defined as the first site of failure. Local/regional failure included the primary tumor and

regional lymph nodes. Distant failure included any site beyond the primary tumor and regional lymph nodes.

Study design and statistics

Two dose levels were set following the results from a preceding phase I study for metastatic esophageal cancer. A recommended dose for phase II was determined using the conventional 3 × 3 method. Dose-limiting toxicities (DLTs) were defined as follows: treatment-related death; grade 4 thrombocytopenia; grade 4 vomiting; PS 3; grade 3 febrile neutropenia persisting ≥4 days; and grade 3 nonhematologic toxicities excluding anorexia, nausea, vomiting, esophageal fistula, esophagitis, and infection due to esophageal fistula. It was also regarded as DLT if radiotherapy could not be completed within 60 days or if protocol treatment could not be completed because of any adverse event. For exploratory evaluation of the efficacy of this treatment, the sample size for phase II part was determined following the assumption that a CR rate of less than 20% would not be promising and a CR rate of 40% or greater with α error of 0.10 and β error of 0.20 would warrant further investigation of this regimen. Taking into account that 10% of the patients may be ineligible, the total sample size including phase I part was determined to be 25 to 40. Survival was measured from the first day of treatment. Death from any cause was included as an event in the overall survival, and any failure and any cause of death were included as events in the progression-free survival. The overall and the progression-free survival curves were calculated by the Kaplan–Meier method [14].

Results

Patient population

Between December 1999 and April 2002, 26 patients were enrolled in the study: 3 patients at level 1 and 23 patients at level 2. Their median age was 60 years (range, 45–69 years), 25 were male, and 1 was female. Patient and tumor characteristics are summarized in Table 1.

Table 1. Patient and tumor characteristics

Number of patients	26
Age	
Median	60
Range	45–69
Sex	
Male	25
Female	1
Performance status	
0	14
1	12
Location	
Ut	13
Mt	12
Lt	1
TNM	
T4	26
N0	5
N1	21
M0	17
M1a	5
M1b	4
Stage	
III	17
IV	9
Involved sites in T4	
Aorta	4
Bronchial tree	19
Both	3

Ut, upper thoracic esophagus; Mt, middle thoracic esophagus; Lt, lower thoracic esophagus; TNM, tumor-node-metastasis classification

Treatment compliance and toxicity

One of 3 patients in the level 1 group and none of the first 3 patients in the level 2 group experienced DLT, and level 2 was determined to be the recommended dose. In total, including patients in the phase II part, 3 of 23 patients in the level 2 group experienced DLT. Twenty-four patients completed the protocol treatment, and 2 patients in the level 2 group could not complete the treatment due to DLT. Eight patients had treatment delay before delivering the second cycle of chemotherapy as a result of hematologic toxicity in 7 patients and pneumonia caused by esophageal fistula in 1 patient. The median overall treatment time of radiotherapy was 44 days (range, 42–56 days), and 21 patients completed radiotherapy within 49 days.

Common grade 3 or greater acute toxicities were leukopenia, 9 (35%); neutropenia, 5 (19%); thrombocytopenia, 4 (15%); esophagitis, 4 (15%); and esophageal fistula, 3 (12%). There was no treatment-related death. The toxicity profile is shown in Table 2. As of the date of this analysis, 1 case with grade 3 pericardial effusion, 1 with grade 3 pleural effusion, and 2 with esophageal stenosis were observed as late toxicities.

Response and survival

Of all 26 registered patients, 3 achieved CR with a CR rate of 12% [95% confidence interval (CI), 2%–30%]. With a minimum follow-up period of 26 months for surviving patients, the median survival and the 1- and 2-year survivals were 12 months (95% CI, 9–22 months), 50% (95% CI, 31%–69%), and 31% (95% CI, 13%–49%), respectively (Fig. 1). The median progression-free survival and the 1-year progression-free survival were 6 months (95% CI, 5–8 months) and 27% (95% CI, 10%–44%), respectively. Two of 3 CR patients and 6 of 23 non-CR patients survived more than 2 years.

Patterns of failure

At the time of this analysis, 22 of 26 patients (85%) showed tumor progression, and 4 patients (15%) were alive without disease progression. The patterns of first failure were local/

Table 2. Acute toxicities[a]

	Level 1 ($n = 3$)					Level 2 ($n = 23$)					Total
	Grade					Grade					≥Grade 3 (%)
	0	1	2	3	4	0	1	2	3	4	
Hemoglobin	0	3	0	0	0	1	13	9	0	0	0
Leukocytes	0	0	2	1	0	1	3	11	7	1	35
Neutrophils	0	0	2	1	0	4	7	8	3	1	19
Platelets	2	0	0	1	0	8	7	5	2	1	15
Creatinine	3	0	0	0	0	22	1	0	0	0	0
Performance status	1	2	0	0	0	4	16	3	0	0	0
Infection	2	0	0	1	0	14	2	5	2[b]	0	12
Diarrhea	1	2	0	0	0	17	5	1	0	0	0
Esophagitis	1	2	0	0	0	5	11	3	4	0	15
Esophageal fistula	3	–	–	0	0	20	–	–	3	0	12
Mucositis/stomatitis	3	0	0	0	0	14	4	3	2	0	8
Nausea	2	1	0	0	0	10	10	3	0	0	0
Vomiting	2	1	0	0	0	14	7	2	0	0	0
Dyspnea	3	0	0	0	0	21	0	2	0	0	0
Pneumonitis	3	0	0	0	0	22	0	1	0	0	0

[a] National Cancer Institute–Common Toxicity Criteria version 2
[b] Both cases were caused by esophageal fistula

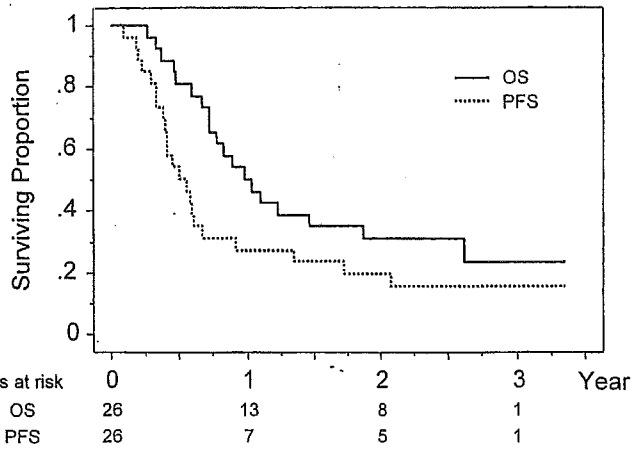

Fig. 1. Overall survival (*OS*) and progression-free survival (*PFS*) for all patients (*Pts*) enrolled in this study

regional only, 12 (46%); local/regional and distant, 3 (12%); and distant only, 7 (27%). Four patients developed local/regional progression after the occurrence of distant metastasis, and two patients developed distant metastasis after local/regional failure. In total, 19 (73%) patients developed local/regional failure and 12 (46%) patients developed distant failure.

Discussion

In the past decade, chemoradiotherapy consisting of CDDP and FU with concurrent radiotherapy has become a standard of care in selected patients with unresectable locally advanced esophageal cancer. Ohtsu et al. [5] reported median progression-free survival, median survival, and 1-year overall survival in patients with T4 and/or M1 lymph no(disease as 6 months, 9 months, and 41%, respectively. Grade ≥3 toxicities were also reported as follows: leukopenia, 24%; anemia, 24%; thrombocytopenia, 17%; esophagitis, 15%; and esophageal fistula, 10%. In our study, median progression-free survival, median survival, and 1-year survival were 6 months (95% CI, 5–8 months), 12 months (95% CI, 9–22 months), and 50% (95% CI, 31%–69%), respectively. Grade ≥3 toxicities were observed as follows: leukopenia, 31%; thrombocytopenia, 15%; esophagitis, 15%; and esophageal fistula, 12%. These results seemed comparable with CDDP and FU with concurrent radiotherapy, showing that the treatment regimen of NDP and FU with concurrent radiotherapy is effective in selected patients with T4 disease. However, these survival figures are far from satisfactory, and patterns of failure showed that about three-fourths of patients developed local/regional failure and about one-half of patients developed distant failure. We should make further efforts to improve local control and to prevent distant metastasis.

The dose-escalation strategy of radiotherapy was o1 way but was not proven to be effective in the INT 0123 study [15], and current approaches of escalating dose of radiotherapy with CDDP and FU could achieve incremental benefit but seem to have reached a plateau. Different combinations with a novel cytotoxic drug are another way to improve survival. Paclitaxel is active for both adenocarcinoma and squamous cell carcinoma of the esophagus. A phase II trial of paclitaxel in patients with advanced esophageal cancer showed a 34% response rate in adenocarcinoma and a 28% response rate in squamous cell carcinoma [16]. There is evidence of synergism between paclitaxel and CDDP or FU [17], and paclitaxel combined with CDDP and FU in patients with advanced esophageal cancer was tested in a phase II study [18]. This trial showed a 46% response rate in adenocarcinoma and a 50% response rate in squamous cell carcinoma. These encouraging results led to trials

employing induction chemotherapy followed by concurrent chemoradiotherapy with paclitaxel, CDDP, and FU, but the advantage of this approach has yet to be proven.

Recently, molecular targeted drugs have been enthusiastically investigated in various malignant diseases [19–22]. Epidermal growth factor receptor is one of the targets, and this has been shown to be effective in patients with head and neck cancer when combined with radiotherapy in a phase III study [23]. It seems reasonable to investigate whether the combination of these agents has a survival impact for esophageal cancer.

There is another concern about the response criteria in the treatment of esophageal cancer. We employed response criteria using endoscopy for the primary tumor, which seemed to be reliable in patients who received nonsurgical treatment [6]. In this trial, the CR rate obtained was far less than expected, and this treatment regimen should be deemed ineffective according to the predefined hypothesis. However, 2 of 3 CR patients and 6 of 23 non-CR patients survived more than 2 years, 3 of these 6 non-CR patients did not show any disease progression, and the median survival obtained was not worse than historical data. This discrepancy suggests that the CR criteria used in this trial was not applicable to T4 disease and thus the CR rate failed to be a surrogate endpoint for survival. We think that overall survival will be appropriate as a primary endpoint in future phase II trials for this patient population.

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