significantly higher than systemic concentration, but also because the drugs are largely extracted by the liver during the first pass, resulting in minimal systemic toxicity [18]. In a number of randomized studies using fluoropyrimidine derivatives, response rates were significantly higher for hepatic arterial infusion (HAI), as compared with intravenous administration. However, the prolongation of survival in patients treated with HAI still remains controversial in the United States and Europe where floxuridine (FUDR) alone has been used for hepatic arterial infusion [2, 5, 16, 19, 20, 22, 26]. FUDR is almost exclusively extracted by the liver, therefore it seems to be difficult to control the micrometastases in the extrahepatic region. Unlike FUDR, a certain level of 5-fluorouracil (5-FU) remains in the systemic when injected into the hepatic artery [6, 23].

The combination of 5-FU and cisplatin (CDDP) exhibits sequence-dependent synergy both in vitro and in tumor-bearing animals [25, 29, 30]. Several in vivo studies including the investigations of human tumor xenografts in nude mice demonstrated that the sequence of 5-FU followed by CDDP was more active than the reverse sequence or either drug alone [21, 25, 32, 33]. The clinical efficacy of 5-FU and CDDP combination has been confirmed. However, such a sequence-dependent antitumor activity has yet to be clinically determined in the treatment of metastatic colorectal cancer confined to the liver. Therefore, the current study was designed to assess the efficacy and tolerability of ambulatory continuous HAI of 5-FU followed by CDDP for such patients who underwent complete resection of primary tumor. The primary objectives of the research were to observe objective response rate, survival, time to progression (TTP) and toxicities in outpatient setting.

Patients and methods

Eligibility criteria

We included patients with histologically confirmed colorectal cancer, who had multiple and/or massive metastases confined to the liver that were not amenable to surgery replacement (the presence of more than 60% liver by unresectable metastasis) after complete resection of primary tumor. Inclusion criteria were as follows: a history of primary colorectal cancer excision; no evidence of extrahepatic metastasis on computed tomography, bone scintigraphy, and magnetic resonance imaging (MRI) if needed; age 20–80 years; Eastern Cooperative Oncology Group performance status ≤ 2; measurable liver lesions; leukocyte count $\geq 3{,}500/mm^3$; neutrophil count $\geq 1{,}500/mm^3$; platelet count $\geq 100{,}000/mm^3$; serum creatinine ≤ 1.5 mg/dl; serum bilirubin ≤ 2.0 mg/dl; AST ≤ 100 IU/l, ALT ≤ 100 IU/l; a life expectancy ≥ 3 months; and adequate cardiac function. Previous fluorouracil-based treatments were eligible if treatment had been completed more than 4 weeks before study entry. Exclusion criteria were: extrahepatic metastases; active uncontrolled infection; unresolved bowel obstruction; known contraindications to fluorouracil (angina pectoris, myocardial infarction in the past 6 months); and portal vein occlusion. This study was approved by the local ethics committee, and patients were informed of the investigational nature of the study and provided their written informed consent before registration in the study.

Treatment plan

Hepatic arterial infusion was given by percutaneous catheterization of the femoral artery. Procedures for pump placement are as follows. A catheter was inserted into common hepatic artery from right femoral artery. Under celiac angiography, the right gastric artery, and gastroduodenal artery were occluded by a steel coil, to avoid inflow of anticancer drugs to other organs. The collateral arteries which feed the liver, if any, were also occluded. After confirming the presence of the tip of a heparin-coated catheter in the common hepatic artery, a reservoir connected to the catheter was implanted in a subcutaneous pocket in the right subinguinal portion and the catheter was secured in the artery. An intraoperative injection of contrast material was used to check the flow immediately after placement.

Patients were treated with 5-FU (450 mg/m^2/day) on days 1–7, which was followed by CDDP (100 mg/body/week) on days 8–14. Each of them was administered continuously using a LV 1.5 Baxter balloon pump (275 ml) at a flow rate of 1.5 ml/h via Infuse-A-Port catheter inserted into common hepatic artery. Dexamethasone 8 mg and heparin 35,000 unit were mixed in balloon pump and concurrently infused. The doses of 5-FU and CDDP were reduced 20% in patients above 70 years. To prevent nausea and vomiting, 5-hydroxytryptamine-3 antagonists were intravenously administered before chemotherapy. G-CSF was used when neutropenia less than $500/mm^2$ or febrile neutropenia less than $1{,}000/mm^3$ were present. Treatment was continued until evidence of progression, unacceptable toxicity, or patient refusal. Treatment was delayed if, on the planned day of treatment, there was leucopenia less than $3{,}000/mm^3$, thrombocytopenia less than $100{,}000/mm^3$, infectious fever, persistent diarrhea, or nonhematological toxicities greater than grade 3, except for nausea and vomiting. If toxicities greater than grade 3 were observed, the doses of both 5-FU and cisplatin were reduced by 20% on the next cycle. This treatment was repeated every 4–6 weeks.

Assessment of treatment and response

Pretreatment evaluation included a complete history, physical examination, performance status assessment and laboratory examinations including hepatic and renal

functions, urinalysis, complete blood count with differential leukocyte profile, serum alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA) and CA19-9. Computed tomography (CT) scans of the chest, abdomen and pelvis were performed before commencement of chemotherapy. During treatment, a physical examination, a complete blood count and urinalysis were performed once a week. Hepatic and renal functions were examined once a month. CEA and CA19-9 were checked every 2 months. Liver metastatic lesions were reevaluated every 8 weeks. A chest radiograph and abdominal ultrasonography or CT scan were repeated at least every 2 month to exclude lung or other abdominal metastases. For evaluating the response to the treatment, the tumors were measured bidimensionally by computed tomography (CT) both before and after chemotherapy. Responses were evaluated every 8 weeks according to World Health Organization Criteria. Toxicities were monitored weekly and scored according to standard NCI-CTC.

Statistical analysis

The data were statistically analyzed using JMP software (SAS Institute Inc, Cary, NC, USA). Survival estimates were calculated using Kaplan-Meier curves and confidence intervals were calculated using Greenwood variance formula. Survival was calculated until death as a result of any cause, and progression-free survival was calculated from start of chemotherapy until progression of disease or death as a result of any cause.

Results

From May 1997 to September 2003, we randomly enrolled 17 patients with extensive and/or massive metastases confined to the liver from colorectal cancer. Patient characteristics were listed in Table 1. There were 4 women and 13 men. The median age was 68 years, with a range from 45-year-old to 80-year-old. Among 17 patients recruited in the group, 15 were in ECOG performance status 0 and the other two in performance status 1 and 2, respectively. Of the five patients who received prior chemotherapy, three were administered CPT-11-based systemic regimen, one 5-FU-based systemic treatment, and one HAI treatment. Patients were given the HAI treatment after we confirmed that they had normal vasculature and could have a catheter inserted to perfuse the liver completely. Infusion via collateral arteries was done in two patients. Total 176 cycles (median 10, range 3–20 per patient) were done so far.

All patients enrolled were assessable for responses. Nine out of 17 patients (53%) showed PR (95% CI, 29.3–76.7%), one of them received a resection of liver metastases after the treatment. Eight patients (47%; 95% CI, 23.3–70.7%) experienced NC. Therefore, disease control rate was 100%. Two patients (11.8%), who showed progression due to collateral feeding arteries, responded to HAI again after occlusion of these vasculature. As shown in Fig. 1, the median overall survival was 26 months (95% CI, 17.5–41 months). The

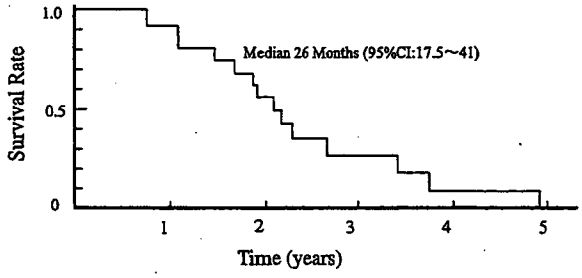

Fig. 1 Kaplan-Meier survival curve of overall survival

Table 1 Patient characteristics and treatment results (As of October 30th, 2004)

Pts	Age/Sex	ECOG PS	Primary site	Histology	Liver metastases	No. of cycles	Response	Survival (Months)
1	56/M	0	Rectum	Well-mod	Multiple	17	PR	27.5
2	70/F	0	Sigmoid	Well-mod	Multiple	18	PR	32
3	77/M	1	Rectum	Well-mod	Multiple	11	NC	13
4	73/M	0	Rectum	Mod	Multiple	9	PR	23
5	73/M	0	Descending	Well	Multiple	10	PR	59
6	80/F	0	Rectum	Well	Multiple	19	PR	45
7	55/M	0	Sigmoid	Mod	Multiple	20	PR	41
8	72/M	0	Sigmoid	Well	Multiple	11	NC	20
9	76/M	2	Sigmoid	Well-mod	Multiple	7	NC	13
10	59/M	0	Rectum	Well-mod	Multiple	6	PR	17.5
11	63/F	0	Ascending	Well-mod	Multiple	6	NC	9
12	70/M	0	Rectum	Mod	Multiple	3	NC	22.5
13	56/F	0	Rectum	Well-mod	Multiple	14	NC	29+
14	67/M	0	Descending	Well-mod	Multiple	3	PR	26
15	45/M	0	Rectum	Well	Massive	12	PR	25+
16	59/M	0	Sigmoid	Mod	Multiple	7	NC	13+
17	68/M	0	Sigmoid	Well	Multiple	10	NC	25

ECOG Eastern Cooperative Oncology Group, *PS* performance status, *Well* well-differentiated adenocarcinoma, *Mod* moderately-differentiated adenocarcinoma, *Well-mod* well to moderately differentiated adenocarcinoma. + alive, *Pts* patients

1-, 2-, and 3-year overall survival rates were 94.1, 57, and 27.1%, respectively. Three patients (17.6%) are alive at present. Median time to progression (TTP) was 14 months (95% CI, 11–20.3 months) (Fig. 2). Four patients (23.5%) progressed at extrahepatic sites, mostly lung (three patients), bone (one patient), brain (one patient).

All the patients were assessable for toxicities and catheter-related complications. There were no treatment-related deaths during the entire courses of study. Five patients experienced the replacement of catheter due to the obstruction. There was no evidence of chemical hepatitis, biliary sclerosis, catheter-induced thrombosis, and duodenal ulceration and hemorrhage that have been associated with HAI administration of chemotherapy. No patient developed severe abdominal pain suggestive of gastroduodenal ulcer or gastroduodenitis. Elevated liver enzymes in documented disease progression were not considered treatment-related toxicities. Non-hematological toxicity was rare with one patient (6%) showing grade 1 vomiting. Hematological and renal toxicities were summarized in Table 2. Grade 3 toxic effects were leukocytopenia (12%) and anemia (24%). No grade 4 toxicities were observed. Cardiac and neurological adverse effects were not encountered in any of the patients. As a result, all the patients received the doses as scheduled.

Discussion

The unique blood supply of the liver allows hepatic arterial infusion active and feasible for patients with liver metastasis [18]. In a number of randomized studies using fluoropyrimidine derivatives, response rates were significantly higher for hepatic arterial infusion (22–62%), as compared with intravenous administration (9–19.6%). However median survivals for HAI treatment groups (12.6–17 month) were not always significantly longer than those for systemic treatment groups, which ranged from 7.5 months to 16 months, indicating that the prolongation of survival remains controversial [2, 5, 16, 19, 20, 22, 26]. Moreover in a randomized study to compare an intrahepatic arterial 5-FU plus leucovorin regimen with the standard intravenous de Gramont fluorouracil plus leucovorin regimen for patients with metastatic colorectal cancer confined to the liver, the objective response rate was 22% and the stable disease rate 32%, median overall survival and TTP were 14.7 and 7.7 months, respectively, 57% of patients were alive at 1 year, and 22% at 2 years in the HAI treatment group. Moreover, there was no evidence of advantage in TTP and overall survival in HAI treatment group, as compared to systemic treatment group [20]. In the present study, sequential hepatic arterial infusion of 5-FU followed by CDDP demonstrated PR and SD in 53 and 47% of treated patients respectively, with disease control rate (PR + SD) of 100%. The median overall survival was 26 months (95% CI: 17.5–41 months) and median TTP 14 months (95% CI: 11–20.3 months). Therefore, sequential hepatic arterial infusion of 5-FU followed by CDDP appears superior to previous HAI treatment employing fluoropyrimidine derivatives. In addition, 3-year overall survival rate was 27.1%, being significantly higher than patients with unresectable liver metastases, who showed a median survival of approximately 9 months and 3 year survival of less than 3% [3, 17, 28].

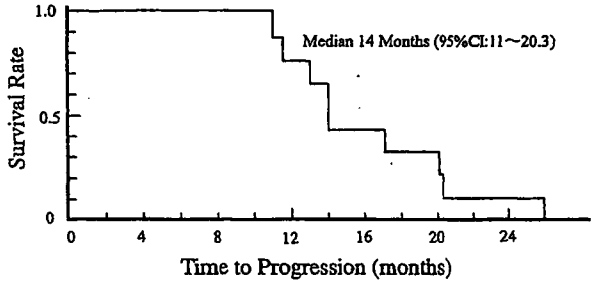

Fig. 2 Kaplan-Meier survival curve of time to progression

Recent progresses have been achieved in the treatment of colorectal cancer by introducing CPT-11 or oxaliplatin. Several phase III trials investigating combination regimens with FU/LV plus CPT-11 or FU/LV plus oxaliplatin as a first-line therapy have achieved overall survival of 14.8–21.5 months [14]. The use of all three active drugs in advanced colorectal cancer produced the longest overall survival [15]. Indeed, triple-combination protocols using FU-LV plus irinotecan plus oxaliplatin have consistently resulted in high response rates of 57–78% in patients with previously untreated advanced CRC and have produced the longest overall survival of 22.5 months in one trial [12, 24, 27, 31]. In comparison with these systemic treatments, we obtained a survival benefit of at least 3.5 months with sequential HAI treatment despite patients having liver metastasis, an extremely poor prognostic factor.

Table 2 Hematological and renal toxicities

NCI-CTC Grade	Number of patients (%)			
	Leucocytopenia	Thrombocytopenia	Anemia	Renal dysfunction
1	1 (6%)	7 (41%)	2 (12%)	1 (6%)
2	1 (6%)	–	4 (24%)	2 (12%)
3	2 (12%)	–	4 (24%)	–
4	–	–	–	–

Data are indicated as the maximum number of patients with the most severe grade of toxicity

Therefore, it seems likely that this sequential treatment would be a much better option for patients with metastatic CRC confined to the liver.

The rationale of HAI is based on increased local drug concentrations and hepatic clearance of the drug before entering systemic flow. Continuous hepatic infusion of 5-FU and CDDP has been shown to yield fivefold to tenfold and fourfold to sevenfold higher local concentration than systemic administration, respectively [7]. In addition, protracted infusion may expose a relatively larger proportion of cycling tumor cells to 5-FU, thereby increasing the efficacy of 5-FU. Moreover, the combination of 5-FU and CDDP has been shown to exhibit a sequence-dependent synergy in vitro and in vivo, with sequence of 5-FU followed by CDDP being the most active schedule. This sequence-dependent synergy can be explained by the mechanism of DNA damage repair and detoxification processes; i.e., pretreatment of 5-FU increased CDDP cytotoxicity and even circumvents CDDP resistance by inhibiting repair of platinum-DNA interstrand cross-links as well as by reducing the cellular GSH levels [8, 9].

Although our treatment improved response rate and prolonged the survival of the CRC patients with liver metastases, further follow-up of these patients and accrual of more numbers of patients are needed. Moreover, four patients (23.5%) experienced extrahepatic metastases which led to the patients' death. Therefore, prevention of extrahepatic micrometastases with systemic chemotherapy appears to be mandatory to further improve overall survival. In conclusion, this sequential combination of 5-FU followed by CDDP through hepatic artery is active and safe in an outpatient setting for patients with colorectal cancer metastasized only to the liver, and warrants further multi-institutional studies.

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Gefitinib, a Selective EGFR Tyrosine Kinase Inhibitor, Induces Apoptosis Through Activation of Bax in Human Gallbladder Adenocarcinoma Cells

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Abstract Although gefitinib, a selective inhibitor of epidermal growth factor receptor (EGFR) tyrosine kinase, has been clinically demonstrated to be effective for certain cancer cell types, the molecular mechanisms of the anti-tumor activity have not been fully elucidated. In this study, we investigated the mechanism of gefitinib-induced growth inhibition and apoptosis in HAG-1 human gallbladder adenocarcinoma cells. Treatment of gefitinib at a dose of 1 μM resulted in a significant growth inhibition, and the cell number irreversibly declined after 72-h incubation, with a progressive expansion of apoptotic cell population over 120-h. Following 2-h treatment, gefitinib significantly inhibited EGFR autophosphorylation and subsequent downstream signaling pathway through Erk and Akt, and induced accumulation of cells in the G0/G1 phase of the cell cycle at 24-h, accompanied by a concomitant increase in p21 transcript and increased expression of p27. Gefitinib did not affect the amount of total and phosphorylated p53 at serine 15, but upregulated the expression of total Bax, with subsequent increase in p18 Bax, an active form of Bax. The expression of Bcl-2 and Bad was unchanged. An increase in gefitinib-induced expression of total Bax might be due to the decreased degradation of Bax, because the level of Bax mRNA has not been altered by gefitinib treatment. Gefitinib promoted the cleavage of full-length p21 Bax into p18 Bax in mitochondrial-enriched fraction, a characteristic feature of Bax activation toward apoptosis. Moreover, blockade of Bax by using anti-Bax small interfering double stranded RNA (siRNA) significantly reduced gefitinib-induced apoptosis. Taken together, these data suggest a critical role of p18 Bax in gefitinib-induced apoptosis. J. Cell. Biochem. 97: 724–734, 2006.

Key words: gefitinib; EGFR; Bax; apoptosis; Akt; Erk

The EGFR, a receptor tyrosine kinase, is overexpressed in a wide variety of epithelial malignancies including non-small cell lung, head, neck, colon, and breast cancers [Salomon et al., 1995; Shirai et al., 1995; Grandis et al., 1998; Brabender et al., 2001] and enhanced expression of epidermal growth factor receptor (EGFR) is associated with more aggressive disease and a poor patient prognosis [Fox et al., 1994; Rusch et al., 1997]. Upon ligand binding, EGFR is activated through autophosphorylation by forming homodimerization or heterodimerization with other members of the HER family tyrosine kinases [Olayioye et al., 1998; Muthuswamy et al., 1999], and transduces a variety of signals to downstream signal transduction cascades that lead to cellular proliferation and survival [Alroy and Yarden, 1997; Schlessinger, 2000].

Gefitinib, a quinazoline derivative that inhibits EGFR tyrosine kinase activity, has been shown to be effective in preclinical studies and in late stages of clinical trials for non-small cell lung cancer [Fukuoka et al., 2003; Sirotnak, 2003], although the activity is associated with

Abbreviations used: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; RTK, receptor tyrosine kinase; MAPK, mitogen activated protein kinase; Erk, extracellular signal-regulated kinase; PI-3K, phosphatidylinositol 3′-kinase.

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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 $1 \sim 2 \times 10^4$ 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

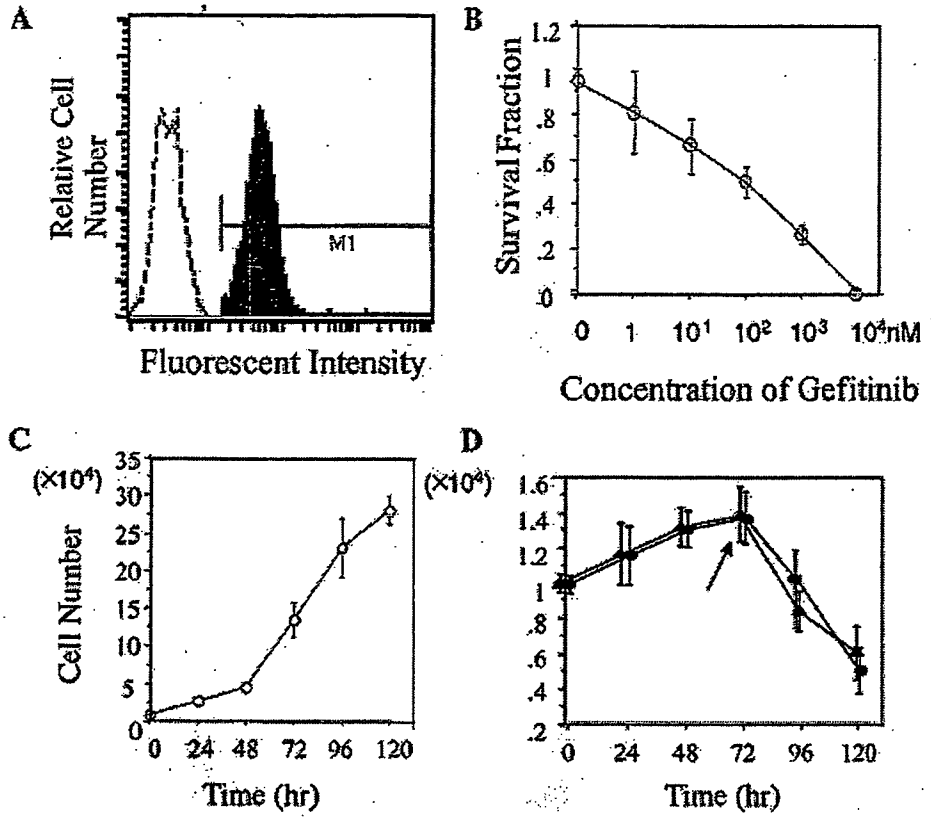

Fig. 1. Expression of EGFR, cytotoxicity of gefitinib, and effects of gefitinib on the growth of HAG-1 cells. A: Expression of EGFR was analyzed by FACS after treatment of cells for 1 h at 4°C with 1 μg of the anti-EGFR antibody. B: Cytotoxicity was determined by using WST-1 assay. Cells were seeded 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

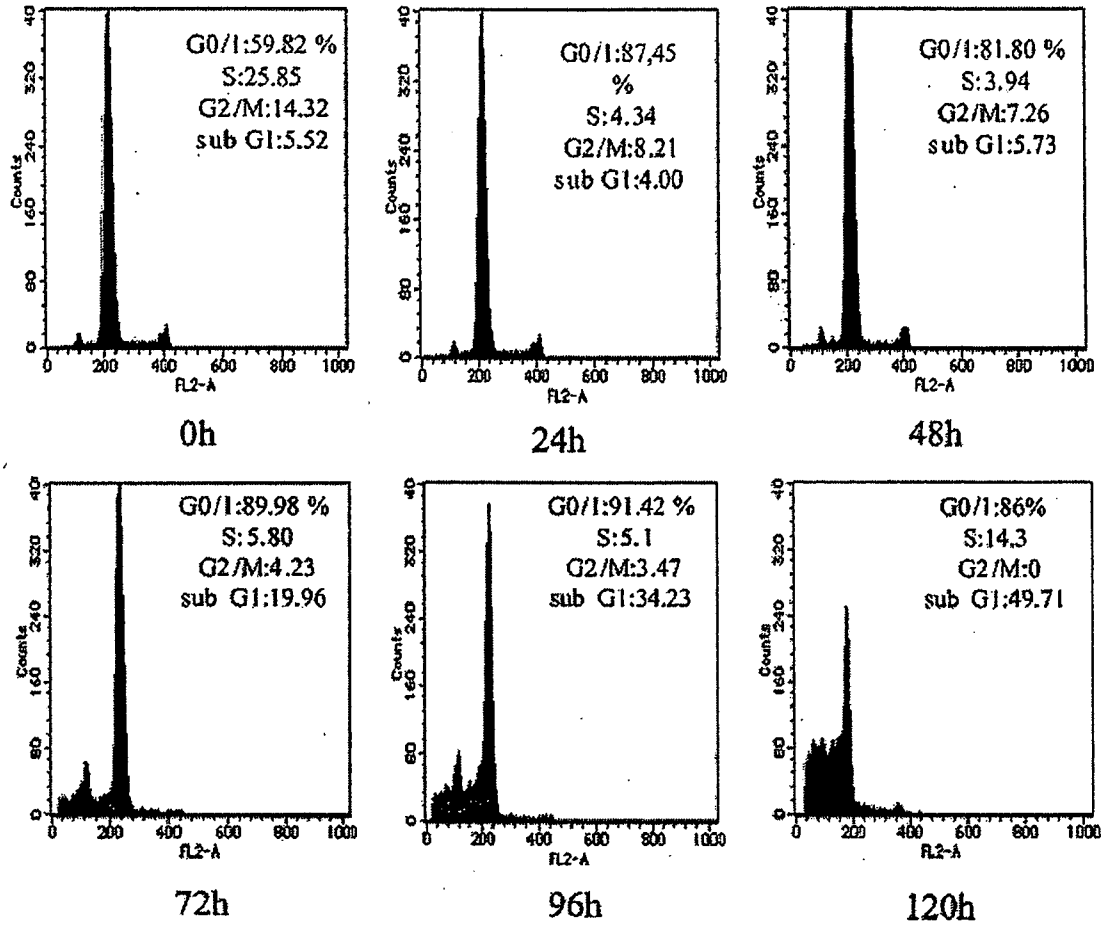

Fig. 2. Time course analysis of the effect of gefitinib on cell cycle progression and apoptosis. HAG-1 cells were stained with propidium iodide after exposure to gefitinib (1.0 μM) for 0, 24, 48, 72, 96, and 120 h, and analyzed by flow cytometry. Percentages of the total cell population in the different 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

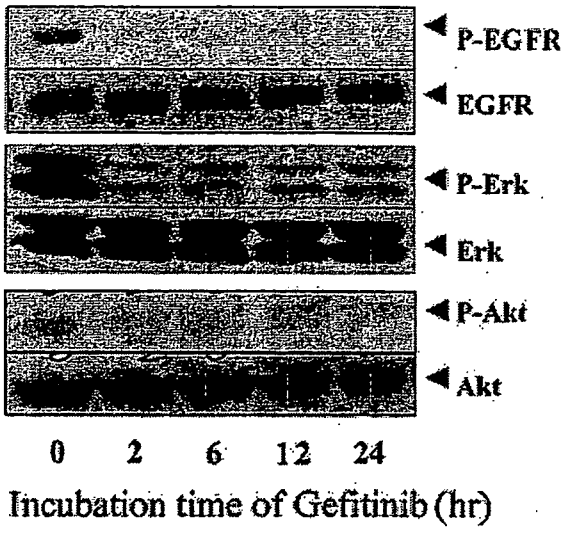

Fig. 3. Effects of gefinitib on the phosphorylation of EGFR and downstream Akt and Erk 1/2. Western blots are shown for phospho- and total EGFR, Erk 1/2, and Akt.

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

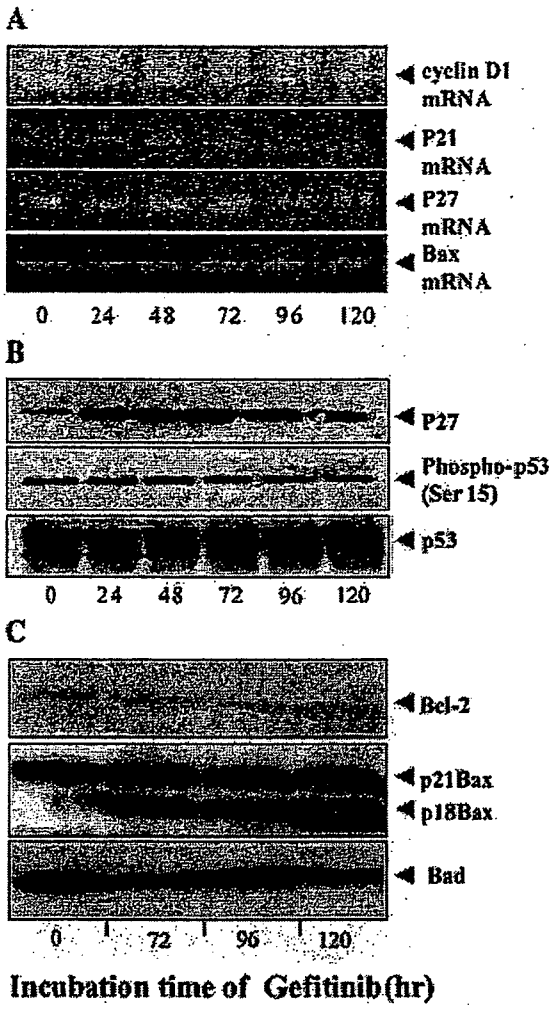

Fig. 4. Quantitative evaluation of apoptosis-associated proteins and RNA transcript in HAG-1 cells treated with gefitinib. The cells were exposed to 1.0 μM gefitinib for indicated times, and processed for RT-PCR and immunoblot analyses as described in Materials and Methods. A: Quantitative analysis of 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

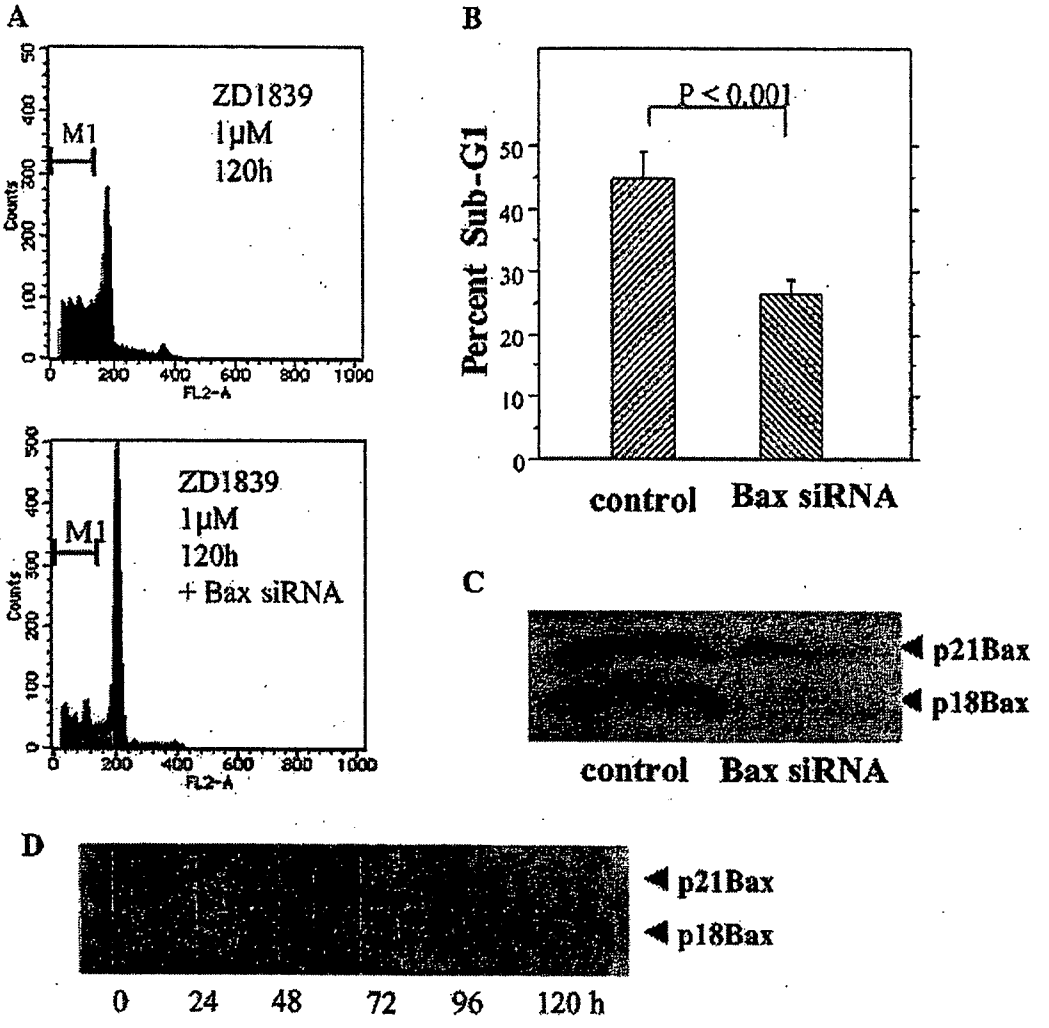

Fig. 5. Attenuation of apoptosis by Bax siRNA and translocation of Bax to mitochondria. HAG-1 cells were transfected with anti-Bax siRNA and processed for FACS analyses as described in Materials and Methods. A: Flow cytometric analysis of cell cycle progression at 120 h following treatment with gefitinib. 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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特集 消化器癌に対する化学療法の進歩

Gastrointestinal Research

消化器癌化学療法の進歩がもたらした臨床上の変化

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Summary
近年の新規抗癌剤や化学放射線療法などの治療法の開発により，消化器癌に対する化学療法は，治癒率，生存期間などの点において着実な進歩が得られている．とくに，大腸癌，膵癌，消化管間質腫瘍(GIST)では新薬の導入と大規模な臨床試験により確立されたエビデンスが日常診療にも導入されている．胃癌では，新薬導入が先行したことにより治療成績は向上したが，第III相試験によるエビデンスがないために日常診療が混沌としており，現在進行中の比較試験の結果を待ちたい．食道癌では，化学放射線療法により治癒が得られるまでになったが，手術療法などを含めた総合的な治療戦略による更なる進歩が期待される．

Key words
化学放射線療法　テガフール・ギメラシル・オテラシルカリウム(S-1)
イリノテカン　タキサン　ゲムシタビン

はじめに

消化器癌に対する新たな化学療法に期待される効果として，治癒率の向上，生存期間の延長，無増悪生存期間の延長，腫瘍縮小効果の向上，毒性の軽減，利便性などがあげられるが，近年の新規抗癌剤の開発によりさまざまな癌種において多くの進歩が得られ，その成果が日常診療に導入されつつある．切除不能・再発症例などの治癒が期待できない症例における生存期間の延長などの点で最も大きな進歩をもたらすのは新薬の導入であるが，既存の抗癌剤でも手術療法・放射線療法と組み合わせることにより，従来治癒が期待できなかった症例の中に治癒が得られる割合も高まりつつある．とくに，大腸癌，消化管間質腫瘍(gastrointestinal stromal tumor：GIST) ではすでに多くのエビデンスが確立されているが，これらの詳細については他稿にそれぞれの新規薬剤別に記載されているので，本稿では食道癌，胃癌，膵癌を中心に記載する．

1 ▌食道癌における化学療法

Radiation Therapy Oncology Group (RTOG) による進行食道癌に対する，放射線単独療法とフルオロウラシル (5-FU)＋シスプラチン (CDDP) と放射線療法の併用 (FP-R) 療法との比較試験により，FP-R 療法のほうがすぐれた延命効果，また高い3年，5年生存率を示した[1]．この結果を受け

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表 1. 切除可能食道癌に対する術前化学放射線療法と手術単独の第III相比較試験

	化学療法	放射線療法	n	MST(月)	3年生存率(%)	p値
Burmeister	FP	35 Gy	128	21.7	33	ns
	—	—	128	18.5	28	
LePrise	FP	20 Gy	41	10	19	ns
	—	—	45	11	14	
Urba	FP	45 Gy	50	16.9	30	ns
	—	—	50	17.6	16	
Walsh (腺癌)	FP	40 Gy	58	16	32	0.01
	—	—	55	11	6	
Walsh (扁平上皮癌)	FP	40 Gy	46	12	36	0.017
	—	—	52	8	11	
Apinop	FP	40 Gy	34	9.7	24	ns
	—	—	35	7.4	10	

FP：5-FU+シスプラチン, ns：not significant, MST：生存期間中央値

て，わが国でも気管などの他臓器浸潤（T 4）を有する，または放射線の照射野内に含まれる遠隔リンパ節転移（M1 Lym）のために切除不能と判断される局所進行食道癌に対するFP-R療法の第II相試験が施行され，5年生存率が17%であった[2]．また，最も進行した食道癌では気管，縦隔などに瘻孔をしばしば合併し，このような症例では放射線療法により腫瘍が縮小しても瘻孔が大きくなるため，従来は瘻孔を有する，または合併した食道癌に対しては放射線療法が禁忌とされていた．しかし，化学放射線療法により著明な腫瘍縮小効果が得られれば，瘻孔の閉鎖だけでなく治癒も得られる可能性が示された．このように，従来は治癒が得られないと考えられてきた局所進行食道癌に対しても，治癒が得られるようになってきた．

5-FU+CDDPを用いた化学放射線療法により，切除可能な食道癌（stage II，III，T 4を除く）に対しても，画像検査にて腫瘍が消失した完全寛解（complete remission：CR）が約60～70%の症例に得られ，5年生存率も40～50%であると報告されている[3]．また，内視鏡的切除不能な早期食道癌（T 1）に対しても95%以上でCRが得られ，3年生存率も80%以上と報告されている．これらの成績は，わが国おける食道癌に対する外科的切除術の全国アンケートの成績に匹敵すると考えられ，従来は開胸・開腹による侵襲の大きな手術がおこなわれてきた症例に対して，切除せずに治癒が得られるようにもなってきた．しかし，外科的切除術に比較して食道癌に対する化学放射線療法は局所制御率において，劣っているといわざるを得ない．また，長期生存例が得られるようになったことを受けて，心・肺に対する放射線の晩期毒性の問題が明らかになりつつある[4]．最近，放射線線量の50.4 Gyと64.8 Gyの比較試験では50.4 Gyのほうが比較的良好な成績を示し[5]，「放射線線量の多いほど，治療効果が高い」という既存の概念にも見直しがかかっており，今後，三次元照射を含めて毒性の軽減の方向性が求められる．

上記の状況のもと，「切除可能な食道癌に対して外科的切除術と化学放射線療法のいずれがすぐれているかを比較すべきである」，または「個々の症例における癌遺伝子を含めた特性を明らかにすることで両者を使い分けるべきである（オーダーメイド医療）」ともいわれている[6]．しかし，欧米で術前に化学放射線療法＋手術群と手術単独群の比較試験が相次いで報告され（表1），有意差を示したもの

表 2. 切除不能・再発胃癌に対する第III相比較試験

年	著者	グループ	症例数	結果
1991	Wils	EORTC	206	FAMTX>FAM
1992	Kelsen	MSKCC	60	FAMTX=EAP
1993	Kim	Korea	298	F=FP=FAM
1994	Cullinan	NCCTG	252	F=FAMe=FAP
1995	Vanhoefer	EORTC	274	FAMTX=FP=ELF
1997	Webb	UK	274	ECF>FAMTX
1999	Ohtsu	JCOG	278	F=FP>UFTM
2005	Moiseyenko		445	DCF>FP
2005	Dank		337	FOLFIRI=FP

FAMTX：5-FU+ドキソルビシン+メトトレキサート, FAM：5-FU+ドキソルビシン+MMC
EAP：エトポシド+ドキソルビシン+CDDP, F：5-FU, FP：5-FU+CDDP
FAMe：5-FU+ドキソルビシン+metyl-lomustine, ELF：エトポシド+5-FU+ロイコボリン
ECF：エトポシド+CDDP+5-FU, UFTM：テガフール・ウラシル+マイトマイシンC, DCF：ドセタキセル+5-FU+CDDP
FOLFIRI：5-FU+ロイコボリン+イリノテカン

は少ないものの，いずれの試験においても術前化学放射線療法＋手術群のほうが良好な成績を示した．また，化学放射線療法単独と化学放射線療法＋手術群の比較でも，同様に化学放射線療法＋手術群のほうが良好な傾向を示した[7]．これらの結果を合わせて考えると，外科的切除術も化学放射線療法もそれぞれの単独療法よりも，化学放射線療法＋手術群のほうがすぐれていると思われる．一方，化学放射線療法の 40 Gy 前後照射時点で腫瘍縮小効果があった症例に対しては，化学放射線療法を 60 Gy まで完遂させても，手術を追加しても，予後に差がないことが報告されている[8]．以上より，今後は切除可能な食道癌に対しては，比較的侵襲の少ない化学放射線療法を施行し，40〜50 Gy 時点で治癒が得られるか否かを判別し，または CR 後の局所再発例など，化学放射線療法では治癒が得られない症例に対して救済手術(salvage surgery)をおこなうといった総合的な治療戦略の構築が必要であると思われる．

食道癌は，疾患頻度が低いことと，化学放射線療法が主体であるため，新規抗癌剤の開発が遅れている．食道癌以外でも，化学放射線療法が治療の主体である頭頸部癌領域では分子標的治療薬も含めた新薬の導入が試みられている．今後，企業ばかりに期待するのではなく，医師主導の治験を展開することなどは腫瘍内科医の果たすべき役割であり，また放射線腫瘍医は局所制御の向上，晩期毒性の軽減，救済手術をおこないやすくすることなどをめざした放射線療法の工夫をおこなうべきであり，さらに外科医は安全にかつ効果的な救済手術の方法を確立することが急務であると思われる．各専門医が力を合わせることにより，更なる治療成績の向上が期待される．

2 ▌胃癌における化学療法

切除不能・再発胃癌に対する全身化学療法は，ベスト・サポーティブ・ケアと比較して，延命効果が得られることは明らかであるが[9]，これまでにおこなわれた抗癌剤治療同士の比較試験において（表 2），再現性をもって延命効果を示した治療法はなく，標準的な化学療法は確立されていない．1990 年後半以降，トポイソメラーゼ I 阻害薬（イリノテカン），経口フッ化ピリミジン系抗癌剤（テガフール・ギメラシル・オテラシルカリウム〔S-

表 3. 切除不能・再発胃癌に対する新薬の第II相試験

薬剤	著者	症例数	奏効率(%)	生存期間(月)
イリノテカン	Futatsuki	60	23	—
S-1	Sakata, Koizumi	101	45	8
カペシタビン	Hong	44	34	—
UFT/LV	Kim	37	27	7
パクリタキセル	Yamada	28	21	11
ドセタキセル	Bang	40	18	12
イリノテカン+CDDP	Boku	29	57	11
イリノテカン+MMC	Yamao	16	63	—
S-1+CDDP	Koizumi	25	76	12
S-1+イリノテカン	Motohiro	24	50	—
S-1+パクリタキセル	Narahara	12	50	—
カペシタビン+CDDP	Kim	42	55	10
カペシタビン+ドセタキセル	Kim	47	40	12
ドセタキセル+CDDP	Mitachi	28	25	10
カペシタビン+エピルビシン+CDDP	Cho	32	59	10
UFT/LV+エピルビシン+CDDP	Jeen	47	58	15
ドセタキセル+カペシタビン+CDDP	Kang	40	68	17
ドセタキセル+UFT/LV+CDDP	Oh	53	53	12
パクリタキセル+5-FU+CDDP	Kim	41	55	6

1]，カペシタビン)，タキサン系抗癌剤(パクリタキセル，ドセタキセル)，新規白金製剤(オキサリプラチン) が胃癌に対しても導入されてきた．これらの新薬を用いた併用療法も検討されたことにより，第I，または第II相試験レベルではあるが，1990年代前半まで30%前後であった奏効率，7～8ヵ月前後であった生存期間の中央値が，それぞれ50%以上，1年近くを示すものが珍しくない(表3)．

しかし，標準的治療が確立されていないために，現場での医療は混沌としているといわざるを得ない．現在，上記の新規薬剤は評価されるべく，第III相試験がわが国を含めた世界中で進行中である．比較のポイントは，タキサン系抗癌剤の上乗せ効果，イリノテカンの位置づけ，5-FU点滴静注に対する経口フッ化ピリミジン系抗癌剤の比較，CDDPに対するオキサリプラチンの比較である．

欧米での比較試験をみると，タキサン系抗癌剤の上乗せ効果については，5-FU+CDDP療法(CF療法) にドセタキセルを追加した3剤 (DCF療法) の比較の結果が報告され，CF療法よりもDCFが有意な生存期間の延長 (ハザード比1.293, p=0.0201) を示したが[10]，生存期間の中央値は8.6ヵ月に対して9.2ヵ月とわずかな差しかなく，毒性が強いこともあって，DCF療法が標準的治療として認識されるには至っていない．CF療法と大腸癌に対する標準的治療の一つであるFOLFIRI療法〔5-FU+ロイコボリン (LV)+イリノテカン〕の比較では，生存期間に差はなかったもののFOLFIRI療法のほうが毒性が低かったと報告されている[11]．経口フッ化ピリミジン系抗癌剤の評価としては，アジアを中心にCF療法とカペシタビン+CDDPの非劣性試験が展開されており，また，米国を中心としてCF療法とS-1+CDDP併用療法の比較試験も進行中である．オキサリプラチンの評価としては，ヨーロッパを中心