Abstract. The epidermal growth factor receptor (EGFR) and a related family member, HER-2, are often overexpressed simultaneously in patients with a variety of malignant tumors, and the combination may cooperatively promote cancer cell growth and survival. Heterodimerization of EGFR and HER-2 has been known to create intense proliferative signals. Lapatinib (GW572016) is a small molecule that is administered orally and functions as a reversible inhibitor of both EGFR and HER-2 tyrosine kinases. In the present study, we evaluated the antitumor effect of lapatinib on head and neck squamous cell carcinoma (HNSCC) cell lines in vitro and in vivo. In vivo we examined the antitumor effects of combined treatment with lapatinib and either cisplatin or paclitaxel. In vitro lapatinib displayed antiproliferative effects on HNSCC cells. The IC50 of lapatinib ranged between 13.6 and 60.2 μM after 24-h exposure to lapatinib. A correlation was not observed between results of in vitro proliferation assays for lapatinib and the expression of EGFR or HER-2. In vivo lapatinib displayed antitumor activity, and induced apoptosis in nude mice bearing an established xenograft of YCU-H891 cells. Lapatinib did not significantly inhibit angiogenesis. Combination treatment of lapatinib with cisplatin or paclitaxel enhanced antitumor activity mainly by inducing apoptosis. Inhibition of angiogenesis was observed only for combination treatment of lapatinib with paclitaxel (compared to vehicle control). These results suggest that: i) lapatinib has antitumor effects in vitro and in vivo; ii) lapatinib may be more effective in combination with cisplatin or paclitaxel; and iii) lapatinib might provide useful clinical benefits to HNSCC patients.

Introduction

Epidermal growth factor receptor (EGFR) and HER-2 belong to the HER family of receptor tyrosine kinases, which include EGFR (ErbB1 or HER-1), HER-2 (ErbB2), HER-3 (ErbB3), and HER-4 (ErbB4). Overexpression of EGFR has been observed frequently in head and neck squamous cell carcinomas (HNSCC) and EGFR overexpression is thought to be correlated with carcinogenesis, metastasis, clinical stage and poor prognosis (1-4). Overexpression of HER-2 has been noted in HNSCC (5-7), and HER-2 heterodimerization with EGFR may mediate disease progression (6,7). Elevated expression of EGFR and/or HER-2 has been associated with tumor cell resistance to chemotherapy and radiotherapy (7-9). Therefore, EGFR and HER-2 are attractive targets for anticancer treatments.

Lapatinib is a dual inhibitor that targets the tyrosine kinase domains of both EGFR and HER-2 by interfering with adenosine triphosphate binding, thus blocking autophosphorylation and resultant downstream signaling activities, including cellular proliferation and survival (10). Lapatinib is approved by FDA for the treatment of metastatic breast cancer in combination with capecitabine. In head and neck cancer, a phase II study of patients with recurrent or metastatic EGFR and/or HER-2 expressing salivary grand carcinoma (11) and a phase I study of lapatinib in combination with chemo-radiation in patients with locally advanced HNSCC (12) were reported. Targeting both EGFR and HER-2 in cancer treatment is reported to be efficacious for breast cancer, non-small cell lung cancer (NSCLC), prostate cancer, and vulvar squamous cell carcinoma (13-16). Based on the above findings, we reported the antitumor effects of gefitinib combined with trastuzumab on HNSCC cells in vitro (17). Cell growth was significantly inhibited by the combination of gefitinib and trastuzumab in some HNSCC cell lines. In the present study, we examined antitumor effects of lapatinib, an inhibitor of tyrosine kinases of both EGFR and HER-2, on HNSCC cell lines in vitro and in vivo.
lines in vitro and in vivo. We also examined antitumor effects of the combined treatment of lapatinib with cisplatin or paclitaxel in a xenograft model of HNSCC cells in vivo.

Materials and methods

Drugs. Lapatinib (GW572016) was provided by GlaxoSmithKline Pharmaceuticals (Research Triangle Park, NC). For the antiproliferative assay, stock solution (10 mM) was made in 100% dimethyl sulfoxide (DMSO) and diluted with culture media. Cisplatin (Briplatin®) and paclitaxel (Taxol®) were purchased from Bristol-Myers Squib (Park Avenue, NY). Cisplatin and paclitaxel were diluted with saline just before use for in vivo studies.

Cell lines and culture conditions. Sixteen HNSCC cell lines were examined in this study. The origins of these cell lines were the oral floor (YCU-OR891), hypopharynx (YCU-H891), mesopharynx (YCU-M862, KCC-M871, and YCU-M911), larynx (KCC-L871, YCU-L891), tongue (KCC-T871, KCC-T873, YCU-T891, and YCU-T892), and maxillary sinus (KCC-MS871, YCU-MS861) and metastatic tumors from different tongue carcinomas (KCC-TCM901, KCC-TCM902, and KCC-TCM903). These cell lines were established in the Department of Otolaryngology, Yokohama City University School of Medicine and Research Institute, Kanagawa Cancer Center. All cell lines were maintained in Dulbecco’s modification of Eagle’s medium (DMEM) (Wako Chemical, Osaka, Japan) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY). These cells were incubated at 37˚C in a moist atmosphere containing 5% CO₂.

In vitro proliferation assays. Cell proliferation assay was performed to assess the effect of treatment on the growth of 16 human HNSCC cell lines. These cells were plated in 96-well flat plates (Sumilon; Sumitomo Bakekite Co., Ltd., Tokyo, Japan) at a concentration of 5x10³ cells/well. Plates were incubated for 24 h prior to drug treatment. After 24-h exposure to lapatinib (5 wells of the 96-well plate per experimental condition), the cell proliferation was assayed by incubating with Tetra Color One (Seikagaku Co., Ltd., Tokyo, Japan). Relative growth inhibition was calculated by dividing the number of recovered drug-treated cells by the number of vehicle-treated control cells.

Evaluation of apoptosis. Terminal deoxynucleotidyl transferase-mediated cUDP nick end-labeling (TUNEL) was done for the evaluation of apoptosis. This was evaluated using an Apoptosis In Situ Detection Kit (Wako Chemical). The apoptotic index was calculated as the percentage of positive cell nuclei stained with peroxidase in the fields at x400 magnification relative to the total number of cells from a minimum of five microscopic fields from each section.

Evaluation of vessel density. In order to estimate the vessel density, the vessels in the tumor were stained with an anti-CD31 antibody (1:200; Dako, Denmark). The tumors were excised and blocked with 2% goat serum and 1% bovine serum albumin in PBS and stained with the antibody. Slides were developed using 3, 3’-diaminobenzidine substrate biotinylated peroxidase reagent (Vector Laboratories, Inc., Burlingame, CA). Vessel density was determined by counting the stained vessels in the fields at x10 magnification. Five fields per histological section were included in the analysis.

Statistical analysis. For statistical analyses of in vivo antitumor activity, apoptotic index, and vessel density, we used Student’s paired t-test.

Results

Antiproliferative effects of lapatinib on 16 HNSCC cells. We determined the antiproliferative effects of lapatinib at different concentrations (0-100 μM) using HNSCC cell lines. As shown in Table I, the IC₅₀ for lapatinib ranged between 13.6 and 60.2 μM.

In vivo studies of the antitumor effects of lapatinib, alone and in combination with cisplatin or paclitaxel, against HNSCC xenografts. We investigated in vivo antitumor activity of lapatinib alone and in combination with cisplatin or paclitaxel in nude mice bearing YCU-H891 squamous cell carcinoma xenografts. Among 16 HNSCC cell lines, YCU-H891 was somewhat more sensitive to lapatinib. As shown in Fig. 1, lapatinib treatment of nude mice bearing established HNSCC tumor xenografts led to dose-dependent inhibition of tumor growth. Significant inhibition of tumor growth was observed in the 200 mg/kg treatment group (P<0.05). But in this group, apparent weight loss of treated mice was found compared with the vehicle control group (data not shown).
We also evaluated the combined effects of lapatinib with cisplatin or paclitaxel (Fig. 2). The combination treatment of lapatinib with cisplatin or paclitaxel reduced the tumor volume significantly compared to either agent alone at the end of the treatment period (P<0.05).

**Effect of treatment on apoptosis in the xenograft model.** Because promotion of apoptosis is a commonly seen anti-tumor mechanism, we did histopathological analysis of the xenograft samples for apoptosis (Fig. 3). Treatment of lapatinib alone led to a dose-dependent increase in the number of apoptotic (TUNEL-positive) cells. The apoptotic index was significantly higher in tumors treated with 100 or 200 mg/kg lapatinib compared to the control (vehicle) mice (P<0.01) (Fig. 4).

The apoptotic index was also significantly higher in tumors from mice treated with lapatinib combined with cisplatin or paclitaxel (compared to either agent alone; P<0.01) (Fig. 4).

**Effect of treatment on microvascular content in the xenograft model.** To determine drug effects on tumor neovascularization, we examined the vessel density of xenograft tumors (Fig. 3). Lapatinib alone inhibited angiogenesis (a lower number of microvessels positive for CD31 staining) compared to the control (vehicle) group. Statistical significance was not observed for the difference between the lapatinib-treated groups and the control group (Fig. 5). The combination treatment of lapatinib with paclitaxel led to a significantly lower number of microvessels positive for CD31 staining (Fig. 5) (P<0.01); it was not significantly better compared to paclitaxel alone. Combination treatment of lapatinib with cisplatin was not significantly better than the control group.

**Discussion**

It is evident that the great majority of HNSCCs express EGFR, and overexpression of EGFR correlates with reduced
survival, increased risk of recurrence and distant metastasis, and resistance to radiotherapy (9,18-21). On the other hand, overexpression of HER-2 is also associated with poor prognosis in cancers of the breast, ovary, bladder and colon (22-24). HER-2 seems to function primarily as a heterodimerization partner for other HER family members (25,26). EGFR heterodimers produce an intense and sustained proliferative signal that is greater than that for EGFR homodimers (27,28). Therapies that target EGFR and HER-2 were reported to be efficacious against breast cancer, non-small cell lung cancer prostate cancer and vulvar squamous cell carcinoma (13-16). We also reported that a therapy that targets HER, gefitinib combined with trastuzumab, has antitumor effect against HNSCC in vitro (17). Accordingly, we examined the antitumor activity of lapatinib, a dual inhibitor of the tyrosine kinase domains of the EGFR and HER-2. We studied HNSCC cells in vitro and in vivo.

In our previous study (17), we studied 16 HNSCC cell lines and were able to calculate an IC_{50} for gefitinib against proliferation of 11 of the lines after 24 h of exposure to the drug. In 5 cell lines an IC_{50} could not be calculated (data not shown). In the present study, we calculated an IC_{50} for lapatinib for all cell lines after 24 h of drug exposure. The IC_{50} for lapatinib ranged between 13.6 and 60.2 μM (Table I). These results suggest that HNSCC cells are more sensitive to lapatinib than gefitinib, and that lapatinib is an effective agent for suppressing growth of HNSCC cells in vitro. A correlation was not observed between: i) our in vitro proliferation assays results for lapatinib; and ii) expression of EGFR or HER-2 in the same cell lines that was reported in our study (17) (data not shown).

About sensitivity of EGFR tyrosine kinase inhibitors such as gefitinib, the effect of an activating mutation and an inactivating mutation of the intracellular domain of EGFR have been reported (29-33). Acquired resistance was associated with the emergence of a second mutation in EGFR (a methionine for threonine substitution at amino acid T790M) (31,34,35). Harboring HER-2 mutations and insertion of HER2 mutations correlated with increased resistance to EGFR tyrosine kinase inhibitors (36-39).

Next we investigated the antitumor effects of lapatinib alone or in combination therapy with two chemotherapeutic agents, paclitaxel or cisplatin, in an in vivo study. Lapatinib treatment led to dose-dependent inhibition of tumor growth.
that activation of the EGFR promotes processes responsible for tumor growth and progression, including proliferation and maturation, angiogenesis, invasion, metastasis, and inhibition of apoptosis (43).

HER-2 seems to function as a heterodimerization partner for other HER family members (25,26). It was reported that EGFR heterodimers produce greater proliferative signal than EGFR homodimers (27,28). Therefore, lapatinib may promote endothelial cell apoptosis via inhibition of the EGFR pathway or heterodimerization of EGFR and HER2. The number of apoptotic events after combination treatment of lapatinib with cisplatin or paclitaxel was higher compared to each single agent administration. Each combination increased the number of apoptotic events. Therefore, both cisplatin and paclitaxel are appropriate partners for lapatinib in combined anticancer therapy.

To evaluate vessel density in vivo, immunohistochemical staining with anti-CD31 antibody was used. The treatment of lapatinib alone decreased the number of CD31-positive endothelial cells, but statistical significance was not observed. The combined treatment of lapatinib with paclitaxel showed significant antiangiogenic activity compared to the control (vehicle) group (P<0.01). But compared to paclitaxel alone, paclitaxel has antiproliferative effects and that in vivo lapatinib is an active antitumor agent against HNSCC due to its ability to induce tumor cell apoptosis. However, it did not observe an antiangiogenic effect. Lapatinib in combination with cisplatin or paclitaxel causes greater inhibition than either agent alone against HNSCC xenografts in a mouse model; the
mechanism appears to be enhancement of tumor cell apoptosis.

Based on results of the combination treatment of lapatinib with cisplatin or paclitaxel, the present study provides an experimental basis for the clinical application for HNSCC patients.

References


