Antitumor effects of lapatinib (GW572016), a dual inhibitor of EGFR and HER-2, in combination with cisplatin or paclitaxel on head and neck squamous cell carcinoma

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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 administrated 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 IC_{50} 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 antiangiogenesis 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.

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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 chemoradiation 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 targeted both EGFR and HER-2 in the treatment of HNSCC. 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*. 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 GlaxoSmith-Kline 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% CO_2.

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 $5x10^3$ 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.

In vivo antitumor activity studies. Female BALB/c nu/nu nude mice, 6-week old, were obtained from Oriental Yeast (Tokyo, Japan). The mice were maintained in a laminar flow room with a constant temperature and humidity. The animals were maintained and experiments were conducted at the Yokohama City University School of Medicine, Laboratory Animal Facility. The experiments were conducted according to the guidelines for animal experiments set by the Animal Experiment Committee of Yokohama City University School of Medicine. Suspensions of YCU-H891 cells (100 μl) (final concentration, $1x10^7$ cells/100 μl) were injected s.c. into the right flank of the mice on day 1. Tumor-bearing mice were randomized (n=6) when the mean tumor volume was 50-100 mm^3. Each group was closely matched before treatment, which began one week after cell transplantation. Lapatinib was dissolved in 0.5% hydroxypropyl-methylcellulose and 0.1% Tween-80 vehicle and was given once daily by oral gavage. The mice were treated with lapatinib (50, 100, 200 mg/kg) from day 8 to day 28. Cisplatin was administered i.p. (2.5 mg/kg/day on day 8). Paclitaxel was administered i.p. (7.5 mg/kg/day 2 times on day 8 and day 15). The control group of mice received hydroxypropylmethylcellulose/Tween-80 vehicle treatment at the same schedule as lapatinib. Treatment group number was 8. Tumor diameters in the control and treated groups were measured weekly with a Vernier caliper. Tumor volume (V) was determined by the equation: $V=ab^2/2$ (a, length; b, width).

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 tissues 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 x100 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 IC_{50} 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).

Table I. The antiproliferative activity of lapatinib.

HNSCC cell lines	Primary site	IC_{50} (μM) Lapatinib
YCC-OR891	Oral floor	13.6
KCC-T871	Tongue	22.8
KCC-T873	Tongue	33.8
KCC-TCM901	Lung[a]	18.7
KCC-TCM902	Lung[a]	33.7
KCC-TCM903	Lung[a]	21.3
YCU-T891	Tongue	22.4
YCU-T892	Tongue	30.5
YCU-MS861	Maxillary sinus	39.0
KCC-MS871	Maxillary sinus	17.8
YCU-M862	Mesopharynx	35.2
KCC-M871	Mesopharynx	54.2
YCU-M911	Mesopharynx	18.8
YCU-H891	Hypopharynx	18.2
KCC-L871	Larynx	60.2
YCU-L891	Larynx	25.1

[a]Metastatic tumors from different tongue carcinomas. In the proliferation assay described in Materials and methods, tumor cells were exposed to lapatinib for 24 h. Data are expressed as IC_{50}, the drug concentration (in μM) that causes 50% inhibition of cell proliferation.

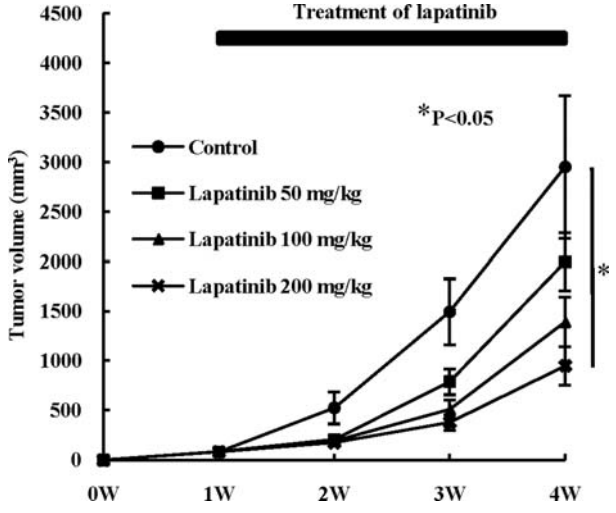

Figure 1. Lapatinib exerts a dose-dependent inhibition on the growth of YCU-H891 tumor xenografts. Data presented are mean values (n=6 per group); error bars indicate SE. *P<0.05 versus control. A significant difference was analyzed by Student's paired t-test.

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

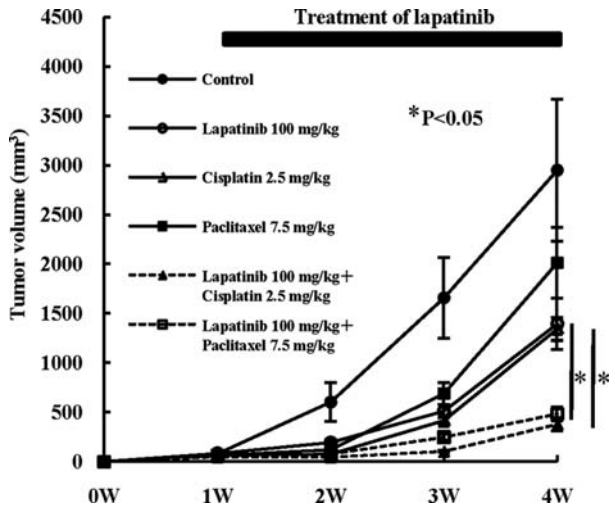

Figure 2. Inhibitory effects of the combination treatment of lapatinib with cisplatin or paclitaxel on the growth of YCU-H891 tumor xenografts. Data presented are mean values (n=6 per group); error bars indicate SE. *P<0.05 versus lapatinib alone or cisplatin alone. A significant difference was analyzed by Student's paired t-test.

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

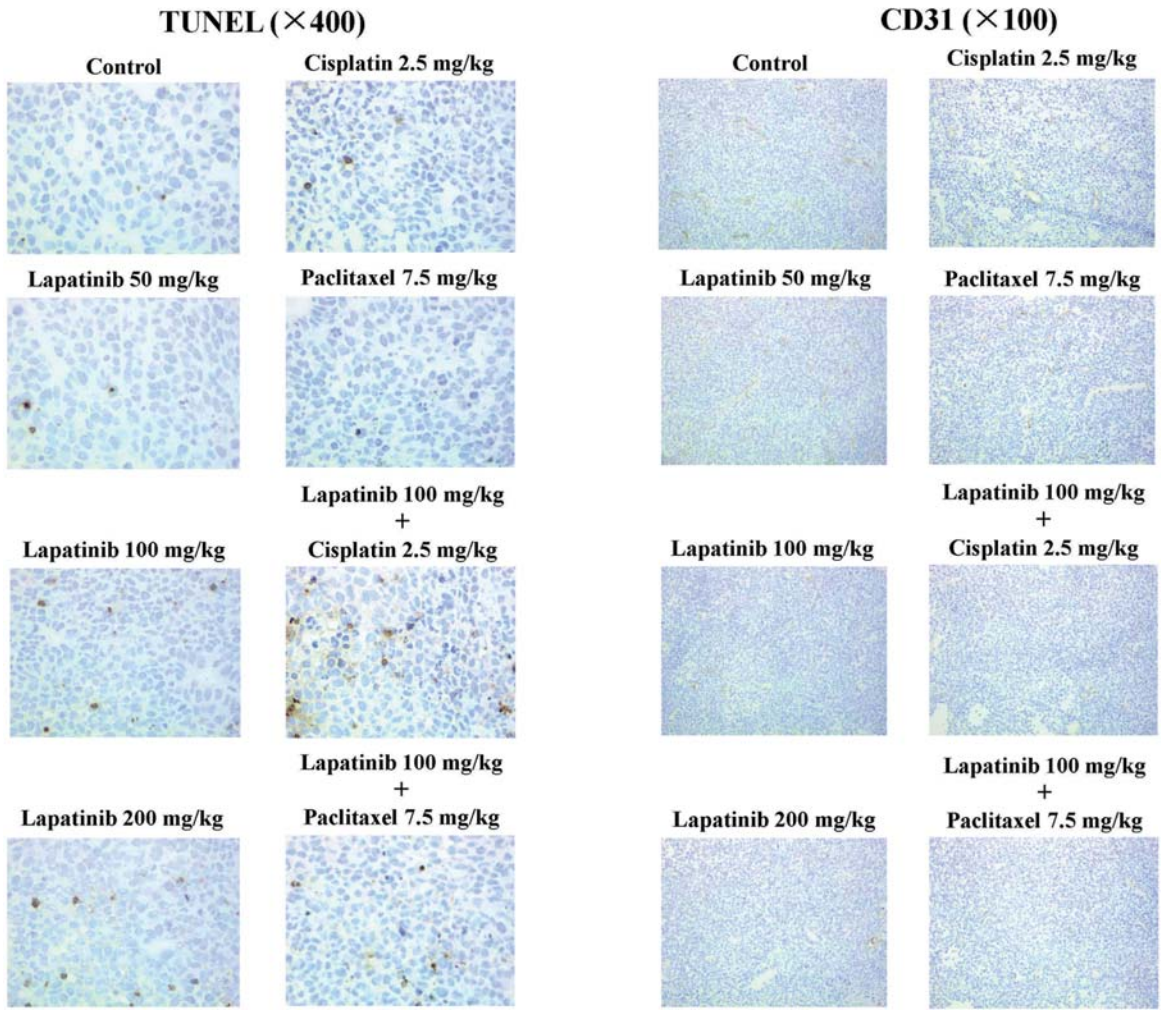

Figure 3. Apoptotic events and vessel density in YCU-H891 tumor xenografts. Terminal deoxynucleotidyl transferase-mediated cUDP nick end-labeling (TUNEL) staining was utilized to evaluate apoptosis (x400). The vessels of tumors were stained with anti-CD31 antibody to evaluate of antiangiogenesis by each treatment (x100).

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

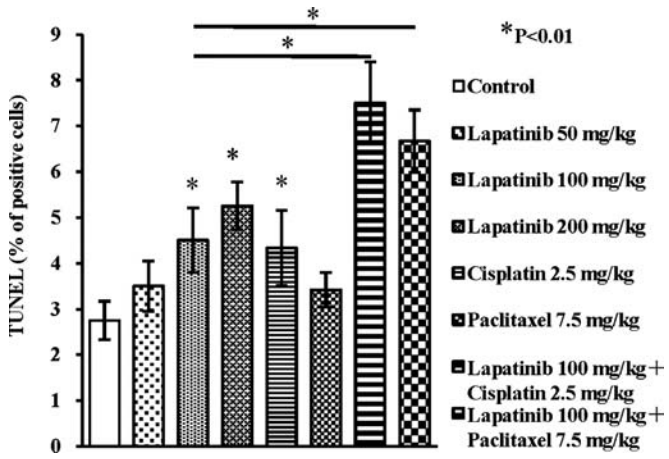

Figure 4. Effects of the treatment with lapatinib alone and combination treatment of lapatinib with cisplatin or paclitaxel on apoptotic index (TUNEL). A statistical significance was observed in the group of lapatinib 100 mg/kg, lapatinib 200 mg/kg and cisplatin 2.5 mg/kg compared to the control (P<0.01). A statistical significance was not observed in the group of the combination treatment of lapatinib with cisplatin or the combination treatment of lapatinib with paclitaxel compared to the lapatinib alone group. Data represent mean values (± SD). A significant difference was analyzed by Student's paired t-test.

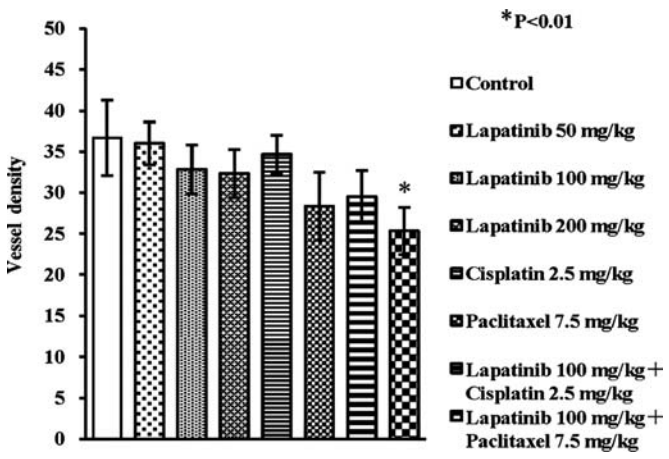

Figure 5. Effects of lapatinib alone and combination treatment of lapatinib with cisplatin or paclitaxel on vessel density (CD31 staining). The treatment of lapatinib alone showed no statistically significat difference at each dose. A significant difference was observed only in the group of the combination treatment of lapatinib with paclitaxel compared to the control (P<0.01). Data represent mean values (± SD). A significant difference was analyzed by Student's paired t-test.

(Fig. 1). Significant inhibition of tumor growth was observed in the 200 mg/kg treatment group (P<0.05), but apparent weight loss of treated mice was observed at this dose. The antitumor effects of lapatinib alone in a breast cancer cell xenograft model *in vivo* have been reported (40). In that study, 75 mg/kg of lapatinib daily for 21 days led to significant antitumor activity compared to vehicle control. Regarding colon cancer, Zhou *et al* reported that lapatinib (200 mg/kg daily) had significant antitumor activity compared to vehicle control. There was <10% weight loss compared to the vehicle control (41). These reports indicated that lapatinib at a dose of 200 mg/kg daily had a significant antitumor effect, but this dose seems to have severe drug toxicity.

Next we investigated the antitumor effect of lapatinib combined with paclitaxel or cisplatin. Each combination decreased tumor growth significantly compared to single agent administration. These chemotherapeutic agents which are available for HNSCC, are good candidates for the combination treatment with lapatinib. A phase I study of lapatinib in combination with chemoradiation in patients with locally advanced HNSCC was reported (12). In that report, cisplatin was used as a chemotherapeutic agent and the overall response rate was 81%. Regarding metastatic breast cancer, a phase II study of lapatinib plus paclitaxel was reported and clinical outcomes improved significantly in HER-2 positive patients (42).

To evaluate apoptosis-mediated antitumor effects of lapatinib *in vivo*, we used the TUNEL assay. After lapatinib alone, the number of TUNEL-positive tumor cells dose-dependently increased and statistical significance was observed compared to the control (vehicle) group. These results indicate that lapatinib induces apoptotic events and has antiproliferative effects on HNSCC cells. It was reported 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, statistical significance was not observed. It has been reported that paclitaxel shows antiangiogenic activity (44-46), but a synergistic effect with lapatinib was not observed. Cisplatin did not show antiangiogenic activity and the combination treatment of lapatinib with cisplatin had no antiangiogenic activity. Synergistic effects against angiogenesis were not expected in combination treatment with cisplatin.

In conclusion, we show that *in vitro* lapatinib 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 anti-angiogenic 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.

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