Lapatinib, a dual inhibitor of EGFR and HER2, has synergistic effects with 5-fluorouracil on esophageal carcinoma

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Abstract. Epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2) amplification occurs in over 30% of esophageal carcinomas. Combination therapies with EGFR and HER2-targeting agents and cytotoxic agents are considered a potential therapeutic option for esophageal cancer. We evaluated the antitumor effects of lapatinib, a dual tyrosine kinase inhibitor which simultaneously inhibits EGFR and HER2, 5-fluorouracil (5-Fu) alone and in combination on esophageal cancer cells. The antiproliferative activity of lapatinib, 5-Fu and lapatinib plus 5-Fu was measured by MTT assay and the combination index (CI) values were calculated. Additionally, cell cycle distribution of lapatinib alone and the combination with 5-Fu were detected by flow cytometry analysis. Annexin V-FITC and propidium iodide stain were used for analyzing the apoptotic cells after cells were treated with either agent alone or in combination. The EGFR and HER2 activated signaling pathways were monitored by western blotting. The combination of lapatinib and 5-Fu synergistically inhibited cell proliferation and exhibited an enhanced proapoptotic effect on esophageal cancer cells. The potentiation effect of combined treatment was associated with downregulation of EGFR and HER2 signaling pathways because data from western blot analysis showed that lapatinib in combination with 5-Fu markedly reduced the phosphorylation of EGFR and HER2, and inhibited the activation of downstream signaling molecules, such as AKT and ERK. A significant G1 arrest was also observed in cell cycle analysis after exposing cells to lapatinib, however, combination with 5-Fu did not enhance G1 arrest. These results indicate that the combination of the lapatinib and 5-Fu is a promising treatment option for esophageal carcinoma with HER2 amplification.

Introduction

Esophageal cancer is the eighth most common cancer and the sixth most common cause of cancer deaths in the world. In China, about 250,000 esophageal cancer cases are diagnosed each year, accounting for half of the world’s total (1-4). The mortality rate associated with esophageal cancer is similar to its incidence rate because of its generally advanced stage at the time of diagnosis, its aggressive characteristics and the deficiency of effective treatment strategies (5). Despite many treatment options for localized esophageal cancer including surgery alone, combined modality strategies such as pre- or post-operative chemotherapy, and definitive chemoradiation developed, the prognosis of long-term survival remains poor. For instance, surgery alone results in a 5-year survival of only 20-25% (6,7). Combined modality therapy increases the 5-year survival to approximately 30-35% (8-10). For metastatic cases, chemotherapy is the mainstay of palliative therapy and results in objective response rates of only 20-40% and median overall survival of 8-10 months (1). Given the poor overall survival in the treatment of esophageal carcinoma with conventional strategies, development of new therapies that would provide more effective responses for a larger number of patients is clearly need. A major challenge in developing effective cancer therapies involves implementation of more effective treatment regimes which overcome the high toxicity of many conventional chemotherapeutic approaches. Recently, new strategies, based on chemosensitisation of tumor cells with relatively low toxicity tyrosine kinase inhibitors (TKIs) have gained much attention (11). Promising targets for such combinatorial treatment of esophageal cancer patients are tyrosine kinase receptors of human epidermal growth factor receptor (HER) family.

The HER family of tyrosine kinase receptors includes HER1 (epidermal growth factor receptor, EGFR), HER2, HER3 and HER4. They were observed overexpressed in a significant proportion of human cancers such as breast, lung, head and neck, colon and prostate, and have been shown to play a crucial role in cell proliferation, survival, migration and differentiation (12-15). In esophageal carcinoma, EGFR overexpression by immunohistochemistry or gene amplification by fluorescent in situ hybridization analysis occurs in 30-90% of tumors and correlated with increased invasion, a
more poorly differentiated histology and a worse prognosis (16-18). Human epidermal growth factor receptor 2 (HER2), is a significant factor in the pathogenesis of aggressive breast cancers. However, it has also been found to be overexpressed in some esophageal cancer cell lines and is associated with several aggressive characteristics, including tumor invasiveness, lymph node metastasis and chemoresistance (19,20). In addition, patients with esophageal cancer may also have a double overexpression of EGFR and HER2, indicating that dual inhibition of the two receptors could be an effective strategy for treatment of esophageal carcinoma (21).

Lapatinib (Tykerb, GlaxoSmithKline) is a dual tyrosine kinase inhibitor with activity against EGFR and HER2 tyrosine kinase domain. In cell-free biochemical kinase assays, lapatinib inhibits the recombinant EGFR and HER2 tyrosine kinases by 50% (IC50) at concentrations of 10.8 and 9.3 nM (22). Treatment with lapatinib has been shown in vitro and in vivo significant inhibition of the downstream effector pathways and the proliferation of EGFR and/or HER2 overexpressing cancer cells (23). In several clinical trials, lapatinib demonstrated activity in HER2 overexpressing breast cancer patients and prolonged progression-free survival in patients with advanced, Herceptin-refractory disease (24). It also was shown that lapatinib in combination with chemotherapy agents, such as cisplatin or paclitaxel, have a synergistic antitumor effect in cancers with EGFR and HER2 amplification (25). Recently, it was reported that lapatinib can inhibit cell growth in EGFR and HER2 overexpressing esophageal cancer cell lines and the combination therapy of lapatinib with cetuximab or trastuzumab is a promising strategy in the treatment of esophageal cancer (26). However, there has still been limited examination of EGFR and HER2-targeting agents in esophageal cancer models, and EGFR and HER2-targeting agents in combination with cytotoxic agents such as 5-fluorouracil have remained unclear. Moreover, the approaches involve application of decreased doses of toxic agents in combination with non-toxic tyrosine kinase inhibitors complementing the efficacy of the treatment have gained much attention as a new strategy.

In the current study, we evaluated the effects of antiproliferative, cell cycle-arrest, proapoptosis of lapatinib either as a single agent or in combination with chemotherapy agent 5-fluorouracil (5-Fu) on esophageal carcinoma. Furthermore, we explored the underlying mechanisms of their synergistic effect on cell growth and apoptosis by focusing on the EGFR and HER2 signaling transduction pathways. These findings will provide useful data for ongoing clinical investigation of lapatinib combined with cytotoxic agents for treatment of esophageal carcinoma.

Materials and methods

Cell culture and reagents. Human esophageal carcinoma cell lines KYSE150 and EC9706 were obtained from cell center of Peking Union Medical College, China and cultured under a humidified atmosphere of 5% CO2 at 37°C in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Lapatinib (GW572016) was obtained from Santa Cruz Biotechnology and 10 mM stock solution was prepared in dimethyl sulfoxide (DMSO). 5-Fluorouracil (5-Fu) (25 mg/ml) was provided by Shanghai Xudong Haipu Pharmaceutical Co., Ltd. 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Aldrich Chemical, Inc.

Cell viability assay. Cells were maintained in medium with 5% FBS and plated in 96-well flat-bottomed plates (2500 cells per well), cultured for 24 h before exposure to various concentrations of 5-Fu or lapatinib as a single agent or in combination for 48 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml, 20 µl) was added to each well and incubated for another 4 h at 37°C. The supernatant was removed and 150 µl DMSO was added to each well. The absorbance at 570 nm was measured using a Multiskan Spectrum instrument (Thermo Labsystems). Absorbance values were expressed as a percentage of that for untreated cells and the concentration of tested drugs resulting in 50% growth inhibition (IC50) was calculated. Data from 5-Fu and lapatinib combination treated cells were analyzed by using the isobologram combination index (CI) method as describe by Chou and Talalay (27). CI values of <1, 1 and >1 indicate synergistic, additive and antagonistic effects, respectively.

Western blot analysis. Cells were lysed for 30 min in radioimmunoprecipitation assay (RIPA) buffer supplement with 1 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 2 mM NaVO3 and 50 mM NaF. Extracts were centrifuged at 10000 g for 15 min at 4°C. Protein was quantitated using bicinchoninic acid kit (Pierce Biochemicals) and 30 µg of each total protein were applied on a 10% SDS-PAGE and then electroblotted onto polyvinylidene difluoride membranes (Millipore). The membranes were incubated with 1% BSA for 2 h at room temperature before incubation overnight at 4°C with primary antibodies including those phosphorylated HER2 (1:1000 dilution; Cell Signaling Technology), HER2 (1:1000 dilution; Neomarkers), phosphorylated EGFR (1:1000 dilution; Cell Signaling Technology), EGFR (1:1000 dilution; Santa Cruz Biotechnology), phosphorylated AKT (1:1000 dilution; Cell Signaling Technology), AKT (1:1000 dilution; Cell Signaling Technology), phosphorylated ERK (1:1000 dilution; Cell Signaling Technology), ERK (1:1000 dilution; Santa Cruz Biotechnology), beta-actin (1:1000 dilution; Santa Cruz Biotechnology), p-ERK (1:1000 dilution; Cell Signaling Technology) and actin (1:1000 dilution; Cell Signaling Technology). The membranes were then incubated with secondary HRP-conjugated antibodies for 1 h after washing with Tris-buffered saline with 0.05% Tween-20 (1:5000 dilution; Cell Signaling Technology). The specific bands were visualized with the Immobilon Western Chemiluminescent HRP Substrate kit (Millipore).

Cell cycle analysis. The effects of lapatinib on cell cycle were evaluated using propidium iodide (PI) staining. Cells were plated evenly in control and experimental wells and allowed to grow to log phase. Cells were then treated with varying doses of lapatinib, 5-Fu or in combination of two drugs for 48 h. At the time of each cell cycle analysis, supernatant of cultured cells was collected, and cells were washed with PBS, digested by trypsin-EDTA, which were then centrifuged at 1000 rpm for 5 min. Supernatant was discarded and cells were fixed in ice-cold 70% ethanol for 24 h. At the time of analysis, cells were washed twice with PBS and then resuspended in 500 µl PBS
with 50 µg/ml PI and 100 µg/ml RNase A. After incubated at 37˚C for 30 min, cells were analyzed for fluorescence with a flow cytometer.

Annexin V-FITC/PI staining assay. The apoptotic cells were measured by an Annexin V-FITC/PI staining kit (BioSea Technology). After treated with 5-Fu and lapatinib alone or combination for 48 h, cells were harvested by exposure to trypsin-EDTA, washed twice with PBS and centrifuged at 1000 rpm for 5 min. The cell pellets were resuspended in 500 µl binding buffer containing of 10 µl Annexin V-FITC and 5 µl PI, incubated at room temperature for 15 min and then analyzed for fluorescence with a flow cytometer.

Statistical analysis. Statistical analysis was carried out with SPSS version 11.5 (SPSS Inc., Chicago, IL, USA). An unpaired two-tailed t-test was used to determine the statistical difference for cell viability and apoptosis. All statistical tests were two-sided and P-values <0.05 were considered statistically significant.

Results

Lapatinib inhibits the growth of esophageal cancer cells with EGFR and HER2 amplification. In order to correlate drug treatment data with tumor EGFR and HER2 expression, we first evaluated the expression of EGFR and HER2 in esophageal cancer cell line KYSE150 and EC9706 by western blot analysis. As shown in Fig. 1, EGFR and HER2 were both overexpressed in KYSE150 cells, whereas EC9706 cells were low in expression of these two receptors. Therefore, the two esophageal cancer cell lines were used for antiproliferative experiments of lapatinib. A dose-dependent growth inhibition was observed following treatment of esophageal cancer cells with lapatinib and the IC_{50} values for KYSE150 and EC9706 cells were 3.84 and 6.44 µM, respectively (Fig. 2). KYSE150 cells were more sensitive to lapatinib possibly due to co-over-expression of EGFR and HER2.

Lapatinib enhanced the cytotoxicity of 5-Fu to esophageal cancer cells. In HER2 overexpressing breast cancer, the effect of chemotherapy can be maximized when combined with trastuzumab or lapatinib. Thus, we next analyzed the combination of lapatinib with chemotherapeutic agent 5-fluorouracil (5-Fu) used for the treatment of esophageal cancer. KYSE150 and EC9706 cells were exposed to either agent alone or in combination for 48 h. The concentrations of lapatinib used for experiments were 5, 10, 15 and 20 µM, and the concentrations of 5-Fu were 0.5, 1, 2 and 4 µg/ml. As shown in Fig. 3, lapatinib significantly enhanced the cytotoxicity of 5-Fu to the esophageal cancer cells. The inhibition rates of 5-Fu at 1 µg/ml and lapatinib at 10 µM on KYSE150 cells were 22.52 and 66.29%, respectively. However, the inhibition rate of 5-Fu (1 µg/ml) combined with lapatinib (10 µM) reached 79.06%. The CI values for 5-Fu and lapatinib were all <1, which also indicating combination of 5-Fu and lapatinib yielded strongly synergistic effects in both KYSE150 and EC9706 cells.

Lapatinib induces a G1 arrest in esophageal cancer cells. The effects of lapatinib on the cell cycle distribution were analyzed since lapatinib had significant growth inhibition on esophageal cancer cell lines. KYSE150 and EC9706 cells were incubated with 1, 5 and 10 µM lapatinib for 48 h and cells were detected by flow cytometry using propidium iodide staining. G1 arrest was seen in KYSE150 cells at all concentrations, but not in EC9706 cells (Fig. 4A and B). However, when cells were exposed to 5-Fu in combination with lapatinib, the cell cycle did not show significant difference compared to untreated controls (Fig. 4C). These results implied that the effect of cell cycle arrest of lapatinib may closely associate with the expression level of EGFR and HER2.

Lapatinib and 5-Fu show synergistic effects on inducing cell apoptosis. To investigate the reason of the synergistic growth inhibition induced by the combination of 5-Fu and lapatinib, we measured the apoptosis-induced effects of each agent alone

Figure 1. Expression of EGFR and HER2 on different esophageal carcinoma cell lines analyzed by western blot analysis.

Figure 2. Effect of lapatinib on cell viability in human esophageal cancer cells. KYSE150 and EC0706 cells were incubated with various concentrations of lapatinib for 48 h and the cell survival rates were determined by MTT assay. Cell viability was expressed as the percentage of cell survival compared with untreated controls. All data were from three independent experiments.
and in combination on esophageal cancer cells. KYSE150 cells were exposed to 5-Fu (4 µg/ml), lapatinib (10 µM) alone or in combination for 48 h. Apoptotic cells were stained by Annexin V-FITC and propidium iodide and then analyzed by flow cytometry. As shown in Fig. 5, 5-Fu and lapatinib induced 10.83 and 11.15% apoptotic cells, respectively, whereas the ratios of apoptotic cells treated with 5-Fu combined with lapatinib were significantly increased than those treated with either agent alone (27.42%, P<0.01). These data indicated that the combination of 5-Fu and lapatinib exhibits an enhanced proapoptotic effect in EGFR and HER2 amplification esophageal carcinoma cells.

**Effects of treatment with lapatinib and 5-Fu on the activation of HER receptors, AKT and ERK signaling.** The molecular mechanisms involved in the synergism of lapatinib and 5-Fu
in the esophageal cancer cell lines KYSE150 and EC9706 were explored. The signaling pathways induced by activated EGFR and HER2 include the MEK/ERK and the PI3K/AKT pathways, both playing an important role in the mitogenic and cell survival responses. KYSE150 and EC9706 cells were exposed to lapatinib (10 µM) or 5-Fu (4 µg/ml) or the combination of the two drugs for 48 h and then the phosphorylated-EGFR, HER2, AKT, and ERK were detected by western blot analysis. In EGFR and HER2 both amplified KYSE150 cells, the treatment of lapatinib markedly reduced the activation of EGFR, HER2, AKT, and ERK without affecting their expression level, which is in agreement with previous reports. Importantly, the combination of lapatinib with 5-Fu led to a more significant reduction of the activation of EGFR, HER2, AKT, and ERK signaling as compared with each single agent (Fig. 6). However, in EC9706 cells with HER2 amplification, lapatinib alone or combined with 5-Fu resulted in reduction in phosphorylation of HER2 while the phosphorylation of EGFR remained unchanged. Lapatinib treatment alone did not affect the activation of AKT, but the combination with 5-Fu decreased the phosphorylation of AKT. The level of phosphorylated ERK was also dramatically reduced after treated with lapatinib alone or combined with 5-Fu (Fig. 6). These results suggest that the inhibition of EGFR and HER2 signaling achieved by the combination of lapatinib and 5-Fu could be augmented beyond that achieved by either agent alone and this inhibition correlated well with the growth inhibitory effects observed in cell proliferation and apoptosis assays.

Discussion

EGFR and HER2 amplification is a frequent molecular abnormality in esophageal cancer as well as in various other cancers (28). Lapatinib is a dual reversible inhibitor of EGFR and HER2 tyrosine kinases and has been demonstrated to inhibit significantly the proliferation of cancer cells with EGFR and/or HER2 overexpression. It has been approved by FDA for the treatment of HER2 positive metastatic breast cancer in combination with capecitabine (29). However, many patients do not experience favorable responses to lapatinib (30). Clinical resistance to lapatinib highlights the need for improved therapeutic strategies. Recently, tyrosine kinase inhibitors are increasingly being used in combination with chemotherapy agents with the aim of improving the antitumor efficacy.

In this study, we performed an initial evaluation of the dual inhibitor of EGFR and HER2 receptors, lapatinib, as a sensitizer for conventional chemotherapy agent 5-Fu. Firstly, the antiproliferative effects of lapatinib alone or in combination with 5-Fu were analyzed on two esophageal cancer cell lines with different EGFR and HER2 expression level. Being consistent with other previous research, the results demonstrated that as a single agent, lapatinib can
effectively inhibit the growth of esophageal cancer cells and its efficacy may associate with the amount of EGFR and HER2 expression. The combination of lapatinib with 5-Fu at all concentrations used (lapatinib at 5, 10, 15 and 20 µM; 5-Fu at 0.5, 1, 2 and 4 µg/ml) exhibited a synergistic antitumor effect on esophageal carcinoma cells with the combination index <1. However, lapatinib at lower concentration (0.5 µM) combined with 5-Fu at higher concentration (50 µg/ml) did not exert synergistic effects with combination index >1 (data not shown). The results indicated that the concentrations of tyrosine kinase inhibitors have to be thoroughly optimized when combined with cytotoxic agents.

In a cell cycle assay, lapatinib induced significant cell cycle arrest for EGFR and HER2 amplification esophageal cancer cells (KYSE150 cells), but it did not affect the cell cycle distribution of esophageal cancer cells with low EGFR and HER2 expression level (EC9706 cells). Moreover, when cells were exposed to 5-Fu in combination with lapatinib, the cell cycle did not show significant difference compared to untreated controls. This result revealed that 5-Fu and lapatinib did not exhibit synergistic effect on cell cycle distribution and the enhanced cytotoxicity of combination of two drugs may not relate to improved cell cycle arrest-induction ability. For further research, the apoptosis caused by either agent alone or in combination were also examined by Annexin V-FITC/PI staining. The results showed that the frequency of apoptosis was markedly greater for cells treated with the combination of lapatinib and 5-Fu than for those treated with either agent alone.

The activation of EGFR and HER2 results in a cascade of downstream substrate activation, leading to a number of processes important to cancer development and progression, including cell proliferation, apoptosis, angiogenesis and metastasis (31,32). Signal transduction through the EGFR family has been shown to be mediated through two major pathways resulting in the activation of AKT and MAPK, which have both been associated with mitogenesis and cell survival (33). In this study, we found that lapatinib markedly reduced the phosphorylation of EGFR, HER2, AKT and ERK in KYSE150 cells amplified EGFR and HER2. The combination of lapatinib with 5-Fu potentiated the blockade of activation of EGFR and HER2 signaling which resulted in a greater degree of growth inhibition and apoptosis induction in KYSE150 cells. In other words, the cooperative effects are because of the enhanced decrease in the activation of EGFR/HER2 signal pathways with combined treatment in KYSE150 cells.

In conclusion, the present study investigated the synergistic efficacy of lapatinib and 5-Fu for treatment of esophageal carcinoma. Lapatinib combined with 5-Fu were more effective in growth inhibition and apoptosis induction of esophageal cancer cells. These findings suggested that such a combination regimen might represent a novel and promising strategy for the clinical therapy to EGFR and HER2 overexpressing esophageal cancer patients. However, additional studies on combination of tyrosine kinase inhibitors and cytotoxic drugs are required to gain further information for clinical application. For example, the in vivo tumor model analysis is necessary to confirm the effectiveness of this therapeutic strategy and the determination of genetic backgrounds of tumor cells responsive to lapatinib will be another focus of further studies. This will allow identification of patients who will benefit from therapies based on combined lapatinib and 5-Fu treatment.

References


