Lapatinib inhibits the growth of esophageal squamous cell carcinoma and synergistically interacts with 5-fluorouracil in patient-derived xenograft models

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Abstract. Lapatinib is a dual tyrosine kinase inhibitor of epidermal growth factor receptor (EGFR) and human EGFR-2 (HER2) tyrosine kinase domains. To explore the potential utility of lapatinib for the treatment of esophageal squamous cell carcinoma (ESCC), we examined the expression profiles of EGFR and HER2 in tumor tissues and in paired adjacent non-neoplastic tissues from patients with ESCC. We evaluated the antitumor effects of lapatinib alone or in combination with oxaliplatin or 5-fluorouracil (5-FU) on a panel of primary ESCC cells in vitro with various levels of EGFR and HER2 expression. The in vivo effect of lapatinib alone or in combination with oxaliplatin or 5-FU was evaluated using a primary ESCC xenograft model. EGFR was overexpressed in 80.9% (76/94) of the ESCC samples, while 24.5% (23/94) of the samples overexpressed HER2. EGFR and HER2 overexpression was detected in 22.3% of samples (21/94). In vitro, the primary ESCC cells were more sensitive to lapatinib combined with 5-FU or oxaliplatin than to lapatinib alone. Lapatinib in combination with 5-FU had more potent antitumor effects in the primary ESCC xenograft model, and markedly reduced the phosphorylation of EGFR and HER2, compared with lapatinib alone or in combination with oxaliplatin. These data indicate that lapatinib has activity in EGFR- and/or HER2-expressing ESCC primary cells, and that lapatinib in combination with 5-FU may be a promising treatment strategy for patients with ESCC.

Introduction

Although adenocarcinoma has replaced squamous cell carcinoma as the most common type of esophageal cancer in Western countries, >90-95% of esophageal cancer cases in Asian countries are esophageal squamous cell carcinomas (ESCCs) (1-3). The prognosis of advanced ESCC patients in China and other Asian countries remains quite poor, despite the use of therapies that combine surgical resection with chemotherapy and/or radiotherapy. The use of tyrosine kinase inhibitors (TKIs) combined with standard chemotherapeutics may provide a novel therapeutic approach for the majority of ESCC patients (4-6).

The human epidermal growth factor receptor (EGFR) family, also known as the HER family, includes four closely related receptors: HER1 (EGFR), HER2, HER3 and HER4. Downstream signaling of the HER family plays a crucial role in cell proliferation, apoptosis, angiogenesis and metastasis (7,8). EGFR overexpression has been identified in several types of human cancer, including gastric, colorectal, breast, lung, prostate and bladder cancer (9,10). The HER2 gene is amplified and overexpressed in ~30% of human breast and ovarian cancers, as well as other tumors, including colorectal and gastric cancer (11-13). Notably, it was previously reported
that EGFR is overexpressed in 33.3% of Japanese ESCC patients, and HER2 is overexpressed in 30.3% of Japanese ESCC patients (14,15). EGFR and HER2 are the main therapeutic targets of TKIs, and oxaliplatin and 5-FU are used as standard chemotherapies (16-18). Thus, anti-EGFR and/or anti-HER2 targeted therapy combined with standard chemotherapies is an attractive approach for the treatment of patients with advanced ESCC.

Lapatinib is a reversible dual TKI that targets the tyrosine kinases in both EGFR and HER2 tyrosine kinases, which in turn inhibits receptor phosphorylation and activation of the downstream signaling pathways, such as extracellular-related kinase (ERK)-1/2 and AKT in cell lines and xenografts (19-21). Lapatinib combined with fluoropyrimidine or trastuzumab exerted synergistic antitumor effects in vitro and in vivo (22), and had clinical activities in several solid tumors (6,23,24). However, no previous studies have described the effects of this TKI combined with standard chemotherapy using ESCC primary xenografts derived from Chinese patients. On the basis of these earlier observations, we explored the potential utility of lapatinib when administered alone or in combination with oxaliplatin and 5-FU for treating ESCC.

Materials and methods

Ethics statement. The present study was conducted according to the principles of the Declaration of Helsinki. The efficacy study was approved by the Ethics Committee of the Third Xiangya Hospital, Central South University, Hunan, China. The collection and use of the archived paraffin tissue blocks in the ESCC study were approved by the Ethics Committee of the Second Hospital, Jilin University, Jilin, China, with prior consent from the patients.

Immunohistochemical analysis. Esophageal cancer tissue microarrays (TMA; Shanghai Outdo, Shanghai, China) comprising 94 malignant esophageal human tumors in which 78 had matched adjacent normal tissues were used for immunohistochemistry (IHC) analysis of EGFR and HER2 protein expression. For antigen retrieval, the tissues were incubated in EDTA buffer with heating for 20 min, and then incubated in Background Sniper (Biocare Medical, Concord, CA, USA) to block any non-specific binding. Sections were then incubated with the anti-EGFR or anti-HER2 monoclonal antibody (Cell Signaling Technology, Beverly, MA, USA) diluted in Dako antibody diluent (DakoCytomation, Carpinteria, CA, USA) overnight at 4°C. The sections were then incubated using the rabbit on rodent polymer system (Biocare Medical) for 30 min at room temperature. Slides were subsequently treated with 3,3-diaminobenzidine chromogen (DakoCytomation) for 5 min to visualize antibody binding. Sections were then counterstained with hematoxylin, dehydrated and mounted. Total rabbit IgG1 substituted for the primary antibodies served as a negative control.

The assessment of EGFR or HER2 staining strength and positivity was carried out simultaneously by a pathologist and two other observers and a consensus was reached for each core. EGFR and HER2 staining was scored as follows: negative, no membranous staining in any of the tumor cells; 1+, membranous staining in <10% of the tumor cells with any intensity or in <30% of the tumor cells with weak intensity; 2+, staining in 10-30% of the tumor cells with moderate to strong intensity or staining in 30-50% of the tumor cells with weak to moderate intensity; and 3+, staining in >30% of the tumor cells with strong intensity or >50% of the tumor cells with any intensity. Tissues scored as 2+ or 3+ were defined as showing positive expression (25).

Animals. Athymic nude mice (Vr: NU-Foxn1nu) aged 5-6 weeks and weighing 18-21 g were purchased from Vital River Laboratories (Beijing, China). The health of all mice was monitored daily by gross observation and analysis of blood samples of sentinel animals. All mice were allowed to acclimatize and recover from any shipping-related stress for ≥72 h prior to experimental use. Autoclaved water and irradiated food were provided, and the mice were maintained on a 12 h light and dark cycle. Cages, bedding and water bottles were autoclaved before use and were changed weekly.

All animal experiments were conducted in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited animal facility after review by the Institutional Animal Care and Use Committees, and in accordance with the GSK policy on the Care, Welfare and Treatment of Laboratory Animals.

Tumors. Excess human esophageal tumor samples were obtained through an institutional review board-approved centralized banking infrastructure at the hospital. Written informed consent was obtained from all participants. None of the samples used in the present study were derived from minors. Biopsies were taken from the luminal surface of resected specimens by a pathologist or surgeon, ensuring that their potential for histopathologic diagnosis and staging was not compromised.

Solid tumor tissues were depleted of necrotic components, cut into 10-15 mg pieces and mixed. The mixed tumor pieces were implanted (single flank) into male Nu/Nu nude mice; 3-5 pieces were mixed with 15-30 µl Matrigel (BD Biosciences, Bedford, MA, USA) per mouse. For continued propagation in mice, the xenograft tumors were excised and processed into mixed tumor pieces. The mixed tumor pieces were re-implanted subcutaneously into new recipient nude mice. All of the primary human esophageal tumors used in this study had undergone 3-4 passages in vivo, and the histologic profiles of all tumors were maintained during serial transplantation.

In vitro ATP tumor chemosensitivity assay. Chemosensitivity was assessed in primary esophageal tumor tissue samples using the CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega, Madison, WI, USA). Briefly, surgical biopsies (1-2 cm³) were obtained during primary surgery. Tumor cells were isolated by mechanical and enzymatic dissociation. Approximately 2x10⁶ cells were then seeded into each well of a 96-well polypropylene microplate. Each concentration of the test drugs was applied in triplicate. The initial concentrations of lapatinib, oxaliplatin and 5-FU were 10, 10 and 100 µM, respectively. Two rows on each plate were reserved for blanks and controls. After preparing the diluted drugs, 135 µl of the cell suspension was added to each well. The plate was incu-
bated for 6 days at 37˚C under high humidity and 5% CO₂. The cells were observed microscopically every 24 h to check for overgrowth or infection. At the end of the incubation period, the cells were lysed by the addition of 75 µl of CellTiter-Glo reagent. Luminescence measurements were made using a FlexStation 3 (Molecular Devices, Sunnyvale, CA, USA).

Immunoblotting. Tumor tissue lysates were prepared by washing the cells with phosphate-buffered saline and subjecting them to lysis with radio-immunoprecipitation assay buffer supplemented with a protease inhibitor cocktail. The protein concentrations were quantified using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Equivalent amounts of proteins were loaded, separated on NuPAGE Novex 4-12% Bis-Tris Gels, and then transferred to polyvinyl difluoride membranes (Invitrogen, Carlsbad, CA, USA). The membranes were blocked for 1 h with 5% nonfat dried milk in Tris-buffer containing 0.1% Tween and were then probed with the diluted primary antibody overnight at 4˚C. The membranes were then washed three times and probed with horseradish peroxidase-linked goat anti-rabbit IgG, and the immunoreactive bands were visualized using an enhanced chemiluminescent detection system (GE Healthcare, Piscataway, NJ, USA). All antibodies were purchased from Cell Signaling Technology.

Test agents and efficacy of study design. Lapatinib (Tykerb®; GlaxoSmithKline, Research Triangle Park, NC, USA) was prepared in 0.5% hydroxypropyl methylcellulose (HPMC) and 0.1% Tween-80 (Sigma, St. Louis, MO, USA). Clinical-grade oxaliplatin (Eloxatin®; Sanofi-Aventis, Bridgewater, NJ, USA) was obtained as a stock solution of 5 mg/ml, diluted with sterile water. 5-FU (Sigma) was freshly dissolved in saline prior to use.

Administration of lapatinib and oxaliplatin in the primary esophageal tumor model. Forty-two mice were selected and randomized into six groups. The mice were treated with vehicle (0.5% HPMC in 0.1% Tween-80, orally, twice daily), lapatinib alone (105 mg/kg, orally, twice daily), oxaliplatin alone (6 mg/kg, intraperitoneal injection, once weekly), or lapatinib (105 mg/kg, orally, twice daily) in combination with oxaliplatin (6 mg/kg, intraperitoneal injection, once weekly) for 3 weeks.

Administration of lapatinib and 5-FU in the primary esophageal tumor model. Forty-two mice were selected and randomized into six groups. The mice were treated with vehicle (0.5% HPMC in 0.1% Tween-80, orally, twice daily), lapatinib alone (105 mg/kg, orally, twice daily), 5-FU alone (15 mg/kg, intraperitoneal injection, once daily for 4 days per week), or lapatinib (105 mg/kg, orally, twice daily) in combination with 5-FU (15 mg/kg, intraperitoneal injection, once daily for 4 days per week) for 3 weeks.

Measurement of tumor growth and body size. Tumor volume was calculated using the following formula: tumor volume = (length x width²)/2. The tumor volume was then used to calculate tumor growth inhibition (TGI), as an index of the antitumor activity of each test drug, as follows: TGI (%) = [1 - (Ti - T₀)/(Vi - V₀)] x 100; where Ti is the mean tumor volume of the treated group, T₀ is the mean tumor volume of the treated group on Day 1 of treatment, Vi is the mean tumor volume of the vehicle-treated group, and V₀ is the mean tumor volume of the vehicle-treated group on Day 1 of treatment. Tumor weight inhibition (TWI) was calculated at the end of the study using the following formula: TWI (%) = (1 - T_TW/V_TW) x 100; where T_TW is the mean tumor weight of the treated group on the final day of the study and V_TW is the mean tumor weight of the vehicle-treated group on the final day of the study. Efficacy data presented as the mean tumor volume ± standard error of the mean (SEM).

The relative change in body weight (RCBW) in each mouse was calculated using the following formula: RCBW
primary tumor cells (ESX001, ESX008, ESX009, ESX026) and primary ESCC cells. As shown in Fig. 3A, five out of seven EGFR and HER2 proteins were highly variable among the models. The levels of unphosphorylated and phosphorylated EGFR and HER2 in primary esophageal tumor ESCCs obtained from Chinese patients. The EGFR and HER2 signaling pathway is activated in these ESCCs (Table I). Of note, the HER2-positive patients were more likely to be EGFR-positive than not, although the difference was not statistically significant. The survival rate was higher than that of patients who were negative for both EGFR and HER2 tended to be no correlation between EGFR and HER2 expression in ESCCs (Table I). Twenty-one samples (22.3%) were positive for both EGFR and HER2 expression was positive in 23 samples (24.5%). Twenty-one samples (22.3%) were positive for both EGFR and HER2. The scatter plots in Fig. 1B show the distribution of EGFR or HER2 IHC scores in ESCC tumor tissues as compared with those in adjacent normal tissues. EGFR expression was significantly higher in ESCC tumor tissues than in adjacent normal tissues (P<0.0001), whereas there was no significant difference in HER2 expression. The distribution of IHC staining of EGFR and HER2 indicated that there was no correlation between EGFR and HER2 expression in these ESCCs (Table I). Of note, the HER2-positive patients were more likely to be EGFR-positive than not, although the difference was not statistically significant. The survival rate of patients positive for both EGFR and HER2 tended to be higher than that of patients who were negative for both EGFR and HER2 (Fig. 2). Taken together, these results suggest that the EGFR and HER2 signaling pathway is activated in these ESCCs obtained from Chinese patients.

EGFR and HER2 expression in primary esophageal tumor models. We next examined the total protein expression levels and phosphorylation levels of EGFR and HER2 in primary tumor cells derived from ESCCs from Chinese patients (n=7). The levels of unphosphorylated and phosphorylated EGFR and HER2 proteins were highly variable among the primary ESCC cells. As shown in Fig. 3A, five out of seven primary tumor cells (ESX001, ESX008, ESX009, ESX026 and ESX030) expressed high levels of EGFR. Five tumor cells (ESX002, ESX007, ESX008, ESX009 and ESX030), particularly ESX007, expressed high levels of total HER2 protein. The level of phosphorylated EGFR or HER2 expression also varied widely. Of the seven primary ESCC tumor cells, most of the cells expressed phosphorylated EGFR or HER2. Very high levels of phosphorylated EGFR (ESX008) or phosphorylated HER2 (ESX007) were observed in one cell line each. These data indicate that EGFR and HER2 are highly expressed and their signaling pathways are activated in ESCCs obtained from Chinese patients.

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<tr>
<th>Her2 (+)</th>
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<tr>
<td>EGFR (+)</td>
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Figure 2. Survival curves of patients according to EGFR and HER2 expression. The survival rates were analyzed by the Kaplan-Meier method, and survival was determined from the time of operation to mortality or the last follow-up.

Inhibitory effects of lapatinib in combination with oxaliplatin or 5-FU on the growth of primary ESCC cells. It is well established that lapatinib inhibits protein phosphorylation in cells overexpressing HER2 (19-21). Therefore, we assessed the inhibitory effects of lapatinib, oxaliplatin, and 5-FU alone or lapatinib in combination with either oxaliplatin or 5-FU on the growth of seven primary ESCC cells. The cells were observed microscopically to assess morphologic changes after 7 days of treatment. In the seven primary ESCC cells, lapatinib and 5-FU both inhibited cell proliferation in concentration-dependent manners, with a calculated IC50 of 1-10 µM. Oxaliplatin also inhibited proliferation with similar IC50 values for six of the seven primary cells; the exception was ESX030, which was resistant to oxaliplatin in vitro (Fig. 3B).

The effect of lapatinib in combination with standard chemotherapy was examined to determine the nature of the interaction (i.e., synergistic, additive or antagonistic). As compared with lapatinib in combination with oxaliplatin, lapatinib in combination with 5-FU showed some evidence of synergy in primary tumor cells, although this was not significant (Table II).
Effects of lapatinib, oxaliplatin and 5-FU on the primary ESCC model

Lapatinib in combination with oxaliplatin. After 21 days of treatment, lapatinib and oxaliplatin administered alone at doses of 105 and 6 mg/kg, respectively, did not appear to inhibit tumor cell growth in the primary ESCC model. The TGIs for lapatinib and oxaliplatin alone were 29.65 and 26.22%, respectively, and the TWIs were 21.95 and 22.88%, respectively. However, administration of lapatinib in combination with oxaliplatin at the same doses had significantly greater inhibitory effects compared with vehicle (P<0.001; Fig. 4A), with a TGI and TWI of 51.42 and 44.55%, respectively (Fig. 4E and F). The antitumor effect of 5-FU alone was significantly greater than that of vehicle (P<0.05). Combining lapatinib with 5-FU at the same doses resulted in greater tumor growth inhibition compared with either drug alone (Fig. 4B). The antitumor effect of lapatinib in combination with 5-FU (TGI=94.05%) was significantly greater than that of vehicle (P<0.001) and compared with lapatinib alone (P<0.05). The TWI for lapatinib in combination with 5-FU (68.82%) was also greater than that of lapatinib or 5-FU alone.

Tolerability. There was no evidence for toxicities with lapatinib alone or in combination with oxaliplatin or 5-FU in athymic mice bearing human primary ESCC tumors. There were no marked changes in RCBW during the study, or any significant differences in RCBW between each treatment group (Fig. 4C and D).

Lapatinib in combination with 5-FU. Consistent with the above experiment, lapatinib administered at a dose of 105 mg/kg had no obvious antitumor effects in the ESCC primary model, with a TGI and TWI of 29.65 and 26.22%, respectively (Fig. 4A, with a TGI and TWI of 51.42 and 44.55%, respectively (Fig. 4E and F). The antitumor effect of 5-FU alone was significantly greater than that of vehicle (P<0.05). Combining lapatinib with 5-FU at the same doses resulted in greater tumor growth inhibition compared with either drug alone (Fig. 4B). The antitumor effect of lapatinib in combination with 5-FU (TGI=94.05%) was significantly greater than that of vehicle (P<0.001) and compared with lapatinib alone (P<0.05). The TWI for lapatinib in combination with 5-FU (68.82%) was also greater than that of lapatinib or 5-FU alone.

Synergistic antitumor effects of lapatinib and 5-FU against ESCCs ex vivo. The phosphorylated and total protein levels of several downstream markers were measured in mice treated with a single agent or lapatinib in combination with oxaliplatin (Fig. 5A) or 5-FU (Fig. 5B). Lapatinib in combination with
5-FU induced a greatest decrease in the phosphorylation of EGFR and HER2 in primary ESCC models as compared with each drug alone or lapatinib in combination with oxaliplatin. The expression levels of pERK were slightly downregulated by lapatinib in combination with either oxaliplatin or 5-FU. There were no considerable increases in the blocking of pAKT expression in mice treated with lapatinib in combination with 5-FU, as compared with each drug alone or lapatinib in combination with oxaliplatin. These results suggest that, in primary ESCC models, blockade of pEGFR and pHER2 is beneficial for the synergistic inhibition of tumor growth following treatment with lapatinib in combination with 5-FU.

Discussion

Lapatinib is currently approved for the treatment of patients with HER2-positive metastatic breast cancer whose disease has progressed following trastuzumab-based therapy, and is licensed for use in these patients in combination with capecitabine (26). Based on these data and the increasing interest in the roles of EGFR and HER2 in gastric and esophageal cancer, we sought to evaluate the therapeutic potential of this dual TKI for the treatment of esophageal cancer. We also sought to compare the antitumor effects of lapatinib with standard chemotherapeutic drugs alone or in combination, and to understand the mechanism of action of these drugs.

In seven primary ESCC cells, the expression levels of EGFR and HER2 (total protein and phosphorylated protein) varied greatly. As previously reported in breast as well as in gastric cancer, there was no correlation between lapatinib activity and EGFR protein expression (27-29). Similarly, no correlation was found between lapatinib activity and HER2 protein expression. Several recent studies have examined the roles of lapatinib administered alone or in combination...
with standard chemotherapies in esophageal cancer cell lines in vitro (21,30). Our study provides further insight into the role of the HER family in esophageal cancer. First, our panel of primary tumor cells was directly derived from ESCC tissue samples obtained from Chinese patients. The physiologic condition differs greatly between primary tumor cells and long-established tumor cell lines. For example, primary tumor cells better reflect the in vivo situation (31-33). Our primary tumor cells were directly derived from ESCCs, and are therefore more clinically relevant than cell lines. Indeed, we found that the activity of ESCC tumor cells can be modulated by blocking the EGFR and HER2 signaling pathways.

The doses of lapatinib, oxaliplatin, and 5-FU used in the present study were determined based on the results of maximum tolerated dose studies performed in nude mice (data not shown). Efficacy studies in primary ESCC xenograft models showed that lapatinib (105 mg/kg) in combination with 5-FU induced near-complete tumor regression in all the mice. The effects of this combination were much greater than those achieved using either drug alone or with lapatinib combined with oxaliplatin. We also found that the synergistic antitumor effects of lapatinib in combination with 5-FU were probably mediated by changes in cell signaling. The levels of phosphorylated EGFR and HER2 were much lower following treatment with lapatinib in combination with 5-FU compared with either agent alone or with lapatinib in combination with oxaliplatin. Therefore, our data suggest that inhibition of the EGFR/HER2 signaling pathway by combining a chemotherapeutic drug and a TKI may augment the effects of both agents on the downstream signaling pathways. The synergy observed for this combination may have important clinical implications.

For example, recent studies using breast cancer models have revealed that the accumulation of inactive HER2 receptor, as induced by lapatinib, enhances trastuzumab activity through antibody-dependent cellular cytotoxicity (34). Although our results are preliminary, they support the ongoing investigation of lapatinib in esophageal cancer as well as its possible combination with 5-FU in tumors overexpressing EGFR and HER2. The results also suggest that the addition of anti-EGFR/anti-HER2 therapy to standard chemotherapeutic drugs could have direct clinical benefits, and makes the investigation of additional anti-EGFR/anti-HER2 therapies in esophageal cancer particularly timely.

In the present study, we focused on the effects of lapatinib alone or in combination with standard chemotherapeutic drugs for esophageal cancer. Several clinical trials have suggested that the activity of anti-EGFR drugs seems to be limited to tumors of the gastroesophageal junction, with the response to both erlotinib and gefitinib being approximately 10% (35,36). In addition, in a clinical study of lapatinib in upper gastrointestinal cancer, disease control (prolonged stable disease) was only detected in patients with HER2-amplified disease. Our in vitro and in vivo observations support these clinical findings and the ongoing development of lapatinib in patients with tumors overexpressing HER2 (37,38).

Esophageal carcinoma is a highly malignant and prevalent cancer in China, for which the existing treatments have limited potential in reducing morbidity and mortality. Therefore, there is an urgent need to develop and refine new combinatorial therapies for this cancer. We have shown that lapatinib in combination with 5-FU has a significant synergistic therapeutic effect against ESCCs overexpressing EGFR and HER2. Lapatinib not only had a direct biological effect in terms of inhibiting the growth of ESCC primary cells in vitro, but also augmented the antitumor effects of 5-FU in primary ESCC models in vivo. Therefore, a regimen in which lapatinib is combined with an established chemotherapeutic drug represents a promising strategy for most patients with ESCCC.

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