Lapatinib acts on gastric cancer through both antiproliferative function and augmentation of trastuzumab-mediated antibody-dependent cellular cytotoxicity

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Abstract

Background Trastuzumab has been recently approved for clinical use to treat HER2-expressing advanced gastric cancer, and anti-HER2-targeting therapy has become a promising option for gastric cancer. Lapatinib is a dual tyrosine kinase inhibitor targeting EGFR and HER2. The aim of the present study was to explore the utility of lapatinib for gastric cancer, with a particular focus on trastuzumab-mediated antibody-dependent cellular cytotoxicity (ADCC).

Methods Nine gastric cancer cell lines were evaluated for the effects of lapatinib on the cell-surface accumulation of HER2 and analyzed for their additional effects on trastuzumab-mediated ADCC. Also, HER2 signaling with Western blot, proliferative function with the MTT assay, and apoptosis-inducing activity with 7ADD/Annexin-V were investigated when a panel of gastric cancer cell lines was treated with lapatinib.

Results Lapatinib inhibited HER2 signaling and cell proliferation in the panel of gastric cancer cell lines. Lapatinib also induced the accumulation of HER2 on the

cell surface, resulting in the enhancement of trastuzumab-mediated ADCC of gastric cancer.

Conclusions Lapatinib exhibits inhibitory activity in gastric cancer cells, and the combination of lapatinib with trastuzumab may be a promising treatment strategy for gastric cancer patients.

Keywords Gastric cancer · HER2 · Lapatinib · Trastuzumab · ADCC

Introduction

Annually, about 110,000 people in Japan develop gastric cancer and an estimated 65,000 die from it, which is second among cancer-related deaths in the country [1]. Despite multimodality therapy such as surgical resection combined with chemotherapy, hyperthermia, and chemoradiotherapy, the prognosis with advanced disease remains poor [2–7]. Therefore, the utilization of molecular-targeted drugs is needed for novel therapeutic approaches for gastric cancer patients.

HER2 is one member of the HER family of tyrosine kinase receptors, and HER2-related downstream signaling plays a crucial role in cell proliferation, survival, migration, and differentiation [8–11]. The HER2 gene is amplified and overexpressed in a variety of tumors, including breast cancers, ovarian cancers, esophageal squamous cell carcinomas, and colorectal cancers [12–14]. We also reported that amplification and overexpression of HER2 were seen in ~20 % of gastric cancers and were correlated with the stage progression of gastric cancer [14, 15].

Trastuzumab is a humanized monoclonal antibody that specifically targets HER2 and exhibits anticancer effects by inducing antibody-dependent cellular cytotoxicity (ADCC)

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and inhibiting tumor cell growth [16, 17]. In particular, we reported that HER2-expressing gastric cancer cells were killed by trastuzumab-mediated ADCC and that activity was correlated with the intensity of HER2 expression [18]. Trastuzumab has been approved for the treatment of patients with HER2-positive metastatic breast cancer and is now being used as a postoperative adjuvant therapy for HER2-positive breast cancer throughout the world. Recently, the ToGA [The Trastuzumab (Herceptin) for Gastric Cancer] study, an international randomized controlled phase III trial, revealed that HER2-positive gastric cancer patients who received combination treatment with trastuzumab and chemotherapy showed significantly improved survival compared with those who received chemotherapy alone [19].

Lapatinib is a dual tyrosine kinase inhibitor (TKI) targeting epidermal growth factor receptor (EGFR) and HER2 tyrosine kinase domains [20, 21], and it significantly inhibits the signaling pathways and proliferation of EGFR- and HER2-overexpressing cancer cells by a mechanism of action distinct from that of trastuzumab [22–25]. Lapatinib has recently been approved by the U.S. Food and Drug Administration for the treatment of HER2-expressing advanced breast cancer [26], and lapatinib prolonged progression-free survival in patients with HER2-overexpressing advanced or metastatic breast cancer in several clinical trials [25, 27–30]. Furthermore, we and others showed that lapatinib induces the accumulation of HER2 at the cell surface and enhances the effects of trastuzumab-mediated ADCC in breast cancer and esophageal squamous cell carcinoma [31–33].

In the present study, we investigated (1) the biological activity of lapatinib against gastric cancer cell lines and (2) whether lapatinib induces the accumulation of HER2 at the cell surface of gastric cancer cell lines in relationship to trastuzumab-mediated ADCC.

Materials and methods

Reagents

RPMI 1640 (Invitrogen, Carlsbad, CA, USA), fetal calf serum (Invitrogen), and penicillin streptomycin (Sigma-Aldrich, St. Louis, MO, USA) were used for cell culture. Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich, and a dual TKI, lapatinib (Tykerb), was purchased from GlaxoSmithKline (Brentford, UK). Anti-HER2 mAb herceptin (trastuzumab; Roche, Basel, Switzerland) and anti-CD20 mAb rituxan (Roche), which is an isotype-matched control monoclonal antibody (mAb), were used for ADCC assays. Primary antibodies

against HER2/ErbB2, phospho-HER2/ErbB2 (Tyr1221/1222), p44/42MAPK (Erk1/2), phospho-p44/42MAPK (Erk1/2) (Thr202/Tyr204), Akt, phospho-Akt (Ser473), β -actin, and horseradish peroxidase (HRP)-linked goat anti-rabbit antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Phycoerythrin (PE)-conjugated mouse anti-HER2/neu antibody, PE-conjugated mouse IgG1 k isotype control immunoglobulin, FITC-conjugated Annexin V, and 7-aminoactinomycin D (7-AAD) were purchased from Becton–Dickinson (Franklin Lakes, NJ, USA). Fluorescein isothiocyanate (FITC)-conjugated trastuzumab and FITC-conjugated CD20 were kindly provided by Prof. Rolf Kiessling (Department of Oncology and Pathology, Immune and Gene Therapy Laboratory, Cancer Center Karolinska, Karolinska Institutet, Stockholm, Sweden).

Cell lines

The human gastric cancer cell lines MKN7, MKN28, MKN45, MKN74, NUGC-3, and OECM-1 were purchased from the Health Science Research Resources Bank (Osaka, Japan). The human gastric cancer cell lines NCI-N87 and KATOIII were obtained from the American Type Culture Collection (Rockville, MD, USA), and OE19 was obtained from the European Collection of Animal Cell Cultures (Sigma-Aldrich). All cell lines were cultured in RPMI 1640 with 5 % fetal calf serum (FCS), 50 U/ml penicillin, and 2 mM L-glutamine.

Western blotting

Gastric cancer cell lines were treated with the indicated doses of lapatinib (100–1,000 nM) for 1 h; DMSO was used as a control. After incubation, collected cells were solubilized and boiled for 10 min and, subsequently, the protein concentration of cell lysates was measured. The same amount of protein was separated by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE), followed by transfer to a polyvinylidene fluoride (PVDF) microporous membrane (Millipore, Billerica, MA, USA) blocked in PBS with 5 % milk powder. After blocking, the membrane was probed with primary antibody to HER2/ErbB2, phosphorylated HER2/ErbB2 (Tyr1221/1222), Erk1/2, phosphorylated Erk1/2 (Thr202/Tyr204), Akt, phosphorylated Akt (Ser473), or β -actin. After washing, the membrane was incubated with HRP-linked goat anti-rabbit antibody (Cell Signalling Technology). The specific bands were visualized by enhanced chemiluminescence according to the manufacturer's protocol (ECL Plus; Amersham Pharmacia Biotech AB, Uppsala, Sweden) and digitally captured by a LAS4000 Lumino Image Analyzer (Fuji Photo Film, Tokyo, Japan).

ADCC assay

Gastric cancer cell lines were cultured in a tissue culture flask and exposed to DMSO or lapatinib for 48 h, after which they were used as targets. HER2-overexpressing cell lines were treated with 500 nM lapatinib and the HER2-low-expressing cell line was treated with 1,000 nM lapatinib. Peripheral blood mononuclear cells (PBMC) from healthy donors obtained by Ficoll-Paque separation (GE Health Care, Uppsala, Sweden) was used as effector cells. After target cells were labeled with 50 μ l ^{51}Cr for 60 min, targets (5×10^3 /well) and various ratios of PBMC were co-cultured in 200 μ l X-VIVO medium. The assays were performed in triplicate in a 96-well U-bottom plate with trastuzumab or the anti-CD20 antibody, with rituximab as an irrelevant control. After 5 h of co-culture, the released ^{51}Cr in the supernatant (100 μ l) was measured with a gamma counter (cpm, counts per minute), and the percentage of specific lysis was calculated according to the formula percent (%) specific lysis = $100 \times (\text{experimental cpm} - \text{spontaneous cpm}) / (\text{maximum cpm} - \text{spontaneous cpm})$. Controls included the incubation of target cells alone with trastuzumab or rituximab.

Flow cytometric analysis

Gastric cancer cell lines were cultured in a tissue culture flask and exposed to different concentrations of lapatinib or DMSO as control for 48 h. After incubation, treated cells were immunofluorescently labeled according to a previously established immunofluorescent staining protocol [34]. Cells were analyzed using FACS Calibur (Becton–Dickinson) and Cellquest software (BD).

MTT cell proliferation assay

Gastric cancer cell lines were cultured in triplicate wells of a 96-well flat-bottomed plate (Corning Ware, Corning, NY, USA) with varying concentrations of lapatinib or DMSO as control in 200 μ l culture medium. After a 52-h incubation, 50 μ l MTT (2 mg/ml; Sigma) was added to wells for the final 4 h of culture. Supernatants were discarded, followed by the elution of the crystallized precipitate using DMSO (50 μ l/well; Sigma). The colorimetric reaction was measured by a spectrophotometer at 450 nm, and lapatinib-

mediated inhibition of proliferation was determined using the formula ($\text{Absorbance}_{\text{lapatinib}} / \text{absorbance}_{\text{DMSO}} \times 100\%$).

Statistics

Student's unpaired *t* test was performed to determine significance. *P* values <0.05 were considered significant.

Results

Lapatinib induced the accumulation of HER2 at the cell surface of gastric cancer cell lines

In the present study, we used a panel of gastric cancer cell lines including KATOIII, MKN-7, MKN28, MKN45, MKN74, NCI-N87, NUGC3, OCUM1, and OE19, and there were variable expressions of HER2 and EGFR in these gastric cancer cell lines (Table 1). As it is well known that MKN7, NCI-N87, and OE19 are HER2-overexpressing and HER2-gene-amplified gastric cancer cell lines [18, 35–38], we used MKN7, NCI-N87, and OE19 as HER2-overexpressing cell lines and the others as HER2-low-expressing cell lines in this study.

A panel of gastric cancer cell lines were tested for HER2 expression on the cell surface by flow cytometry following exposure to DMSO as control or several doses of lapatinib for 48 h (Fig. 1). Lapatinib enhanced the surface expression of HER2 on all the tested gastric cancer cell lines in a dose-dependent manner (Fig. 1). We used two HER2-overexpressing cell lines, MKN7 and OE19, and three HER2-low-expressing cell lines, MKN45, MKN74, and KATOIII, for the following experiments as representative cases.

Next, we used the FITC-conjugated trastuzumab mAb for flow cytometry, which directly corresponds to the levels of the trastuzumab-binding sites on the tumor cells. As shown in Fig. 2, lapatinib could upregulate the trastuzumab-binding sites of the HER2 molecule on all tested cell lines, although upregulation in the HER2-low-expressing cell lines MKN45, MKN74, and KATOIII was marginal.

Taken together, lapatinib induced the accumulation of cell-surface HER2 expression of gastric cancer cell lines along with the increase in trastuzumab-binding sites.

Table 1 Epidermal growth factor receptor (EGFR) and HER2 expression on gastric cancer cell lines assessed by flow cytometry

Cell lines	NCI-N87	MKN7	OE19	KATOIII	OCUM-1	MKN74	MKN45	MKN28	NUGC3
EGFR (MFI)	27.5	58.3	21.0	38.7	66.2	68.1	29.0	29.7	54.0
HER2 (MFI)	1,555.1	1,191.6	959.9	281.0	273.8	184.0	174.2	132.4	54.4

MFI mean fluorescence intensity

Fig. 1 Lapatinib induced the surface accumulation of HER2 on gastric cancer cell lines. Nine gastric cancer cell lines (MKN7, MKN28, MKN45, MKN74, KATOIII, NCI-N87, NUGC3, OCUM1, and OE19) were treated with DMSO or indicated doses of lapatinib (1–1,000 nM) for 48 h. In this study, MKN7, NCI-N87, and OE19 were HER2-overexpressing cell lines and the other six cell lines were

HER2-low-expressing cell lines. After dead or apoptotic cells were excluded using Annexin V and 7-AAD staining, HER2 expression on these cell lines was assessed by flow cytometry. Data are from one representative experiment of three independent experiments, each with comparable results

Lapatinib inhibits HER2 signaling and proliferation in gastric cancer cell lines

Next, we confirmed the inhibition of HER2 signaling induced by lapatinib in gastric cancer cells with Western blot analysis. As shown in Fig. 3, lapatinib could inhibit HER2 phosphorylation in OE19, MKN7, and MKN45 in a dose-dependent manner, whereas the expression of phospho-HER2 in HER2-low-expressing MKN74 and KATOIII was not detected or was not downregulated. Furthermore, lapatinib reduced the phosphorylation of Erk1/2 and Akt, which are more downstream events of the HER2-signaling pathway in a dose-dependent manner (Fig. 3), especially in HER2-overexpressing OE19 and MKN7, and to a lesser degree in MKN45. However, although lapatinib could reduce the phosphorylation of HER2 in MKN45, there was no decrease in phosphorylation of Akt in MKN45 (Fig. 3). Furthermore, there was faint downregulation of phospho-Erk in MKN74 and KATOIII when treated with lapatinib at 1,000 nM, whereas there was no clear inhibition of phospho-Akt in both cell lines. These observations showed that

lapatinib could inhibit HER2 signaling on gastric cancer cells, especially in HER2-overexpressing cells.

As a functional consequence of lapatinib treatment, gastric cancer cell lines were evaluated for proliferative and apoptosis-inducing activity. As shown in Fig. 4a, lapatinib induced apoptosis in most gastric cancer cell lines. Moreover, lapatinib inhibited 50–60 % of the cell growth in OE19, MKN7, and MKN45 at 1,000 nM; 20 % growth inhibition was seen in MKN74 and KATOIII at 1,000 nM (Fig. 4b). Taken together, lapatinib could induce antiproliferative and apoptosis-inducing activity in gastric cancer cells.

Lapatinib enhanced trastuzumab-mediated ADCC

As we already described that lapatinib increased the surface accumulation of HER2, we next examined whether lapatinib could enhance trastuzumab-mediated ADCC in the gastric cancer cell lines. In the ADCC assay, HER2-overexpressing cell lines were treated with 500 nM lapatinib and the HER2-low-expressing cell lines were treated with 1,000 nM lapatinib to avoid the direct cytotoxic effect

Fig. 2 Lapatinib increased binding sites of trastuzumab on gastric cancer cell lines. Five gastric cancer cell lines (OE19, MKN7, MKN45, MKN74, and KATOIII) were treated with DMSO or lapatinib for 48 h. HER2-overexpressing cell lines were treated with 500 nM lapatinib and the HER2-low-expressing cell line was treated with 1,000 nM lapatinib. After dead or apoptotic cells were excluded

using Annexin V and 7-AAD, the levels of trastuzumab-binding sites of the HER2 molecule were analyzed with the FITC-conjugated Herceptin monoclonal antibody (mAb), which directly corresponds to the levels of trastuzumab-binding sites on tumor cells. Data are from one representative experiment of three independent experiments, each with comparable results

of lapatinib in HER2-overexpressing cells (Fig. 4a). As shown in Fig. 5, pretreatment of target cells with lapatinib significantly increased the trastuzumab-mediated ADCC by 20–30 % compared to DMSO-treated cells in the HER2-overexpressing cell lines, OE19 and MKN7. On the other hand, lapatinib-induced enhancement of ADCC was marginal in the HER2-low-expressing cell lines (MKN45, MKN74, and KATOIII) in comparison to those in HER2-overexpressing OE19 and MKN7 (Fig. 5). The enhancement of trastuzumab-mediated ADCC by lapatinib was confirmed in three independent experiments using PBMC from three different healthy donors. Thus, lapatinib could enhance the trastuzumab-mediated ADCC paralleling with the upregulation of surface HER2 expression in gastric cancer cell lines.

Discussion

In the present study, we indicated that a dual TKI targeting epidermal growth factor receptor (EGFR) and HER2,

lapatinib, has the ability to induce growth inhibition as well as augment trastuzumab-mediated ADCC against gastric cancer cells. The percentage of patients with overexpression of HER2 was 22.1 % in the ToGA population [1, 19], and we also reported that the overexpression of HER2 was seen in ~20 % of gastric cancer patients [14, 15], indicating that significant populations of gastric cancer cells may be eligible for HER2 targeting. Thus, anti-HER2 targeting with a combination of lapatinib and trastuzumab for gastric cancer is a novel and promising strategy.

The original ToGA study revealed that HER2-positive gastric cancer patients who received combination treatment with trastuzumab and chemotherapy showed significantly improved survival compared with those who received chemotherapy alone [19]. Furthermore, Sawaki et al. [1] recently analyzed a subgroup of 101 Japanese patients enrolled in the ToGA study and showed that the overall survival of Japanese patients with HER2-positive advanced/metastatic gastric cancer who received trastuzumab plus chemotherapy was improved compared with

Fig. 3 Lapatinib inhibited phosphorylation of HER2, Erk1/2, and Akt in gastric cancer cell lines. Five gastric cancer cell lines (OE19, MKN7, MKN45, MKN74, and KATOIII) were treated with DMSO or indicated doses of lapatinib (100–1,000 nM) for 1 h. Western blotting

to detect HER2, Erk1/2, and Akt, including phosphorylated (p-HER2, p-Erk1/2, and p-Akt) and nonphosphorylated forms, was performed. Data are from one representative experiment of three independent experiments, each with comparable results

patients who received chemotherapy alone. These results clearly indicate that trastuzumab combined with chemotherapy may provide a standard strategy for gastric cancer with HER2 expression, and the improvement of HER2-targeting therapy may provide an even more successful strategy for HER2-positive gastric cancer.

In several recent reports on preclinical and clinical models of HER2-positive breast cancer, lapatinib combined with trastuzumab enhanced apoptosis and growth inhibition as a result of the synergistic inhibitory effect of HER2 signaling and significantly improved the progression-free survival rate compared to lapatinib alone [22, 25, 39]. As a novel action of lapatinib, we and Scaltriti et al. recently reported that lapatinib induced the accumulation of HER2 at the surface of cancer cells, resulting in the enhancement of trastuzumab-mediated ADCC [31–33]. As

it is clear that ADCC is a key factor in the therapeutic action of trastuzumab [40, 41], the enhancement of trastuzumab-mediated ADCC induced by lapatinib is ideal and promising. In fact, in a HER2-positive xenograft model, lapatinib combined with trastuzumab triggered complete tumor regression through the enhanced trastuzumab-mediated ADCC with the accumulation of HER2 at the cell surface caused by lapatinib [31]. Furthermore, we recently reported a case in which a trastuzumab-refractory tumor was converted to trastuzumab-sensitive tumor following lapatinib treatment [33]. In this case, because we did not use lapatinib and trastuzumab in the same period, there would appear to be no synergistic effects between the two drugs, and we believe that lapatinib might alter tumor sensitivity to trastuzumab by the accumulation of HER2 at the cell surface, resulting in the enhancement of

Fig. 4 Lapatinib induced apoptosis of and inhibited cell proliferation in gastric cancer cell lines. Five gastric cancer cell lines (OE19, MKN7, MKN45, MKN74, and KATOIII) were treated with DMSO or indicated doses of lapatinib (1–1,000 nM) for 48 h. After incubation, apoptosis assays by flow cytometry were performed using Annexin V and 7-AAD (**a**) and the MTT cell proliferation assay (**b**).

trastuzumab-mediated ADCC [33]. In line with this case report of breast cancer, we demonstrated in the present study that lapatinib induced the accumulation of HER2 at the cell surface of gastric cancer cells, resulting in the enhancement of trastuzumab-mediated ADCC. Thus, lapatinib combined with trastuzumab might be a novel promising approach for gastric cancer patients.

Wainberg et al. [38] recently reported that the combination of lapatinib and trastuzumab was highly synergistic in inhibiting cell growth of HER2-expressing gastric cancer in in vitro and in vivo xenograft models. The present study is, to our knowledge, the first report describing the action of lapatinib relating to the augmentation of trastuzumab-mediated ADCC through the upregulation of surface HER2 on gastric cancer cells. The upregulation of surface HER2 expression was dramatically observed in HER2-overexpressing gastric cancers to be greater than

Representative data of three independent experiments, each with comparable results, are shown. Data between cells treated with DMSO and cells treated with indicated doses of lapatinib were analyzed using a non-paired Student's *t* test; significance was set at $p < 0.05$ (*) or $p < 0.01$ (**).

that in HER2-low expressing tumors, paralleling the augmentation of trastuzumab-mediated ADCC. However, the antiproliferative and apoptosis-inducing activities induced by lapatinib were seen in both HER2-overexpressing and HER2-low-expressing gastric cancers, although we could not show a marked inhibitory effect of HER2 signaling (MAPK and Akt pathway) in HER2-low-expressing tumors by Western blot. There are some explanations to describe this discrepancy: the sensitivity of the Western blot was not sufficient to detect alternation of HER2-signaling molecules in HER2-low-expressing tumors, or the other HER2-signaling pathways rather than the MAPK or Akt pathway are involved in the inhibitory effect induced by lapatinib in HER2-low-expressing tumors. Regardless of unknown underlying mechanisms, the observations that lapatinib could have an inhibitory effect against HER2-low-expressing gastric cancer may prompt us to apply lapatinib

Fig. 5 Lapatinib enhanced trastuzumab-mediated ADCC in gastric cancer cell lines. Five gastric cancer cell lines (OE19, MKN7, MKN45, MKN74, and KATOIII) were treated with DMSO or lapatinib for 48 h. After the 48-h incubation, trastuzumab-mediated antibody-dependent cellular cytotoxicity (ADCC) was performed as described in “Materials and methods.” Representative data of three

independent experiments, each with comparable results, are shown. Data between (DMSO + trastuzumab + PBMC) and (lapatinib + trastuzumab + PBMC) at the same T:E ratio were analyzed using an unpaired Student's *t* test; significance was set at $p < 0.05$ (*) or $p < 0.01$ (**)

not only for HER2-overexpressing tumors but also for HER2-low or -negative tumors.

In conclusion, we presented several actions of lapatinib against gastric cancer, which include antiproliferative and apoptosis-inducing functions, as well as the enhancement of trastuzumab-mediated ADCC through the accumulation of HER2 at the cell surface. The combination of trastuzumab with lapatinib may be one of the ideal options for gastric cancer.

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