Pertuzumab in combination with trastuzumab shows significantly enhanced antitumor activity in HER2-positive human gastric cancer xenograft models.

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Running title: Pertuzumab and trastuzumab combination in gastric cancer
**Translational Relevance**

Gastric cancer shows a poor prognosis. Although various therapies including fluoropyrimidine- and platinum-based combination chemotherapy have been used for gastric cancer, these therapies have not improved the rate of overall survival. Recently, the effectiveness of trastuzumab for gastric cancer has been demonstrated in a clinical PIII study, ToGA, revealing how meaningful anti-HER2 therapy is for gastric cancer. This is the first report showing the usefulness of a new HER2 antibody, pertuzumab, for HER2 over-expressing gastric cancer in vivo and in vitro. These results were a scientific rationale for developing pertuzumab for HER2-positive gastric cancer.
Abstract

Purpose: We investigated the antitumor activity of the combination of two different humanized monoclonal human epidermal growth factor receptor (HER) 2 antibodies, pertuzumab and trastuzumab, for gastric cancer.

Experimental Design: Tumor mouse xenograft models were used to examine antitumor activity. Cell proliferation was examined using crystal violet staining. HER family proteins’ expression was analyzed by ELISA and IHC. Phosphorylated proteins and heterodimers were detected by western blotting and in situ PLA™, respectively. Apoptosis activity was examined by caspase 3/7 activity. ADCC activity was detected by xCELLigence™. Microvessel density (MVD) was examined by CD31 staining.

Results: Pertuzumab in combination with trastuzumab showed significant antitumor activity compared with each monotherapy in NCI-N87, a HER2-positive human gastric cancer xenograft model. The efficacy was stronger than that of the maximum effective dose with each monotherapy. Similar antitumor activity was shown in 4-1ST, another HER2-positive gastric cancer model, but not in MKN-28, a HER2-negative model. Combining pertuzumab with trastuzumab enhanced cell-growth inhibition and apoptosis activity by inhibiting EGFR-HER2 heterodimerization, and the phosphorylation of these receptors and their down-stream factors. This effect was also seen in HER2-HER3 signaling. Furthermore, pertuzumab in combination with trastuzumab potentiated the ADCC activity of those antibodies and reduced tumor MVD.

Conclusions: We demonstrated the significantly enhanced efficacy of pertuzumab combining with trastuzumab for HER2 over-expressing gastric cancer through the potentiation of cell-growth inhibition, apoptosis activity, cell-killing activity by ADCC, and anti-angiogenic activity. This study suggests the clinical benefit of combination therapy with pertuzumab and trastuzumab for patients with HER2-positive gastric cancers.
Introduction

Gastric cancer shows a poor prognosis and is the second-leading cause of cancer-related deaths. Its incidence is estimated at 934,000 cases, 56% of new cases are in Eastern Asia, 41% in China, and 11% in Japan (1). Although fluoropyrimidine- and platinum-based combination chemotherapy is the most widely accepted in the world at present, its benefit does not translate into higher overall survival rates. Therefore, more effective therapies for gastric cancer are required.

The human epidermal growth factor receptor (HER) family is composed of EGFR, HER2, HER3, and HER4. They regulate cell proliferation, differentiation, and apoptosis through the activation of their signal transduction by forming homodimers or heterodimers (2). The over-expression of HER family protein is often related to tumor malignancy. In gastric cancer, EGFR, HER2, and HER3 overexpression has been identified and a relationship with prognosis is suggested (3-7). Therefore, inhibiting the signal transduction through heterodimers including HER2 possibly provides more benefit to patients with gastric cancer. Recently, the ToGA trial (a phase III study of trastuzumab (Herceptin®) in HER2-positive advanced and inoperable gastric cancer) showed a survival benefit when trastuzumab was added to chemotherapy in HER2-overexpressing gastric cancer patients (8) and the FDA has approved trastuzumab for HER2-positive metastatic gastric and gastroesophageal junction cancer. Thus, anti-HER2 therapy has been identified to be of clinical significance.

Trastuzumab is a humanized anti-HER2 antibody and is suggested to show its antitumor activity through inhibiting ligand-independent HER2 signaling, enhancing antibody-dependent cellular cytotoxicity (ADCC) activity (9, 10), blocking formation of p95HER2, a constitutively active form of HER2 (11), and suppressing tumor angiogenesis (12). Trastuzumab has demonstrated survival benefit in the treatment of HER2-overexpressing metastatic breast cancer and in the adjuvant therapy of HER2-overexpressing breast cancer (13-15). For gastric cancer, our previous
study showed that trastuzumab significantly inhibited tumor growth in HER2-overexpressing human gastric cancer mouse xenograft models (16).

Pertuzumab, which is a new humanized anti-HER2 antibody, is thought to exert antitumor activity in a different manner from trastuzumab. Pertuzumab binds to domain II of HER2, the region of dimer formation, whereas trastuzumab binds to domain IV of HER2. Thus, pertuzumab could inhibit the dimerization of HER2 with other HER family proteins and prevent ligand-dependent HER2 signaling (17). In HER2-positive breast cancer, the usefulness of pertuzumab and trastuzumab was shown in preclinical and clinical reports (18, 19). To investigate the effect of pertuzumab and trastuzumab for patients with previously untreated HER2-positive metastatic breast cancer, a phase III trial of pertuzumab in combination with trastuzumab and docetaxel is underway (20). Clinical benefit of combination therapy of pertuzumab and trastuzumab is expected.

Pertuzumab and trastuzumab in combination might provide more effective antitumor activity than either single agent for HER2-positive tumors including gastric cancer, because of their different mechanisms of HER2-signal inhibition. This combination therapy could provide clinical benefit for HER2-positive gastric cancer patients. In the present study, we investigated the antitumor efficacy of pertuzumab in combination with trastuzumab as well as its mechanism of action as a new therapy for gastric cancer by using HER2-overexpressing human gastric cancer mouse xenograft models.

**Materials and Methods**

**Molecular modeling of trastuzumab/pertuzumab/HER2 ternary complex**

The binding position of the Fab of pertuzumab or trastuzumab to HER2 was assigned using the crystallographic structure of the Fab (pertuzumab)/HER2 complex (Protein Data Bank (PDB) ID: 1s78) (17) or the Fab (Trastuzumab)/HER2 complex (PDB ID: 1n8z) (21). We used the
3D structure of IgG1 monoclonal antibody (PDB ID: 1IGY) (22) as a surrogate for that of each whole antibody because the 3D structures of the whole antibodies are not known. The position of pertuzumab or trastuzumab whole antibody was estimated by superposing the 3D structure of IgG1 (PDB ID: 1IGY) onto that of the Fab (pertuzumab or trastuzumab)/HER2 complex (PDB ID: 1s78 or 1n8z respectively). The superposition of the Fab of IgG1 and the Fab of pertuzumab or trastuzumab was done using the software PyMOL (The PyMOL Molecular Graphics System, Schrödinger, LLC, New York, NY, USA). Finally the pertuzumab whole antibody/HER2 complex model and trastuzumab whole antibody/HER2 complex model were merged by superposing the 3D structures of HER2 of the two models.

**Construction of the transfectants of NK-92 expressed human FcgRIIIa-158V allotypes**

CD16-negative NK-92 tumor line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cDNA encoding FcgRIIIa was amplified by PCR from the fetal spleen cDNA Library (Clontech, Mountain View, CA, USA) using a specific primer set (CD16F1: TAA GAA TTC CCA CCA TGT GGC AGC TGC TCC TCC C, CD16F2: TAA GCG GCC GCT TAT CAT TTG TCT TGA GGG TCC TTT CTC C). The cDNA encoding FcgRIIIa was subcloned into pGEM® T-Easy vector (Promega, Madison, WI, USA). Since cDNA sequencing revealed that the obtained FcgRIIIa were all 158F allotype, the FcgRIIIa-158V allotype sequence was generated by site-directed mutagenesis (Quick change®: Stratagene, La Jolla, CA, USA). The cDNA encoding FcgRIIIa-158V allotype was cloned at the EcoRI and NotI sites of the pCXND3 expression vector, which is a derivative of pCXN (23) containing a CAG promoter and a neomycin-resistant gene. The resulting plasmid was designated pCXND3/CD16(V). To establish transfectants of NK-92 cells that produce human FcgRIIIa-158V allotype, pCXND3/CD16(V) was transfected by the electroporation method using GENE-PULSER II (BioRad Laboratories, Hercules, CA).
CA, USA) under the conditions of 1.5 kV and 25 μFD. Flow cytometry-based screening was performed using αCD16-FITC (Beckman Coulter, Brea, CA, USA), and ⁵¹Cr-release assay was performed to confirm the ADCC activity in pCXND3/CD16(V)-transfected NK-92 cells. The resulting FcγRIIIa-158V-positive NK-92 cells exerting ADCC activity were designated CD16(158V)/NK-92 cells.

Test agents

Trastuzumab and pertuzumab were provided by F. Hoffmann-La Roche (Basel, Switzerland) as a fine powder and a liquid, respectively. Trastuzumab was dissolved in purified water. Both antibodies were diluted with saline or culture medium in in vivo or in vitro experiments. Human immunoglobulin G (HuIgG) was purchased from MP Biomedicals, Inc. (Aurora, OH, USA) and was reconstituted with water and diluted with saline.

Animals

Male, 5-week-old BALB-nu/nu mice (CAnN.Cg-Foxn1<nu>/CrI/Crlj nu/nu) were obtained from Charles River Japan (Yokohama, Japan). All animals were allowed to acclimatize and recover from shipping-related stress for 1 week prior to the study. The health of the mice was monitored by daily observation. Chlorinated water and irradiated food were provided ad libitum, and the animals were kept under a controlled light-dark cycle (12h-12h). All the animal experiments were conducted in accordance with the Institutional Animal Care and Use Committee.

Cell lines and culture

Three human gastric cancer cell lines were used in the present study. NCI-N87 cells were purchased from ATCC. MKN-28 cells were purchased from immuno-Biochemical Laboratories Co.,
Ltd. (Fujioka, Japan). NCI-N87 and MKN-28 were maintained in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS at 37°C under 5% CO₂. 4-1ST cells were purchased from the Central Institute for Experimental Animals (Yokohama, Japan) and maintained in BALB-nu/nu mice by subcutaneous (sc) inoculation of pieces of tumor. CD16(158V)/NK-92 was maintained in MEMα (Wako, Osaka, Japan) supplemented with 12.5% FBS, 12.5% horse serum, 0.02 mM folic acid, 0.1 mM 2-mercaptoethanol, 0.2 mM inositol, and 0.5 mg/mL G418, and 20 ng/mL recombinant human IL-2 at 37°C under 5% CO₂.

**Immunohistochemistry of EGFR and HER2**

Tumor xenograft tissues were resected and processed as formalin-fixed, paraffin-embedded specimen sections. These were examined for the expression of EGFR or HER2 protein by immunohistochemistry (IHC) using EGFR pharmDx kit™ and HercepTest®.

**In vivo tumor growth inhibition studies**

Each mouse was inoculated sc into the right flank with either 5 × 10⁶ cells/mouse of human gastric cancer cell lines MKN-28 or NCI-N87, or an 8-mm³ piece of 4-1ST tumor tissue. Several weeks after tumor inoculation, mice were randomly allocated to control and treatment groups. The administration of anticancer agents was started when the tumor volumes reached approximately 0.2 to 0.3 cm³. To evaluate the antitumor activity of the test agents, tumor volume was measured twice a week. The tumor volumes (V) were estimated and the percentage of tumor growth inhibition (TGI%) was calculated as described previously (24). Trastuzumab and pertuzumab were administered intraperitoneally once a week for three weeks.

**In vitro anti-proliferation assays**
NCI-N87 cells were seeded on 96-well plates at $5 \times 10^3$ cells/well and pre-cultured for 24 hours. The cells were treated with pertuzumab, trastuzumab or both and cultured for three days. The cells were fixed with 10% formalin neutral buffer solution. Crystal violet staining and extraction were performed as described previously (16). Their absorbance was measured at 595 nm. The cells after 24 hours pre-culture were also detected by crystal violet staining as the absorbance of the pre-cultured well. The percentage of cell proliferation inhibition (% Inhibition) was calculated as follows: % Inhibition = \{1 – (absorbance of treatment well – absorbance of pre-cultured well) / (absorbance of non-treatment well – absorbance of pre-cultured well)} × 100. In case of ligand-dependent proliferation, cells were pre-cultured in RPMI-1640 with 0.1% FBS and treated with pertuzumab, trastuzumab or both 30 min before EGF or HRGα stimulation. The percentage of cell proliferation (% Proliferation) was calculated as follows: % Proliferation = (absorbance of treatment well – absorbance of pre-cultured well) / (absorbance of unstimulated well – absorbance of pre-cultured well) × 100.

**Apoptosis assay**

NCI-N87 cells were seeded on 96-well plates at $1 \times 10^4$ cells/well and cultured in the same way as the ligand-dependent anti-proliferation assay (see above). Twenty-four hours after treatment, Caspase-Glo™ 3/7 Assay (Promega) was used to measure caspase 3/7 activity. Caspase 3/7 activity was calculated as follows: Caspase 3/7 activity = (luminescent unit of treatment well – luminescent unit of blank well) / (mean luminescent unit of control well).

**Cell stimulations**

Cells were precultured for 1 day after seeding. After that the cells were starved in RPMI-1640 supplemented with 0.1% FBS for 20-24 hours and treated with pertuzumab, trastuzumab
or both. After 3.5 hours incubation, cells were exposed to EGF or heregulin (HRG) α for 5 min.

**Western blotting**

Cells were washed with ice-cold PBS and lysed with Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA, USA) with 10 mM NaF, 1 µg/mL aprotinin and 1 mM PMSF. After centrifugation (4°C, 14,000g, 5 min.), the resultant supernatant was used for the assays. Protein concentration of the supernatant was quantified using the DC protein assay kit (BioRad). Cell lysates (20 µg protein/lane) were electrophoresed on SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked in SuperBlock® T20 (TBS) Blocking Buffer (Thermo Scientific, Waltham, MA, USA) and probed with each antibody against HER2 (sc-31153), HER3 (sc-285), (Santa Cruz Biotechnology, Santa Cruz, CA, USA), EGFR (#2963), pEGFR (Tyr1068) (#2236), pHER2 (Tyr1248) (#2247), pHER3 (Tyr1289) (#4791), Akt (#2920), pAkt (Ser473) (#4058), ERK1/2 (#9102), pERK1/2 (Thr202/Tyr204) (#9106) (Cell Signaling) and actin (A2228) (Sigma-Aldrich, St. Louis, MO, USA) as the first antibodies. These proteins were detected by horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). The bands were visualized using ECL plus (GE Healthcare Life Sciences, Buckinghamshire, UK).

**ELISA**

Tumor samples were taken when the tumors had reached a volume of approximately 0.3 to 0.5 cm³ and were immediately frozen in liquid nitrogen and stored at -80°C. Tumor samples were homogenized in PBS containing 0.05% Tween 20 and centrifuged (4°C, 10,000 × g, 20 min.). The resultant supernatant was used for the assays. We used Duo Set® IC (R&D Systems, Minneapolis, MN, USA) to detect human total EGFR, HER2, and HER3. Quantikine®
(R&D systems) was used to detect human VEGF. Total protein levels in the tumor tissue samples using a DC protein assay kit (BioRad Laboratories).

**in situ proximity ligation assay**

in situ proximity ligation assay (PLA) was done to detect EGFR-HER2 heterodimer. We used Duolink® in situ PLA™ (Olink Bioscience, Uppsala, Sweden). In the assay, oligonucleotide-conjugated ‘PLA probe’ antibodies are directed against primary antibodies for EGFR or HER2. Annealing of the ‘PLA probes’ occurs when EGFR and HER2 are in close proximity, which initiates the amplification of repeat sequences recognized by the fluorescently-labeled oligonucleotide probe. Cells were seeded on 8-well chamber slides at $1 \times 10^4$ cells/well and stimulated as described above. Cells were washed with ice-cold PBS and fixed with 4% (w/v) paraformaldehyde PBS 30 min at RT. The cells were then washed with ice-cold PBS and permeabilized with 0.2% Triton X-100 PBS 5 min at RT. After that the cells were washed with ice-cold PBS and assayed with Duolink® in situ PLA™. Anti-HER2 Ab (OP15, Calbiochem, Darmstadt, Germany) and Anti-EGFR Ab (sc-03, Santa Cruz Biotechnology) were used as primary antibodies. PLA™ probe anti-Mouse MINUS and PLA™ probe anti-Rabbit PLUS were used as two PLA™ probes. In a negative control, primary antibodies were not included. For detection, Duolink® detection kit 563 was used. ProLong Gold Antifade Reagent with DAPI (Molecular Probes, Eugene, OR, USA) was used as the mounting medium. The specimens were observed using a fluorescence microscope (BX50: Olympus, Center Valley, PA, USA).

**Immunoprecipitation**

Streptavidin-coupled magnetic beads (Invitrogen, Carlsbad, CA, USA) were incubated with biotinylated HER2 antibody (ab79205, Abcam, Cambridge, MA, USA) at 4°C 1 h. Cell lysates
(1 mg) were added for 2 h at 4°C. Beads were washed 3 times with lysis buffer (20 mM HEPES, 15 mM NaCl, 2 mM EDTA, 10 mM NaF, 0.5% NP-40 and 0.1 mM Na3VO4 supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail (Sigma-Aldrich)), resuspended in SDS sample buffer, and boiled. The supernatant was used for western blotting.

**ADCC assays**

We used RTCA (Real-time Cell Analyzer) (xCELLigence, Roche Diagnostics K. K., Tokyo, Japan) to monitor ADCC activities in real time. The system measures electrical impedance on the bottom of the tissue culture E-Plates, which contain interdigitated electrodes, as the Cell Index. NCI-N87 cells were seeded on E-plates at $5 \times 10^3$ cells/well and were precultured for 24 hours. CD16(158V)/NK-92 were added as the effector at a target ratio of 1:1. Cells were treated with pertuzumab, trastuzumab or both at the same time. The Cell Index was measured for three days after treatment. The Normalized Cell Index was calculated as follows: (Cell Index at each point) / (Cell Index at the point of pertuzumab and trastuzumab treatment).

**Measurement of microvessel density in tumor tissues**

Microvessel density (MVD) in tumor tissue was evaluated with immunohistochemical staining of CD31. Tumor samples were collected 96 hours after administration of trastuzumab, pertuzumab or both. Immunohistochemical staining was performed as described previously (25). MVD (%) was calculated from the ratio of the CD31-positive staining area to the total observation area in the viable region. Three to six fields per section (0.4856 mm² each) were randomly analyzed, excluding necrotic areas. Positive staining areas were calculated using imaging analysis software (WinROOF; Mitani Corporation, Fukui, Japan).
Results

Pertuzumab in combination with trastuzumab inhibits tumor cell growth more than each does as a single agent.

Pertuzumab has a different binding site from trastuzumab and could bind to HER2 without competing with trastuzumab (Fig. 1A). Therefore we hypothesized that pertuzumab would show significant antitumor activity in combination with trastuzumab. To examine whether pertuzumab could provide efficacy in combination with trastuzumab, we first analyzed tumor cell growth inhibition of pertuzumab in combination with trastuzumab in vitro. Pertuzumab alone inhibited the growth of NCI-N87, the HER2-positive human gastric cancer cell line (Fig. 1B open rhombus). In combination with trastuzumab, pertuzumab could potentiate its anti-proliferation activity (Fig. 1B filled rhombus). Trastuzumab alone at 20 μg/mL inhibited the growth of the cells to 55.2 ± 1.3% (mean ± SD, n = 3).

Pertuzumab has antitumor activity as a single agent in a mouse xenograft model.

We examined the efficacy of pertuzumab in NCI-N87 tumor xenograft. On day 22 (21 days after starting treatment), tumor growth inhibition rates (TGI%) were 18%, 31%, 46% and 41% at doses of 10, 20, 40 and 80 mg/kg, respectively. Pertuzumab showed significant antitumor activity at 40 and 80 mg/kg (Fig. 2A). The maximum effective dose was 40 mg/kg.

Pertuzumab in combination with trastuzumab enhances antitumor activity.

Next, we investigated the antitumor activity of pertuzumab in combination with trastuzumab in NCI-N87 tumor xenograft. The combination of pertuzumab and trastuzumab dramatically enhanced the antitumor activity (TGI% was 145%) compared with pertuzumab or trastuzumab alone (TGI% was 31% and 52% respectively.) (Fig. 2B). We also examined the efficacy
of pertuzumab and trastuzumab in two other human gastric cancer xenografts, HER2-positive 4-1ST and HER2-negative MKN-28. The antitumor activity of pertuzumab in combination with trastuzumab in 4-1ST was also significantly higher than either pertuzumab and trastuzumab as single agents (TGI% was 22%, 57% and 109% respectively) (Fig. 2C). On the other hand, no combined effect was seen in MKN-28, (TGI% was -2%, and 27% in the pertuzumab treatment group and the combination treatment group, respectively) (Fig. 2D). MKN-28 was insensitive for trastuzumab in a separate experiment (16). We examined the expression levels of HER family protein in these gastric tumor tissues of mice xenograft models by ELISA and IHC (Table1). All cell lines expressed EGFR and HER3 by ELISA. All cell lines were also identified as EGFR-positive cells by IHC according to clinical diagnosis. We also stained HER3 immunohistochemically; however HER3 was only slightly detected on the plasma membrane in all examined cell lines (data not shown). The expression level of HER2, but not EGFR or HER3, was considered to be a key factor of the efficacy.

**Pertuzumab in combination with trastuzumab strongly reduces EGFR-HER2 signaling.**

To examine the mechanism of combination efficacy of pertuzumab and trastuzumab, we next analyzed HER2 signal transduction. We focused on the EGFR-HER2 signaling because HER3 expression level was very low in our gastric cancer models. First, we examined the phosphorylation of HER2 under serum starvation or under EGFR stimulation to evaluate ligand-independent or ligand-dependent signals, respectively. In the absence of EGF, phosphorylation of HER2 and the down-stream factor Akt and ERK1/2 were strongly reduced by combination of pertuzumab and trastuzumab. In the presence of EGF, pHER2 was also more attenuated by the combination than by either antibody alone. Phosphorylation of EGFR, Akt and ERK1/2 were increased by EGF stimulation and the combination of pertuzumab and trastuzumab strongly reduced these phosphorylations (Fig. 3A). Next, we examined whether pertuzumab in combination with
trastuzumab down-regulated the phosphorylation of EGFR and HER2 by inhibiting their heterodimerization. We evaluated the dimerization level of EGFR-HER2 induced by EGF in the presence of pertuzumab, trastuzumab or both by in situ PLA. EGFR-HER2 heterodimers were slightly decreased by pertuzumab. However pertuzumab and trastuzumab in combination remarkably reduced the EGFR-HER2 heterodimers on the plasma membrane (Fig. 3B). The inhibition of EGFR-HER2 heterodimerization by the combination was also detected by immunoprecipitation (Fig. 3B). We also examined the cell proliferation and apoptosis activity under EGF-stimulation. The cells proliferated EGF-dependently and both pertuzumab and trastuzumab inhibited this EGF-dependent cell growth. The combination of pertuzumab and trastuzumab significantly enhanced the cell growth inhibition (Fig. 3C). Additionally, the combination of pertuzumab and trastuzumab significantly enhanced apoptosis activity (Fig. 3D). These results suggest that pertuzumab in combination with trastuzumab inhibits EGFR-HER2 dimerization and its down-stream signaling, although neither pertuzumab nor trastuzumab does it sufficiently alone.

**Pertuzumab in combination with trastuzumab enhances the ADCC activity.**

Both pertuzumab and trastuzumab are reported to have ADCC activity. We investigated the ADCC activity induced by pertuzumab and trastuzumab as another mechanism of the combined effect by using RTCA. We observed the Cell Index of NCI-N87 for three days after the addition of pertuzumab, trastuzumab and CD16(158V)/NK-92 cells. The Cell Index was normalized at the point of pertuzumab and trastuzumab addition. Normalized Cell Index after NK cells were added was significantly reduced by the addition of pertuzumab and trastuzumab compared with the addition of each single agent alone. No reduction of the Normalized Cell Index was seen after the addition of pertuzumab and trastuzumab without NK cells (Fig. 4A). These results suggest that combination therapy of pertuzumab and trastuzumab has more potent antitumor activity through the enhancement
of ADCC activity.

**Pertuzumab in combination with trastuzumab shows anti-angiogenic activity.**

There are some reports on the anti-angiogenic activity of trastuzumab (12, 26). As another combination mechanism, we examined whether pertuzumab enhances the anti-angiogenic activity of trastuzumab. In our study, neither pertuzumab nor trastuzumab could significantly decrease MVD. However, a significant reduction in MVD was seen if the tumor tissue was treated with pertuzumab in combination with trastuzumab (Fig. 4B, C). We also quantified the VEGF protein levels in those tumor tissues. Trastuzumab reduced VEGF levels in tumor tissues significantly. Pertuzumab also tended to reduce the VEGF levels, although it was not significant. When mice were treated with pertuzumab in combination with trastuzumab, the VEGF levels decreased more than they did with pertuzumab alone or trastuzumab alone (Fig. 4D). These results show that pertuzumab in combination with trastuzumab has an effect not only by inhibition of EGFR-HER2 signaling and enhancement of ADCC activity, but also by inhibition of tumor angiogenesis.

**Pertuzumab in combination with trastuzumab strongly reduces HER2-HER3 signaling.**

Finally, we also examined the signal transduction of HER2-HER3 in NCI-N87. Although NCI-N87 showed low expression levels of HER3 by ELISA and IHC assay, it has been reported that HER3 triggered strong signal transduction by dimer formation with HER2. Therefore it is possible that a few HER3 proteins in NCI-N87 still play a role as mediators of the growth signal. We examined the phosphorylation levels of HER2, HER3, Akt and ERK1/2 under HRGα stimulation after treatment with pertuzumab and trastuzumab. HRGα increased pHER3 and pAkt, and the combination of trastuzumab and pertuzumab reduced them as well as pHER2 (Fig. 5A). We also examined the contribution of HRGα to cell proliferation. The cells proliferated HRGα-dependently.
and both pertuzumab and trastuzumab inhibited the HRGα-dependent cell growth. The combination of pertuzumab and trastuzumab significantly enhanced the cell growth inhibition. (Fig. 5B). The apoptosis activity of pertuzumab and trastuzumab in combination also significantly increased compared to that of pertuzumab or trastuzumab alone (Fig. 5C). Therefore, HER3 would also play a role in showing the efficacy of the combination even though the HER3 protein expression is low.

**Discussion**

It is reported that pertuzumab and trastuzumab bind to different domains of HER2. A previous study showed that pertuzumab and trastuzumab were oriented at different angles with respect to HER2 after binding to HER2, by using a 3D-structure model of the Fab region of pertuzumab and trastuzumab and p185HER2 (27). As shown in Fig.1A, binding of whole IgG to HER2 ECD protein, pertuzumab would not interfere with the binding of trastuzumab on HER2, even though both antibodies are displayed in their whole IgG conformation. This result indicates that pertuzumab and trastuzumab could have combination effects on antitumor activity.

We examined the efficacy of pertuzumab and trastuzumab in vivo. The reason for in vivo study is that the in vivo models indicate the efficacy of this antitumor activity more accurately, because trastuzumab or pertuzumab have a mechanism through ADCC activity. We used NCI-N87 and 4-1ST as HER2-positive models. NCI-N87 and 4-1ST were determined as HER2 2+ and 3+ respectively by IHC (HercepTest®) and FISH (Pathvysion®) in our previous study (16). MKN-28 was determined to be a HER2-negative cell line (16), so we used it as a negative control model. At first, we examined the dose dependent activity of pertuzumab on tumor growth in the xenograft model to determine the combination dose. In the NCI-N87 model, 3-week treatment with pertuzumab showed a significant antitumor activity at 40 and 80 mg/kg. The maximum effective dose of pertuzumab was determined as 40 mg/kg, because the efficacy of 40 mg/kg of pertuzumab
was the same as that of 80 mg/kg (TGI% was 46% and 41%, respectively). Next, we examined the antitumor activity of pertuzumab in combination with trastuzumab. In the NCI-N87 HER2-positive xenograft model, remarkable tumor regression was observed. The antitumor activity of the combination group was more potent than that of the maximum effective dose of both antibodies as single agents (the TGI% of our current combinational study was 145% and the maximum TGI% of pertuzumab and trastuzumab were 46% in our current study and 89% in a separate experiment (16) respectively.). Another HER2-positive model 4-1ST also showed a potent effect of the pertuzumab and trastuzumab combination. Meanwhile, in MKN-28, a HER2-negative tumor model expressing HER1 or HER3, an effect of the combination was not shown. Therefore, the usefulness of this combination would only be observed in HER2-positive tumors. We could not examine the safety profile except for body weight in the present study because of species difference. No significant loss of body weight was observed. In the present study, we used subcutaneous xenograft models. However, orthotopic models might reflect actual tumors better and it would be interesting to know if the same efficacy would be shown in the orthotopic models.

In order to explain the strongly enhanced antitumor efficacy of pertuzumab and trastuzumab combination treatment, we hypothesized two mechanisms, direct action on tumor cells and a specific mechanism caused by the tumor environment. Actually, pertuzumab showed more potent cell-growth inhibition and apoptosis activity in combination with trastuzumab than either pertuzumab or trastuzumab monotherapy showed in vitro, and the efficacy of the combination in mouse xenograft models was more remarkable than in vitro. As the direct action on tumor cells, we examined whether pertuzumab in combination with trastuzumab enhanced the inhibition of HER2 signal transduction. In the analysis of inhibition of the signaling pathways through HER1 and HER2, down-regulation of pHER1, pHER2, pAkt and pERK1/2 was observed in the combination group compared to the single agents. We also showed that pertuzumab in combination with trastuzumab
reduced EGFR-HER2 heterodimers with Duolink® in situ PLA™, which was reported to detect heterodimers (28, 29). Others reported that pertuzumab could enhance the endocytic down-regulation of EGFR by counteracting the EGFR-HER2 heterodimerization (30). In our HER2-positive gastric cancer model, only a little decrease in EGFR-HER2 heterodimers was detected by pertuzumab alone. Anyway, we consider that one mechanism of the effect of the combination of pertuzumab and trastuzumab is the reduction of EGFR-HER2 heterodimers and their signal transduction. In NCI-N87, HER3 expression was low and we considered that EGFR and HER2 were mainly functional. However, HER2-HER3 heterodimer is considered the most active HER signaling dimer (20, 31) and there are some reports suggesting that pertuzumab show antitumor activity through HER2-HER3 signal inhibition in non-small cell lung cancer and breast cancer (32, 33). In fact, HRGα induced the phosphorylation of HER3 and Akt in NCI-N87, and pertuzumab in combination with trastuzumab reduced them strongly; therefore, it is possible that low expression of HER3 was functional in this model and that pertuzumab in combination with trastuzumab also showed antitumor activity by inhibiting HER2-HER3 signaling as well as EGFR-HER2 signaling. In our study, total HER2 level of the combination was decreased. It is reported that anti-HER2 antibodies like trastuzumab led to degradation through the c-Cbl-regulated proteolytic pathway (34, 35). We could not examine the signal inhibition in 4-1ST model because it was an in vivo-maintained cell line. In this study, it was suggested that both the EGFR-HER2 and the HER2-HER3 signaling were involved in the tumor growth inhibition of the combination of pertuzumab and trastuzumab. Comparing the degree of cell growth, the HER3-dependent cell growth was inhibited more strongly than the EGFR-dependent cell growth. However, it should not be concluded that HER3 is more important than EGFR for the clinical therapy of HER2-positive gastric cancer, and the contributing rate of EGFR and HER3 to HER-signaling might be different between types of cancer (e.g. gastric cancer and breast cancer). In a HER2-positive breast cancer cell line, KPL-4, where pertuzumab in...
combination with trastuzumab showed strongly enhanced antitumor activity (19), EGF did not stimulate phosphorylation of EGFR, but HRGα did phosphorylate HER3 (data not shown). It is necessary to examine exhaustively whether EGFR or HER3 is valuable as a secondary biomarker in clinical research from many points of view, such as the expression level of ligands or HER family proteins.

As an in vivo-specific mechanism caused by the tumor environment, we considered the potentiation of ADCC activity and anti-angiogenic activity. ADCC activity is one of the key mechanisms of the antitumor activity of trastuzumab (36). Moreover, recent reports showed that pertuzumab also had ADCC activity (19, 37). Anti-angiogenic activity is also shown as the mechanism of antitumor activity of trastuzumab (12). We examined the potentiation of ADCC, because both trastuzumab and pertuzumab showed antitumor activity through ADCC activity. ADCC was measured using RTCA, reported as a new method by which it is possible to observe cell adhesion areas according to time (38). The degree of ADCC measured with RTCA was equivalent to that of measured with ^51^Cr-release assay. We tried to use CD16-transfected NK-92 as the effector cell. The ADCC of trastuzumab was reported using the NK-92 cell, a human NK cell line (39). However, our NK-92 cell did not show ADCC activity to the target cell NCI-N87. Using this CD16-transfected cell line, the ADCC activity of pertuzumab or trastuzumab on NCI-N87 was observed, and that of the combination group was significantly more potent. In a breast cancer model, another report showed that pertuzumab in combination with trastuzumab did not enhance ADCC activity of either antibody alone (19). This difference may be due to the different materials and methods. Secondly, we tried to clarify the anti-angiogenic activity of these antibodies. In the combination group, a significant decrease in MVD and the VEGF protein in tumor tissue was observed. VEGF has been reported as a key angiogenic factor in tumors (40). Therefore, it was considered that pertuzumab in combination with trastuzumab decreased MVD by reduction of VEGF
as a result of HER2 signal inhibition. Based on these results, the potent activity of the combination was due to enhancement of the ADCC activity and anti-angiogenic activity in addition to direct cell growth inhibition through HER2 signal inhibition.

In conclusion, compared to single agent treatment, pertuzumab in combination with trastuzumab showed significantly stronger antitumor activity in HER2-positive human gastric cancer mouse xenograft models through the enhancement of direct cell growth inhibition, ADCC activity, and anti-angiogenesis activity. The combination therapy of pertuzumab and trastuzumab is worth examining as a new therapy for HER2 positive gastric cancer.

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Figure legends

Fig. 1
A, 3D structure of the trastuzumab/pertuzumab/HER2 ternary complex. B, anti-proliferative activity of pertuzumab in combination with trastuzumab. Cells were treated with pertuzumab (0.1–100 µg/mL), with or without trastuzumab (20 µg/mL), for three days in the culture medium. Open rhombus: without trastuzumab; and filled rhombus: with trastuzumab. Data points are mean ± SD (n = 3). The 0% point was defined as the point of 0 µg/mL of pertuzumab of both open and filled rhombuses. Statistically significant differences are shown as *: P < 0.05 by the two-way analysis of variance.

Fig. 2
Antitumor activity of pertuzumab as a single agent and in combination with trastuzumab. A, mice bearing NCI-N87 tumors were randomly divided into 5 groups (n = 6/group) and treated with HuIgG or pertuzumab once a week for 3 weeks. Open square: HuIgG; filled triangle: Pertuzumab 10 mg/kg; filled square: Pertuzumab 20 mg/kg; filled circle: Pertuzumab 40 mg/kg and asterisk: Pertuzumab 80 mg/kg. B, C, D, tumor-bearing mice were randomly divided into 4 groups (B,C) or 2 groups (D) (n = 6/group) and treated with HuIgG, pertuzumab, trastuzumab or both pertuzumab and trastuzumab once a week for 3 weeks. B: NCI-N87 tumors (HER2 positive); C: 4-1ST tumors (HER2 positive) and D: MKN-28 tumors (HER2-negative). Open square: HuIgG; filled square: Pertuzumab at 20 mg/kg (B) or 40 mg/kg (C, D); open rhombus: Trastuzumab at 20 mg/kg and filled rhombus: Combination of pertuzumab and trastuzumab at 20 mg/kg and 20 mg/kg (B) or 40 mg/kg and 20 mg/kg (C, D respectively). Data points are mean ± SD of the tumor volume (mm³). Statistically significant differences are shown as *: P < 0.05 vs. the control group, **: P < 0.05 vs. the single agent.
groups by the Wilcoxon test.

Fig. 3
Effect of pertuzumab in combination with trastuzumab on the signal transduction of HER family proteins. A, NCI-N87 cells were treated with pertuzumab (40 µg/mL), trastuzumab (40 µg/mL) or both for 3.5 h followed by EGF (100 ng/mL) stimulation for 5 min. The pHER2, HER2, pEGFR, EGFR, pAkt, Akt, pERK1/2, ERK1/2 and actin were detected by western blotting. B, EGFR-HER2 heterodimer was detected by Duolink® in situ PLA™ and immunoprecipitation. Red: EGFR/HER2 heterodimer; Blue: Nuclear. EGFR/HER2 heterodimers were slightly decreased by pertuzumab and strongly decreased by the combination of pertuzumab and trastuzumab (white arrows). Objective × 20. C, the cell growth inhibition in the presence of EGF was examined. NCI-N87 cells were treated with pertuzumab (20 µg/mL), trastuzumab (20 µg/mL) or both 30 min before the addition of EGF (100 ng/mL). Control was untreated cells. The cell proliferation was examined three days after the treatment. Data points are mean ± SD (n = 3). Statistically significant differences are shown as *: P < 0.05 by the t-test. D, the caspase 3/7 activity of pertuzumab and trastuzumab combination was measured. NCI-N87 cells were treated with pertuzumab (20 µg/mL), trastuzumab (20 µg/mL) or both 30 min before the addition of EGF (100 ng/mL). Control was untreated cells. The caspase 3/7 activity was measured 24 h after treatment. Data points are mean ± SD (n = 5). Statistically significant differences are shown as *: P < 0.05 by the t-test.

Fig. 4
Effect of pertuzumab in combination with trastuzumab on ADCC activity and anti-angiogenic activity. A, ADCC activity was examined with RTCA. NCI-N87 cells were pre-cultured in the culture medium for 24 h and treated with pertuzumab (10 ng/mL), trastuzumab (2 ng/mL) or both.
NK cells were added as the effector at target ratios of 1:1 simultaneously. Cell Indices were measured every 5 min for the first 4 h, every 15 min for the next 44 h, and every hour thereafter. Cell Indices were normalized at the treatment point. Red: Control (non-treatment) group; Green: Pertuzumab group; Blue: Trastuzumab group; Magenta: Combination group. Data points are mean ± SD (n = 3). Statistically significant differences are shown as *: P < 0.05 vs. the control group, **: P < 0.05 vs. the single agent groups by the t-test. B, tumor microvessels was stained immunohistochemically with anti-CD31 antibody at 96 h after the treatment with HuIgG (40 mg/kg) (as a control), pertuzumab (20 mg/kg), trastuzumab (20 mg/kg) or both (n = 5-6/group). C, the MVD of NCI-N87 was determined as described above. D, the VEGF protein levels of each treatment groups at 96 h were quantified by ELISA (n = 5-6/group). Data points are mean ± SD of tumor volume (mm³). Statistically significant differences are shown as *: P < 0.05 vs. control group by the Wilcoxon test.

Fig. 5
Effect of pertuzumab in combination with trastuzumab on the signal transduction of HER2 and HER3. A, NCI-N87 cells were treated with pertuzumab (20 µg/mL), trastuzumab (20 µg/mL) or both for 3.5 h followed by HRGα (100 ng/mL) stimulation for 5 min. The phosphorylation levels of HER2 and HER3 in the cell lysates were detected by western blotting. B, the cell growth inhibition in the presence of HRGα was examined. NCI-N87 cells were treated with pertuzumab (20 µg/mL), trastuzumab (20 µg/mL) or both 30 min before the addition of HRGα (100 ng/mL). Control was untreated cells. The cell proliferation was examined three days after treatment. Data points are mean ± SD (n = 3). Statistically significant differences are shown as *: P < 0.05 by the t-test. C, the caspase 3/7 activity of pertuzumab and trastuzumab combination was measured. NCI-N87 cells were treated with pertuzumab (20 µg/mL), trastuzumab (20 µg/mL) or both 30 min before the addition of...
HRGα (100 ng/mL). Control was untreated cells. The caspase 3/7 activity was measured 24 h after treatment. Data points are mean + SD (n = 5). Statistically significant differences are shown as *: P < 0.05 by the t-test.
References


activity of trastuzumab in combination with chemotherapy in human gastric cancer xenograft models.


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Table 1. HER family protein expression levels in human gastric tumor tissues.

<table>
<thead>
<tr>
<th>Xenograft</th>
<th>ELISA (ng/mg protein)†</th>
<th>IHC‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EGFR</td>
<td>HER2</td>
</tr>
<tr>
<td>NCI-N87</td>
<td>9.0 ± 1.2</td>
<td>180 ± 76</td>
</tr>
<tr>
<td>4-1ST</td>
<td>0.94 ± 0.10</td>
<td>330 ± 72</td>
</tr>
<tr>
<td>MKN-28</td>
<td>3.5 ± 0.37</td>
<td>1.7 ± 0.39</td>
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†Means ± SD (n = 4)
‡IHC was according to clinical diagnosis (EGFR pharmDx kit™ and HercepTest®)
Fig. 3
Fig. 5
Clinical Cancer Research

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