Cancer Therapy: Preclinical

Pertuzumab in Combination with Trastuzumab Shows Significantly Enhanced Antitumor Activity in HER2-Positive Human Gastric Cancer Xenograft Models

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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 immunohistochemistry. Phosphorylated proteins and heterodimers were detected by Western blotting and in situ proximity ligation assay (PLA), respectively. Apoptosis activity was examined by caspase 3/7 activity. Antibody-dependent cellular cytotoxicity (ADCC) activity was detected by xCELLigence. Microvessel density was examined by CD31 staining.

Results: Pertuzumab in combination with trastuzumab showed significant antitumor activity compared with each monotherapy in NCI-N87, an 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, an 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 downstream 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 microvessel density.

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

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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 epidermal growth factor receptor (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 overexpression 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...
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 shown in a clinical phase III 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 overexpressing gastric cancer in vivo and in vitro. These results were a scientific rationale for developing pertuzumab for HER2-positive gastric cancer.

when trastuzumab was added to chemotherapy in HER2-overexpressing gastric cancer patients (8) and the Food and Drug Administration 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 cytolysis (ADCC) activity (9, 10), blocking formation of p95HER2, a constitutively active form of HER2 (11), and suppressing tumor angiogenesis (12). Trastuzumab has shown 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 benefits 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; ref. 17] or the Fab (trastuzumab)/HER2 complex (PDB ID: 1n8z; ref. 21). We used the three-dimensional (3D) structure of IgG1 monovalent antibody (PDB ID: 1IGY; ref. 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). 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 2 models.

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

CD16-negative NK-92 tumor line was purchased from the American Type Culture Collection (ATCC). The cDNA encoding FcγRIIIa was amplified by PCR from the fetal spleen cDNA library (Clontech) 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 FcγRIIa was subcloned into pGEM T-Easy vector (Promega). Because cDNA sequencing revealed that the obtained FcγRIIa were all 158F allele, the FcγRIIa-158V allele sequence was generated by site-directed mutagenesis (QuickChange; Stratagene). The cDNA encoding FcγRIIIa-158V allele 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 FcγRIIIa-158V allele, pCXND3/CD16(V) was transfected by the electroporation method using GENE-PULSER II (Bio-Rad) under the conditions of 1.5 kV and 25 μF. Flow cytometry–based screening was conducted using αCD16-FITC (Beckman Coulter), and 51Cr-release assay was conducted to confirm the ADCC activity in pCXND3/CD16(V)-transfected NK-92 cells. The resulting FcγRIIa-158V-positive NK-92 cells were cloned and designated as NK-92(R1). NK-92(R1) cells were then infected with Sendai virus to make stable transfectants containing high levels of human FcγRIIIa-158V and were designated as NK-92(R1)-158V.
exerting ADCC activity were designated CD16(158V)/NK-92 cells.

**Test agents**

Trastuzumab and pertuzumab were provided by F. Hoffmann-La Roche 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 (HulG) was purchased from MP Biomedicals, Inc. and was reconstituted with water and diluted with saline.

**Animals**

Male, 5-week-old BALB-<sup>nu/nu</sup> mice (CAnN.Cg-Foxn1<sup>nu/nu</sup>/CrlCrlj nu/nu) were obtained from Charles River 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 (12–12 hours). 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. NCI-N87 and MKN-28 were maintained in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS at 37°C under 5% CO<sub>2</sub>. 4-1ST cells were purchased from the Central Institute for Experimental Animals and maintained in BALB-<sup>nu/nu</sup> mice by subcutaneous inoculation of pieces of tumor. CD16(158V)/NK-92 was maintained in α-minimal essential medium (MEMα; Wako) supplemented with 12.5% FBS, 12.5% horse serum, 0.02 mmol/L folic acid, 0.1 mmol/L 2-mercaptoethanol, 0.2 mmol/L insulin, and 0.5 mg/mL G418, and 20 mg/mL recombinant human interleukin (IL) 2 at 37°C under 5% CO<sub>2</sub>.

**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 subcutaneously into the right flank with either 5 × 10<sup>6</sup> cells per mouse of human gastric cancer cell lines MKN-28 or NCI-N87, or an 8-mm<sup>3</sup> 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 cm<sup>3</sup>. 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 3 weeks.

**In vitro antiproliferation assays**

NCI-N87 cells were seeded on 96-well plates at 5 × 10<sup>3</sup> cells per well and precultured for 24 hours. The cells were treated with pertuzumab, trastuzumab, or both and cultured for 3 days. The cells were fixed with 10% formalin neutral buffer solution. Crystal violet staining and extraction were conducted as described previously (16). Their absorbance was measured at 595 nm. The cells after 24-hour preculture were also detected by crystal violet staining as the absorbance of the precultured well. The percentage of cell proliferation inhibition (% inhibition) was calculated as follows: % Inhibition = [1 – (absorbance of treatment well – absorbance of precultured well)/(absorbance of nontreatment well – absorbance of precultured well)] × 100. In case of ligand-dependent proliferation, cells were precultured 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 precultured well)/absorbance of unstimulated well × 100.

**Apoptosis assay**

NCI-N87 cells were seeded on 96-well plates at 1 × 10<sup>4</sup> cells per well and cultured in the same way as the ligand-dependent antiproliferation 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 to 24 hours and treated with pertuzumab, trastuzumab or both. After 3.5 hours of incubation, cells were exposed to EGF or heregulin (HRG) α for 5 minutes.

**Western blotting**

Cells were washed with ice-cold PBS and lysed with Cell Lysis Buffer (Cell Signaling Technology) with 10 mmol/L NaF, 1 μg/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride. After centrifugation (4°C, 14,000 × g, 5 minutes), the resultant supernatant was used for the assays. Protein concentration of the supernatant was quantified using the DC Protein Assay Kit (Bio-Rad). Cell lysates (20 μg protein per lane) were electrophoresed on SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked in SuperBlock T20 (TBS) Blocking Buffer (Thermo Scientific) and probed with
each antibody against HER2 (sc-31153), HER3 (sc-285; Santa Cruz Biotechnology), 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 Technology), and actin (A2228; Sigma-Aldrich) 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).

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 minutes). The resultant supernatant was used for the assays. We used Duo Set IC (R&D Systems) 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 (Bio-Rad).

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). 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 × 10⁴ cells per well and stimulated as described above. Cells were washed with ice-cold PBS and fixed with 4% (w/v) paraformaldehyde PBS for 30 minutes at room temperature. The cells were then washed with ice-cold PBS and permeabilized with 0.2% Triton X-100 PBS for 5 minutes at room temperature. After that, the cells were washed with ice-cold PBS and assayed with Duolink in situ PLA. Anti-HER2 Ab (OP15; Calbiochem) 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 2 PLA probes. In a negative control, primary antibodies were not included. For detection, Duolink detection kit 563 was used. Anti-HER2 Ab (OP15; Calbiochem) were used as secondary 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. The specimens were observed using a fluorescence microscope (BX50; Olympus).

Immunoprecipitation
Streptavidin-coupled magnetic beads (Invitrogen) were incubated with biotinylated HER2 antibody (ab79205; Abcam) at 4°C for 1 hour. Cell lysates (1 mg) were added for 2 hours at 4°C. Beads were washed 3 times with lysis buffer (20 mmol/L HEPES, 15 mmol/L NaCl, 2 mmol/L EDTA, 10 mmol/L NaF, 0.5% NP-40, and 0.1 mmol/L 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.) 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 × 10³ cells per 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 3 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 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 conducted as described previously (25). Microvessel density (%) was calculated from the ratio of the CD31-positive staining area to the total observation area in the viable region. Three to 6 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).

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 antiproliferation activity (Fig. 1B, filled rhombus). Pertuzumab 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% = 145%) compared with pertuzumab or trastuzumab alone (TGI% = 31% and 52%, respectively; Fig. 2B). We also examined the efficacy of pertuzumab and trastuzumab in 2 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 or trastuzumab as single agents (TGI% = 22%, 57%, and 109%, respectively; Fig. 2C). On the other hand, no combined effect was seen in MKN-28, (TGI% = 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 (Table 1). 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
Pertuzumab and Trastuzumab Combination in Gastric Cancer

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>IHCb</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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</tbody>
</table>

aData are presented as mean ± standard deviation (SD) (n = 4).

bIHC was according to clinical diagnosis (EGFR pharmDx kit and HercepTest).

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 EGF stimulation to evaluate ligand-independent or ligand-dependent signals, respectively. In the absence of EGF, phosphorylation of HER2 and the downstream factor Akt and extracellular signal–regulated kinase (ERK) 1/2 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 downregulated 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 significantly reduced 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). In addition, 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 downstream 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 3 days after the addition of pertuzumab, trastuzumab, and CD16(150V)/NK-92 cells. The cell index was normalized at the point of pertuzumab and trastuzumab addition. Normalized cell index after natural killer 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 natural killer 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 antiangiogenic activity

There are some reports on the antiangiogenic activity of trastuzumab (12, 26). As another combination mechanism, we examined whether pertuzumab enhances the antiangiogenic activity of trastuzumab. In our study, neither pertuzumab nor trastuzumab could significantly decrease microvessel density. However, a significant reduction in microvessel density was seen when the tumor tissue was treated with pertuzumab in combination with trastuzumab (Fig. 4B and 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 immunohistochemical 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 pertuzumab and trastuzumab 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 with 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 Figure 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 an 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% = 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, respectively; ref 16). Another HER2-positive model 4-1ST also showed a potent effect of the pertuzumab and trastuzumab combination. Meanwhile, in MKN-28, an 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 whether the same efficacy would be shown in the orthotopic models.

To explain the strongly enhanced antitumor efficacy of pertuzumab and trastuzumab combination treatment, we hypothesized 2 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, downregulation of pHER1, pHER2, pAkt, and pERK1/2 was observed in the combination group compared with 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 downregulation 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. Anyways, 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.
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 showed antitumor activity through HER2-HER3 signal inhibition in non-small-cell lung cancer and breast cancer (32, 33). In fact, HGRαc 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 an HER2-positive breast cancer cell line, KPL-4, where pertuzumab in combination with trastuzumab showed strongly enhanced antitumor activity (19), EGFR did not stimulate phosphorylation of EGFR but HGRαc 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 antiangiogenic 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). Antiangiogenic 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 51Cr-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 natural killer 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. Second, we tried to clarify the antiangiogenic activity of these antibodies. In the combination group, a significant decrease in microvessel density 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 microvessel density by reduction of VEGF as a result of HER2 signal inhibition. On the basis of these results, the potent activity of the combination was due to enhancement of the ADCC activity and antiangiogenic activity in addition to direct cell growth inhibition through HER2 signal inhibition.

In conclusion, compared with 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 antiangiogenesis activity. The combination therapy of pertuzumab and trastuzumab is worth examining as a new therapy for HER2-positive gastric cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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