Strongly Enhanced Antitumor Activity of Trastuzumab and Pertuzumab Combination Treatment on HER2-Positive Human Xenograft Tumor Models

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Abstract
The human epidermal growth factor receptor (HER) family plays an important role in cell survival and proliferation, and is implicated in oncogenesis. Overexpression of HER2 is associated with aggressive disease and poor prognosis. Trastuzumab is a humanized monoclonal antibody targeting HER2 and has proven survival benefit for women with HER2-positive early and metastatic breast cancer. Pertuzumab, another monoclonal antibody, is a HER2 dimerization inhibitor that binds to a different epitope on HER2 than trastuzumab and inhibits HER2 dimer formation with other HER family members such as HER3 and HER1. We investigated the antitumor activity of these agents alone and in combination in HER2-positive breast and non–small cell lung cancer xenografts. Our data show that the combination of trastuzumab and pertuzumab has a strongly enhanced antitumor effect and induces tumor regression in both xenograft models, something that cannot be achieved by either monotherapy. The enhanced efficacy of the combination was also observed after tumor progression during trastuzumab monotherapy. Near-IR fluorescence imaging experiments confirm that pertuzumab binding to tumors is not impaired by trastuzumab pretreatment. Furthermore, we show by in vitro assay that both trastuzumab and pertuzumab potently activate antibody-dependent cellular cytotoxicity. However, our data suggest that the strongly enhanced antitumor activity is mainly due to the differing but complementary mechanisms of action of trastuzumab and pertuzumab, namely inhibition of HER2 dimerization and prevention of p95HER2 formation. [Cancer Res 2009;69(24):OF1–7]

Introduction
The human epidermal growth factor receptor (HER) family comprises HER1, HER2, HER3, and HER4. These proteins are capable of forming heterodimers and homodimers, which activate signal transduction pathways that regulate many cellular processes, including growth, proliferation, and survival (1). As such, the HER proteins have been implicated in oncogenesis (2); in particular, tumors that overexpress the HER2 protein or have amplification of the HER2 gene are associated with aggressive disease and poor prognosis (3–6). HER2 overexpression has been identified in numerous tumor types, including 20% to 25% of breast cancers (7–10), 4% to 6% of non–small cell lung cancers (NSCLC; refs. 3, 11), and 20% to 24% of gastric cancers (5, 12).

Trastuzumab (Herceptin) is a humanized monoclonal antibody that binds to the extracellular domain of HER2, inhibiting HER2-mediated proliferation by activating antibody-dependent cellular cytotoxicity (ADCC; refs. 13, 14), preventing formation of p95HER2 (a truncated and constitutively active form of HER2; ref. 15), blocking ligand-independent HER2 signaling (16), and inhibiting HER2-mediated angiogenesis (17). Trastuzumab has a proven survival benefit for women with HER2-positive breast cancer both in advanced metastatic disease and in early disease when administered for 1 year as adjuvant therapy (18–22). A number of phase II clinical trials have also been carried out in patients with HER2-positive NSCLC with some success, although the number of patients in these trials has been small (23–25).

Pertuzumab, a humanized monoclonal antibody, is the first in a new class of HER2 dimerization inhibitors that block HER2 dimerization with other HER family members, thus inhibiting the downstream signaling processes that are associated with tumor growth and progression (26–28). Trastuzumab and pertuzumab bind to distinct epitopes on the HER2 extracellular domain, and it has been hypothesized that a combination of the two agents might provide a more effective inhibition of tumor growth than either agent alone (27, 29). Initial in vitro data suggest that this hypothesis is correct, although in vivo data have been lacking (30). The aim of this study was to investigate the antitumor effect of the combination of trastuzumab and pertuzumab in HER2-overexpressing NSCLC (Calu-3) and breast cancer (KPL-4) xenograft models.

Materials and Methods

Cell lines and culture. Calu-3 cells were obtained from Chugai Pharmaceuticals Co., Ltd. KPL-4 cells were provided by Professor J. Kurebayashi (Kawasaki Medical School, Kurashiki, Japan). The cells were cultured as previously described (31).

Tumor xenografts. To initiate tumor xenografts, 5 × 10⁶ Calu-3 cells were injected s.c. into BALB/c nu/nu female mice and 3 × 10⁶ KPL-4 cells were implanted orthotopically into the right penultimate inguinal mamma of severe combined immunodeficient (SCID) beige mice. Tumors were allowed to establish growth (21 d for Calu-3 and 20 d for KPL-4) after implantation before initiation of treatment.

Treatment of animals. Calu-3 or KPL-4 tumors (100 mm³) were treated with trastuzumab (30 mg/kg loading dose, then 15 mg/kg weekly), pertuzumab (30 mg/kg loading dose, then 15 mg/kg weekly), or both, administered i.p. for the duration of the study. Tumor volumes and body weights were measured twice weekly. For the acute study, advanced Calu-3 tumors of ~400 mm³ were treated once with trastuzumab and/or pertuzumab at a dose of 30 mg/kg. Samples were harvested 7 d later for immunohistochemistry (IHC) and Western blot analysis.
Western blot analysis. For Western blot analysis, protein lysates (20 μg protein/lane) were electrophoresed on TRIS NuPage Ready-Gel (1.0 mm; Invitrogen) for 1 h at 60 V, transferred to nitrocellulose membrane (Hybond enhanced chemiluminescence, Amersham) and incubated with a polyclonal rabbit anti-human c-erbB2 antibody (Dako) at 1 μg/mL. HER2 protein was detected using a labeled secondary antibody diluted at 1:5,000 (goat anti-rabbit POD labeled; Bio-Rad Laboratories GmbH) and Lumi-Light Western Blotting Substrate (Roche Diagnostics GmbH). The Odyssey detection system (LI-COR Bioscience) was used for quantification.

For control of siRNA experiments (Fig. 5), 60 μg protein/lane was electrophoresed and blotted onto a polyvinylidene difluoride membrane (Invitrogen). Polyclonal rabbit anti-human ErbB-3 antibody (C-17, sc-285, Santa Cruz) at 1 μg/mL and polyclonal rabbit anti-human epidermal growth factor receptor (EGFR) antibody (06-847, Upstate) at 1 μg/mL were used. Anti-rabbit IgG-HRP–linked antibody (7074, Cell Signaling) was used as secondary antibody at 1:2,000 dilution. Luminescence was detected with Luminescence was detected with Lumi-Light Western Blotting Substrate on the LumiImager Instrument (Roche Diagnostics GmbH).

Assessment of metastases by quantification of human DNA. Genomic DNA was isolated using the High Pure PCR Template Preparation kit (Roche Diagnostics GmbH) and quantified using the PicoGreen Quantification kit (Molecular Probes). Primers for human Alu repeats were synthesized by TIB MOLBIOL GmbH and quantitative PCR (qPCR) was performed using the LightCycler System (Roche Diagnostics GmbH), as previously described (32).

Near IR fluorescence imaging in vivo. Trastuzumab and pertuzumab were labeled with the fluorescent dye Cy5 (General Electric) according to the manufacturer’s instructions. When KPL-4 tumors reached a volume of 200 mm³, mice were injected i.v. with a single 50-μg dose of either trastuzumab-Cy5 or pertuzumab-Cy5. Near IR fluorescence (NIRF) measurements were performed using the Bioscan Imaging System (Siemens Medizintechnik) equipped with an inhalation mask for anesthesia. Fluorescence acquisition time was 4 s for all experiments. For the evaluation of binding competition, mice were injected i.v. with a single dose of trastuzumab (30 mg/kg). After 48 h, Cy5-labeled trastuzumab or pertuzumab (50 μg/mouse) was injected i.v. NIRF signals were measured as described above.

Figure 1. Antitumor activity of trastuzumab and/or pertuzumab in NSCLC (Calu-3) and breast cancer (KPL-4) xenograft tumor models. Mice with (A) Calu-3 and (B) KPL-4 xenograft tumors were treated for the duration of the study with vehicle (violet circle), pertuzumab (pink diamond), trastuzumab (orange triangle), trastuzumab + pertuzumab (green circle). Points, mean tumor volume (mm³) (n = 10 mice/group); bars, SEM. C, investigation of KPL-4 metastatic tumor spread to the lungs of mice. Genomic DNA from lung samples of mice with KPL-4 tumors that had been treated with trastuzumab, pertuzumab, or trastuzumab with pertuzumab was extracted and qPCR performed to investigate any KPL-4 tumor infiltrate into the lung. Data are the mean of 9 to 10 mice per group. D, mice with KPL-4 xenograft tumors were treated for the duration of the study with vehicle (violet circle), trastuzumab (orange triangle), and trastuzumab followed by pertuzumab (trastuzumab then pertuzumab added on day 35; green circle).
ADCC assays. Twenty-four hours before the assay, effector cells [peripheral blood mononuclear cells (PBMC)] were prepared from a healthy blood donor by Ficoll gradient centrifugation. PBMCs were diluted to a final concentration of 2.5 × 10^6 cells/mL in RPMI/10% fetal bovine serum (FBS) and cultured overnight. The following day, the target KPL-4 and Calu-3 cells grown in RPMI/10% FBS were detached using Accutase (PAA Laboratories), sedimented by centrifugation, and resuspended in RPMI/10% FBS at a final concentration of 1 × 10^5 cells/mL. Cells were labeled by adding 1 μL bis (acetoxyethyl) 2,6-terpyridine-6,6-dicarboxylic acid (Perkin-Elmer LAS GmbH) per milliliter cell suspension, mixing carefully and incubating for 25 min at 37°C and 5% CO₂. Cells were washed thrice with 10 mL cell culture medium and diluted to 1.0 × 10^3 cells/mL.

To perform the assay, 50 μL of serial dilutions of either trastuzumab or pertuzumab was added to each well of a 96-well plate (Greiner Bio-One GmbH). To test the combination of trastuzumab and pertuzumab, 25 μL of each antibody were added; instead, the total final concentration of the antibody mixture was the same as in the single antibody experiments. Fifty microliters of target cells and PBMCs were added, resulting in a final antibody concentration that ranged from 0.4 to 50 ng/mL and an effector/target ratio of 25:1. Controls included effector cells alone, only target cells and effector cells, and target cells with 3% Triton X-100. The antibody mixture was the same as in the single antibody experiments. Pertuzumab was added to each well of a 96-well plate (Greiner Bio-One GmbH) per milliliter cell suspension, mixing carefully and incubating for 25 min at 37°C and 5% CO₂. Cells were washed thrice with 10 mL cell culture medium and diluted to 1.0 × 10^3 cells/mL.

Results
Antitumor Activity of Trastuzumab and Pertuzumab Both Alone and in Combination
Trastuzumab in combination with pertuzumab has strongly enhanced antitumor activity. We investigated the efficacy of trastuzumab and pertuzumab in Calu-3 and KPL-4 tumor xenografts. Mice were treated with either: vehicle control, pertuzumab or trastuzumab alone, or trastuzumab and pertuzumab in combination. In Calu-3 NSCLC xenografts, monotherapy with pertuzumab or trastuzumab was able to significantly inhibit tumor growth, with treatment-to-control ratios (TCR) of 0.23 and 0.27, respectively. The combination of trastuzumab and pertuzumab produced a dramatically enhanced antitumor activity compared with single-agent treatments (TCR 0.05; Table 1; Fig. 1A), resulting in tumor regression and, in 3 of 10 animals, complete tumor remission (Supplementary Fig. S1; Table 1).

Treatment of KPL-4 breast cancer xenografts with either trastuzumab or pertuzumab inhibited tumor growth with TCRs of 0.67 and 0.65, respectively. Again, combining trastuzumab and pertuzumab induced strongly enhanced antitumor activity compared with either agent alone (TCR 0.04; Table 1; Fig. 1B), resulting in tumor regression and, in 6 of 10 animals, complete tumor remission (Table 1).

Combination of trastuzumab and pertuzumab can inhibit metastatic tumor spread. Genomic DNA from lung samples of host animals with KPL-4 xenografts was analyzed by qPCR to investigate if the tumor had metastasized to the lung. Monotherapy with either trastuzumab or pertuzumab did not prevent metastases forming in the lungs of the mice by day 43; however, no human DNA was detected in the lungs of mice treated with the combination of both trastuzumab and pertuzumab, even at day 99, suggesting that this combination completely prevented metastatic tumor spread to the lungs of these animals (Fig. 1C). Findings for the liver were similar (data not shown).

Table 1. Synergistic antitumor activity by trastuzumab and pertuzumab combination therapy

<table>
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<th>Xenograft</th>
<th>Treatment</th>
<th>Response, TCR</th>
<th>Confidence interval</th>
<th>TGI,%</th>
<th>Tumor-free animals, n</th>
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<td>Calu-3</td>
<td>P</td>
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<td>0.08–0.53</td>
<td>85</td>
<td>0/10</td>
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<td></td>
<td>T</td>
<td>0.27</td>
<td>0.06–0.57</td>
<td>82</td>
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<tr>
<td></td>
<td>P + T</td>
<td>0.05</td>
<td>0.02–0.11</td>
<td>&gt;100*</td>
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</tr>
<tr>
<td>KPL-4</td>
<td>P</td>
<td>0.67</td>
<td>0.45–0.94</td>
<td>38</td>
<td>0/10</td>
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<tr>
<td></td>
<td>T</td>
<td>0.65</td>
<td>0.43–0.91</td>
<td>45</td>
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<tr>
<td></td>
<td>P + T</td>
<td>0.04</td>
<td>0.00–0.25</td>
<td>&gt;100*</td>
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Abbreviations: P, pertuzumab; T, trastuzumab; TCR, treatment:control ratio; TGI, tumor growth inhibition.

*Tumor regression.
Pertuzumab maintains antitumor activity after progression on trastuzumab. To investigate the combination of trastuzumab and pertuzumab after progression on trastuzumab, SCID mice bearing KPL-4 xenograft tumors were treated with trastuzumab (30 mg/kg loading dose followed by 15 mg/kg weekly) until progression (day 35). At this point, mice either continued treatment with trastuzumab alone or with trastuzumab in combination with pertuzumab. The combination of the two agents was sufficient to inhibit tumor growth and, indeed, reduce tumor mass for a further 45 days (Fig. 1D). A similar result was obtained using the Calu-3 xenograft model: addition of pertuzumab to continued trastuzumab treatment–induced tumor shrinkage despite progressive growth on trastuzumab single-agent treatment (Supplementary Fig. S2).

Trastuzumab with pertuzumab induces tumor cell loss. To determine how trastuzumab and pertuzumab were inhibiting tumor growth, an acute study, using just one dose of either trastuzumab and pertuzumab monotherapy or combination therapy, was performed in mice with advanced Calu-3 xenograft tumors. Western blot analysis of HER2 protein levels in tumor lysates showed that both trastuzumab and pertuzumab as single agents reduced HER2 protein levels significantly (TCR, 0.48 and 0.56, respectively). However, combination of trastuzumab with pertuzumab was clearly superior [TCR, 0.17 (confidence interval, 0.00–0.39); Fig. 2A]. Similar protein decreases were found for HER1, HER3, phosphoHER2, and downstream signaling components (data not shown). A single treatment with combined trastuzumab and pertuzumab resulted in a more than additive effect on the level of HER2 protein (Fig. 2A).

In vivo visualization of trastuzumab and pertuzumab binding to xenograft tumors. Using NIRF optical imaging, we showed that single-agent trastuzumab-Cy5 and pertuzumab using NIRF technology. Comparable NIRF signals were obtained at the tumor site when KPL-4 xenograft–carrying mice were treated with either (A) trastuzumab-Cy5 or (B) pertuzumab-Cy5. When mice were pretreated with unlabeled trastuzumab, no tumor staining was seen with (C) trastuzumab-Cy5, whereas (D) pertuzumab-Cy5 treatment showed similar staining intensity to tumors not pretreated with trastuzumab.

Figure 2. Investigation of the synergistic antitumor effects of trastuzumab and pertuzumab. Mice with Calu-3 tumors were treated with a single dose of antibody, and tumors were explanted 1 wk after antibody treatment and analyzed by A, Western blot and B, IHC for HER2 protein expression.

Figure 3. In vivo imaging of KPL-4 tumor xenografts, with labeled trastuzumab and pertuzumab using NIRF technology. Comparable NIRF signals were obtained at the tumor site when KPL-4 xenograft–carrying mice were treated with either (A) trastuzumab-Cy5 or (B) pertuzumab-Cy5. When mice were pretreated with unlabeled trastuzumab, no tumor staining was seen with (C) trastuzumab-Cy5, whereas (D) pertuzumab-Cy5 treatment showed similar staining intensity to tumors not pretreated with trastuzumab.
ADCC assays with trastuzumab and/or pertuzumab. To identify the mechanism of action for the strongly enhanced activity of trastuzumab and pertuzumab, we performed in vitro ADCC assays using human PBMCs (effector cells) and either cultured Calu-3 or KPL-4 cells (target cells). Both trastuzumab and pertuzumab applied as single agents effectively activated ADCC against Calu-3 or KPL-4 cells (target cells). Both trastuzumab and pertuzumab have shown that these agents bind to different epitopes on the HER2 protein (27, 37); however, concomitant HER1, HER2, and HER3 expression in human NSCLC and breast cancer cell lines used for xenograft models and siRNA downregulation of HER1 and HER3. The HER2-positive cell lines KPL-4 and Calu-3 were analyzed for HER-family receptor expression by Western blotting. Whereas Calu-3 showed strong HER1 and moderate HER3 coexpression, KPL-4 cell lysates produced a very strong HER3 and strong HER1 band (see Fig. 5B and D).

To confirm the HER2 status according to clinical standards, IHC was performed on Calu-3 and KPL-4 xenograft tumors. Both Calu-3 and KPL-4 xenograft tumors were strongly positive for HER2 staining (IHC 3+ using the HercepTest scoring recommendations). Because HER2 dimerization with either HER1 or HER3 can promote cell survival and proliferation, and act as a potential escape mechanism from trastuzumab inhibition, we performed a knockdown of HER1 and HER3, respectively, and investigated the effect on proliferation of Calu-3 and KPL-4 cells. Calu-3 and KPL-4 cells were transfected with siRNAs targeting either HER1 or HER3, and proliferation was measured. HER1 and HER3 targeting siRNAs effectively downregulated expression of the respective mRNAs roughly by about 60% to 80% in both cell lines (Fig. 5A and C). Western blot data confirmed that HER1 and HER3 protein expression was effectively reduced after siRNA treatments (Fig. 5B and D). In Calu-3 cells, HER1 siRNA treatment inhibited cell growth by ~30%, whereas downregulation of HER3 reduced growth only by ~7%, an effect that was no longer statistically significant (Fig. 5A). Both results on Calu-3 cell growth are consistent with the relative coexpression of HER1 and HER3 in this HER2-positive cell line. In KPL-4 cells, HER3 expression turned out to be more important for cell growth than HER1: whereas HER1-specific siRNA reduced cell growth by ~10%, HER3 siRNA treatment entailed ~25% growth inhibition (Fig. 5C). Again, this result is consistent with the relative coexpression of HER1 and HER3 in the HER2-positive cell line KPL-4.

Discussion

This study has shown that monotherapy with either trastuzumab or pertuzumab has a moderate growth inhibitory effect on NSCLC (Calu-3) and breast (KPL-4) xenograft tumors. The combination treatment with trastuzumab and pertuzumab induced a strongly enhanced antitumor activity in both Calu-3 and KPL-4 xenograft models, resulting in tumor regression and a complete inhibition of metastatic tumor spread in host animals. Moreover, addition of pertuzumab to trastuzumab therapy enhanced antitumor activity in KPL-4 and Calu-3 xenograft tumors that had progressed on trastuzumab. Reduction in the levels of HER2 protein following treatment with trastuzumab and/or pertuzumab was due to a loss of cells expressing HER2 rather than a downregulation of HER2 protein at the cell surface.

The synergistic action of trastuzumab and pertuzumab combination treatment was previously shown in vitro (30) using the calculation of the combination index as outlined by Chou and Talalay (35, 36). In the present work, combination treatment with trastuzumab and pertuzumab dramatically enhanced the antitumor effect compared with each drug alone: whereas single agent treatments could only delay tumor growth (transiently in KPL-4), the combination treatment induced effective tumor regressions and cures in both xenograft models. We believe that this qualitative difference in antitumor efficacy is a consequence of the synergistic action of both antibodies described in vitro.

In vitro studies investigating the mechanism of action of trastuzumab and pertuzumab have shown that these agents bind to different epitopes on the HER2 protein (27, 37); however, concomitant...
binding to HER2-positive tumors has not been shown in vivo. Using NIRF imaging, we show for the first time that in vivo binding of trastuzumab and pertuzumab is not mutually exclusive. Similar amounts of pertuzumab and trastuzumab, respectively, can bind to HER2-positive tumors when used either in monotherapy or in combination.

Activation of ADCC has been described as a major mechanism of action of trastuzumab (13, 14, 38) and the present study confirms that this is also a property of pertuzumab. Since trastuzumab and pertuzumab are able to bind simultaneously to tumors in vivo, we investigated if the strongly enhanced antitumor effect of these two agents was due to enhanced ADCC. Each antibody alone mediated a strong activation of ADCC, reaching comparable levels of maximal specific target cell lysis. The combination of trastuzumab and pertuzumab (final total antibody concentration equivalent to traditional mechanisms of chemotherapy resistance. The strongly enhanced activity of trastuzumab and pertuzumab is likely attributable to their alternative, differing mechanisms of action. Previous studies have reported that trastuzumab can sterically inhibit access for the proteases that cleave the extracellular domain and, therefore, prevent the formation of p95HER2, the truncated and very active form of HER2 (15, 42). However, trastuzumab is unable to prevent ligand-activated HER2/HER3 or HER2/HER1 heterodimerization, which may provide tumor cells with an escape mechanism from the inhibitory effects of trastuzumab. In the current study, downregulation of HER3 and HER1 mRNA and protein reduced the proliferation of both Calu-3 and KPL-4 cells. This illustrates the importance of these pathways in the propagation of HER2-positive tumors and may even become more important when ligand-independent HER2 activation is inhibited by trastuzumab. Pertuzumab prevents the HER2 protein from dimerizing with HER3 and/or HER1 (26, 27, 29, 43), thus blocking these potential escape pathways. Alternatively, pertuzumab monotherapy can obstruct ligand-activated HER2 dimerization and signaling but would be unable to prevent formation of p95HER2, which is readily achieved by trastuzumab.

Thus, the differing mechanisms of action of trastuzumab and pertuzumab can act in a complementary fashion to provide a more complete blockade of HER2-mediated signal transduction than either agent alone.

In the clinical setting, dramatically enhanced antitumor activity of trastuzumab and pertuzumab has been shown in a phase II clinical trial that investigated the efficacy and safety of a trastuzumab/pertuzumab combination in 66 patients with HER2-positive metastatic breast cancer whose disease had progressed during trastuzumab therapy. Of these patients, 8% had a complete response, with an overall response rate of 24.2% and a complete clinical benefit rate of 50% (39). Data from the current study also indicate a potential role for pertuzumab and trastuzumab in HER2-positive NSCLC, although it should be noted that HER2 overexpression is rare in NSCLC.

Conclusions

Trastuzumab and pertuzumab in combination have a strongly enhanced antitumor effect and promote tumor regression in xenograft models of HER2-positive breast cancer and NSCLC. Although both agents potently activate ADCC, this is unlikely to be the main mechanism by which the strongly enhanced effects of this combination are mediated. Instead, the differing but complementary mechanisms of action of trastuzumab and pertuzumab may explain the significantly enhanced antitumor activity produced by...
combined treatment with these agents. The combination of these agents may prove to be of substantial benefit to patients who currently respond suboptimally to trastuzumab therapy; further trials are warranted.

Disclosure of Potential Conflicts of Interest
All authors are employees of Roche Diagnostics GmbH.

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Acknowledgments
Received 12/9/08; revised 10/5/09; accepted 10/6/09; published OnlineFirst 11/24/09. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. R. Juchem for the histopathologic analysis of the tumor samples; Professor J. Kurebayashi for kindly providing KPL-1 cells; U. Haupt, F. Ols, S. Baumann, and C. Biehler for excellent technical assistance with respect to in vivo experiments; P. Kern for performing ADCC experiments; K. Palme and M. Hoch for siRNA experiments; and L. Chang for medical writing assistance.

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Cancer Res  Published OnlineFirst November 24, 2009.

Updated version  Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-08-4597

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