Trastuzumab and Pertuzumab Produce Changes in Morphology and Estrogen Receptor Signaling in Ovarian Cancer Xenografts Revealing New Treatment Strategies

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Abstract

Purpose: The aim of this study was to investigate the antitumor effects of HER2-directed combination therapy in ovarian cancer xenograft models to evaluate their potential. The combinations of trastuzumab and pertuzumab, and trastuzumab and aromatase inhibitor therapy were investigated.

Experimental Design: The effects of trastuzumab, pertuzumab, and letrozole on growth response, apoptosis, morphology, and gene and protein expression were evaluated in the SKOV3 ovarian cancer cell line xenograft and a panel of five human ovarian xenografts derived directly from clinical specimens.

Results: The combination of HER2-directed antibodies showed enhanced antitumor activity compared with single antibody therapy in the SKOV3 xenograft model. Apoptosis, morphology, and estrogen-regulated gene expression were modulated by these antibodies in both spatial and temporal manners. A panel of ovarian cancer xenografts showed differential growth responses to the combination of trastuzumab and pertuzumab. High HER2 expression and increasing HER3 protein expression on treatment were associated with growth response. In trastuzumab-treated SKOV3 tumors, there was a change in tumor morphology, with a reduction in frequency of estrogen receptor alpha (ERα)-negative clear cell areas. Trastuzumab, but not pertuzumab, increased expression of ERα in SKOV3 xenografts when analyzed by quantitative immunofluorescence. ERα and downstream signaling targets were modulated by trastuzumab alone and in combination. Trastuzumab enhanced the responsiveness of SKOV3 xenografts to letrozole when given in combination.

Conclusions: These data suggest that trastuzumab in combination with pertuzumab could be an effective approach in high HER2-expressing ovarian cancers and could also enhance sensitivity to endocrine therapy in ERα-positive ovarian cancer.

Introduction

Survival figures for epithelial ovarian cancer remain poor, with 65% of women dying of this disease within 5 years of diagnosis (1) despite generally good initial response rates to chemotherapy (2, 3). This is partly because women with ovarian cancer present with late-stage disease, but also because advances in targeted therapy in ovarian cancer have not matched developments in other solid-organ tumors, such as the use of tamoxifen/ aromatase inhibitors in breast cancer (4) or cetuximab in colorectal cancer (5). There is good evidence that platinum–taxane first-line chemotherapy is superior to other chemotherapy regimens for ovarian cancer (2, 6–9), but 20% to 30% of patients do not respond to this therapy and chemoresistance develops in the majority of tumors. Targeted agents with lower toxicity, such as trastuzumab (Herceptin) and pertuzumab, directed against HER2, are not routinely used in clinical practice for ovarian cancer but may benefit some women (3, 10). There is therefore a clinical need to identify patients who will benefit from targeted therapy and to establish which combinations are likely to show greatest efficacy.

HER2 (erbB2) expression in ovarian cancer is associated with advanced stage, higher recurrence frequency, shorter survival time, and lower sensitivity to platinum-based chemotherapy (11, 12). Trastuzumab and pertuzumab are humanized anti-HER2–targeted antibodies with different...
Translational Relevance

There is currently interest in identifying targeted therapies for the treatment of ovarian cancer. HER2- and ERα-targeted therapies would be readily translatable options, with well-established use in other cancers. The complex interplay between ERα and HER2 signaling means that detailed understanding of these pathways is required to personalize therapy. We evaluated the use of HER2-targeted approaches in preclinical xenograft models of ovarian cancer and showed that the combination of trastuzumab and pertuzumab are effective in high HER2-expressing xenografts. In addition, trastuzumab treatment results in increased expression of ERα, which, in turn, resulted in enhanced sensitivity to the antiestrogen letrozole. These data indicate that treatment can change target receptor expression, which may lead to increased sensitivity to alternative therapies. These results support the consideration of combination HER2 treatment in ovarian cancer and also combined anti-HER2 and anti-ERα therapy, perhaps as part of sequential therapy.

Materials and Methods

In vivo tumor models

For xenograft studies, adult female CD-1 nude mice were implanted subcutaneously in the flank with SKOV3 tumor fragments (previously established from the cell line) or fragments from xenografts established from resected primary ovarian cancers or ascites. Tumors were allowed to grow to 4 to 6 mm in diameter (over a period of approximately 1 month). Animals were then allocated to treatment (5 mice/group) or control (10 mice/group) groups and treatment was commenced (defined as day 0). Mice were treated with trastuzumab (20 mg/kg), pertuzumab (20 mg/kg), or trastuzumab + pertuzumab (20 mg/kg each). Drugs were given via the intraperitoneal (i.p.) route in saline on days 0, 3, 7, and 10. In experiments with letrozole, the drug was given i.p. at a dose of 10 μg/kg/d for 14 days. Tumor size was measured twice weekly by using calipers, and the volume was calculated according to the formula π/6 × length × width². Relative tumor volumes (%) were then calculated for each individual tumor by dividing the tumor volume on day t (Vt) by the tumor volume on day 0 (Vo) and multiplying by 100. Tumors were excised at the end of the experiment and divided, with half of the material fixed in 10% buffered formalin and half snap frozen in liquid nitrogen.

RNA extraction

Total RNA was prepared from 10 to 50 mg of frozen tissue from SKOV3 xenografts after 4 days of treatment, preincubated with RNAlater-ICE (Ambion), using the miRNeasy Mini Kit (Qiagen) and TissueRuptor (Qiagen)
following the manufacturers’ instructions. The RNA quality was checked by the RNA 6000 Nano Assay on the Agilent Bioanalyzer (Agilent Technologies). RNA integrity numbers were more than 8.5.

**Tissue microarray construction**

Tissue microarrays (TMA) were constructed for both tumor xenografts and primary ovarian tumors. For xenografts, 2 replicate TMAs were constructed and each TMA contained two 0.6-mm cores of each primary tumor xenograft under different treatment conditions. The construction and clinicopathologic details of the primary tumor TMA have already been described (26). Four replicate TMAs were constructed using established techniques (27), and 3 replicates were used for analysis within this study. Each TMA contained a representative 0.6-mm core from each of the 122 cases included within the study. Where primary lesions showed a mixed histologic pattern, representative areas of each histologic type were targeted so that all types were adequately represented within the analysis.

**Immunohistochemistry**

Immunohistochemistry was carried out on TMAs, using a standard immunoperoxidase procedure. Antigen retrieval was done by microwaving the slides under pressure for 5 minutes in Tris-EDTA buffer (10 mmol/L Tris, 1 mmol/L EDTA, 0.05% Tween 20, pH 9), and slides were treated with 3% H2O2 for 10 minutes to quench peroxidase activity. Slides were then incubated in serum-free block solution (Dako) for 20 minutes to eliminate any nonspecific background staining. Primary antibody incubations were Ki-67 (M7240 Dako; 1:50), ERα (VP-E613 Vector Laboratories; 1:50), HER2 (A0485 Dako; 1:400), HER3 (MS-201-PABX Labvision; 1:500) for 1 hour at room temperature, and cleaved caspase-3 (9661 Cell Signaling Technology; 1:400) overnight at 4°C. EnVision [horseradish peroxidase (HRP) rabbit/mouse; Dako] was used as a secondary antibody, and positive staining was detected by incubation with 3,3′,5-diaminobenzidine solution (DAB + chromogen; Dako) with hematoxylin counterstain. Quantification of proliferation (using Ki-67) or apoptosis (cleaved caspase-3; 0%–100%) was based on the number of immunoreactive nuclei divided by the total number of tumor cell nuclei in each TMA core ×100.

**Immunofluorescence**

Immunofluorescence for ERα was carried out using methods previously described (28). For slide staining, 3-μm TMA slides were deparaffinized and antigen retrieved by pressure cooking in antigen retrieval buffer (sodium citrate, pH 6.0). Endogenous peroxidases were blocked with 3% hydrogen peroxide for 15 minutes, and nonspecific binding was blocked with serum-free protein block for 15 minutes. Slides were then incubated with primary antibodies (ERα, 1:50; Vector Laboratories), diluted in 0.05% PBS Triton X-100 (PBST) for 1 hour at room temperature, and finally diluted in Dako antibody diluent. After washing in 0.05% PBST, sections were incubated for 1 hour at room temperature with secondary antibodies, which included an Alexa 555–conjugated goat anti-mouse antibody diluted 1:100 in 0.1 mol/L TBS, and prediluted goat anti-rabbit antibody conjugated to an HRP-decorated dextran-polymer backbone (EnVision; Dako). Slides were then incubated for 10 minutes with Cy5-tyramide, which is activated using HRP, to visualize target phosphorylation protein expression. 4′,6-Diamidino-2-phenylindole (DAPI; Invitrogen) was used to stain the nuclear compartment.

**Image analysis for morphologic analysis**

For image analysis, hematoxylin and eosin (H&E)-stained sections of tumors were scanned onto the Ario SL-50 image analysis system (Applied Imaging Genetix). Briefly, 3-color-filtered images were captured on a black-and-white charge-coupled device camera and reconstructed by the supplied proprietary software. Total tumor areas and clear cell areas were marked, and the areas were automatically calculated by the proprietary analysis software.

**AQUA automated image analysis**

A detailed description of the AQUA HistoRx methodology is available elsewhere (28, 29). Pan-cytokeratin antibody was used to identify infiltrating tumor cells, DAPI counterstain to identify nuclei, and Cy5-tyramide detection to target for compartmentalized (tissue and subcellular) analysis of tissue sections. Monochromatic images of each TMA core were captured at 20× objective with an Olympus AX-51 epifluorescence microscope, and high-resolution digital images were analyzed by the AQUA analysis software. When whole-tissue sections of xenograft tumors were analyzed, the whole section was divided into 20× objective magnification images and an AQUA score was generated for each frame for heterogeneity analysis. Briefly, a binary epithelial mask was created from the cytokeratin image of each TMA core. If the epithelium comprised less than 5% of total core area, the core was excluded from analysis. Similar binary masks were created for cytoplasmic and nuclear compartments on the basis of DAPI staining of nuclei. Target expression was quantified by calculating the Cy5 fluorescent signal intensity on a scale of 0 to 255 within each image pixel, and an AQUA score was generated by dividing the sum of Cy5 signal within the epithelial mask by the area of the cytoplasmic compartment. The same primary antibodies against HER2 and HER3 were used under the same conditions as for the immunohistochemistry.

**Gene expression profiling**

A full account of the microarray analysis is given in the parallel study (Sims and colleagues, manuscript in preparation). Briefly, total RNA (0.5 μg) was amplified and biotinylated using the Illumina TotalPrep RNA Amplification Kit (Ambion) according to manufacturers’ standard procedure as 2 identical aliquots for independent labeling and hybridization. The quality and quantity of cRNA in the samples were checked with an Agilent Bioanalyzer 2100 (Agilent), and samples were diluted to 150 ng/μL before hybridizing to Illumina HT-12 BeadChips. This was undertaken in the
Wellcome Trust Clinical Research Facility (Edinburgh). Microarray data analysis was carried out using the statistical programming language R and Bioconductor packages (30). Heat maps were generated using the TreeView program, as described previously (31). All raw gene expression files and clinical annotation are publicly available from the caBIG database in the Edinburgh Experimental Cancer Medicine Centre (https://catissuesuite.ecmc.ed.ac.uk/caarray/).

Quantitative reverse transcriptase PCR
RNA (1 μg) was reverse transcribed (RT-PCR) using a QuantiTect Reverse Transcription kit (Qiagen). The amount of cDNA was quantified using Rotorgene (Corbett Research) and the QuantiTect SYBR Green system (Qiagen). Primers were from Qiagen. The housekeeping gene GUSB was used as a reference gene and its level was constant across the microarray results.

Western blot analysis
Xenografts were lysed in 50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1% Triton X-100, 5 mmol/L EGTA, 10 μg/mL aprotinin (Sigma), complete protease inhibitor cocktail (Roche), and phosphatase inhibitor cocktails 1 and 2 (Sigma) and spun for 10 minutes at 16,000 × g at 4°C. The protein content of the resulting supernatant was determined by the biocinchoninic acid protein assay (Sigma). Protein lysates were electrophoretically resolved on 7.5% to 10% SDS-PAGE and transferred to Immobilon-P membranes. After transfer, membranes were blocked with 1% blocking agent (Roche) in TBS before probing overnight at 4°C. noreactive bands were detected using enhanced chemiluminescence film (Amersham). Integrated absorbance values were obtained by densitometric analysis with a gel scanner and analyzed by Labworks gel analysis software (UVP Life Sciences).

Statistics
Relationships between variables were analyzed by 1-way ANOVA. Analysis of the microarray data was carried out using the statistical program R and the lumi package of the Bioconductor software. The Simpson diversity index for heterogeneity (32) was calculated as the sum of the squares of the proportion of AQUA scores belonging to each AQUA score range (0–50, 51–100, 101–150, etc.). A Simpson index of 1 indicates total homogeneity and 0 indicates total heterogeneity.

Results
Anti-HER2 antibodies inhibit the growth of SKOV3 xenografts and have differential effects on proliferation and apoptosis
To determine whether treatment with anti-HER2 monoclonal antibody therapy was more effective when administered alone or in combination, HER2-amplified SKOV3 xenografts were treated with trastuzumab, pertuzumab, or a combination of both (Fig. 1A). In the first experiment, treatment was initiated when mean tumor volume was 44 mm³. Pertuzumab slowed xenograft tumor growth markedly (Fig. 1A, left) but did not result in complete tumor regression in any of the xenografts. Trastuzumab alone caused a marked regression, and combined treatment with trastuzumab and pertuzumab resulted in complete regression in all tumors (10 of 10). In a second experiment using larger SKOV3 xenograft tumors (Fig. 1A, right; mean tumor volume = 150 mm³), similar differential results were obtained. Pertuzumab slowed tumor growth, whereas trastuzumab alone or combination therapy resulted in stable disease. Combination treatment produced a more rapid cytostatic effect, but trastuzumab alone produced comparable activity after 10 days (Fig. 1A).

We next investigated the effects of single- or combined-agent therapy on xenograft proliferation and apoptosis. Proliferation and apoptosis were estimated by calculating a Ki-67 and activated caspase-3 index on TMAs of xenograft tissue taken from each time point (Fig. 1B). No significant differences in Ki-67 proliferation were noted between treatment groups or over time. In contrast, the apoptotic index differed significantly (P < 0.05; 1-way ANOVA) between treatment groups and over time. The apoptotic index was significantly higher in the combination group 2 days after the initial treatment. These results suggest that tumor inhibition in the combination group is associated with induction of apoptosis rather than inhibition of proliferation.
SKOV3 xenografts show both morphologic and molecular heterogeneity after treatment with HER2 inhibitors, suggesting that therapy changes the phenotype of constituent cell populations

Histologic examination of SKOV3 xenograft tumors showed distinct differences in morphologic appearance, both within the same tumor and between treatment groups. Xenografts showed a range of ovarian histologic patterns, including endometrioid and clear cell patterns, similar to mixed ovarian tumors seen in clinical tumor samples (Fig. 3A). Quantitative image analysis showed that clear cell areas were markedly reduced following treatment with trastuzumab and significantly reduced after combination therapy–treated tumors (Fig. 3B), suggesting that trastuzumab can selectively target different populations of cells within morphologically heterogeneous tumors.

To verify the change in morphology at the molecular level, we examined changes in expression of genes that had been identified as the most differentially expressed in clear cell versus non–clear cell carcinoma from a previous study (33) following treatment with HER2 inhibitors, using data from a parallel microarray study (Sims –and colleagues, manuscript in preparation). The majority of genes that were more highly expressed in clear cell tumors than other ovarian histologic subtypes were reduced following treatment, and those that were expressed lower in clear cell tumors showed predominantly higher expression following treatment (Fig. 3C). These changes were of greater magnitude and more significant for trastuzumab alone or in combination than with pertuzumab treatment alone, consistent with the changes in morphology. Two of the top 3 genes that have previously been shown to be more highly expressed in ovarian clear cell carcinomas than other ovarian epithelial carcinomas (33), SPP1 and NNMT, were significantly downregulated in the trastuzumab and combination therapy groups (P < 0.002, P < 0.0001; Fig. 3C).

We reasoned that because HER2 expression had been assessed and was constant both across the tumors and different histologic subtypes, trastuzumab sensitivity must be mediated by differential activation of other pathways that are known to cross-talk with HER2 signaling and impact on therapeutic response. We therefore assessed the expression of ERα across the tumors, as cross-talk with HER2 signaling is known to mediate sensitivity to endocrine therapies in breast cancer and more recently it has been shown that ERα may be upregulated by trastuzumab (35). Clear cell areas, but not other morphologic patterns, were ERα negative/low by immunohistochemistry (Fig. 4A), consistent with previous reports (25).

We next investigated whether ERα gene and protein expression levels change in response to HER2-directed therapy in ovarian cancer. We used quantitative immunofluorescence to measure ERα protein expression on tissue...
sections to establish both the magnitude and distribution of ER expression (Fig. 4A and B and Supplementary Fig. S4). ERα nuclear protein expression was significantly increased in SKOV3 xenografts treated with either trastuzumab or trastuzumab and pertuzumab in combination but not in tumors treated with pertuzumab alone (ANOVA \( P < 0.05 \); Fig. 4A). Western blot analysis indicated similar changes (Fig. 4B). Because ERα-negative clear cell areas were reduced in trastuzumab-treated tumors, we reasoned that this could be responsible for an average increase in ERα expression in these tumors. Immunofluorescence confirmed that clear cell areas were negative for ERα but also that the intensity of fluorescence increased after treatment with trastuzumab in non–clear cell areas (Fig. 4A). When the distribution of ERα expression was assessed using an index of heterogeneity (the Simpson index), both the trastuzumab-treated and combination groups were more heterogeneous than control or pertuzumab-treated groups (Fig. 4A), suggesting that despite a reduction in clear cell areas, these tumors became more variable in ERα expression and showed an overall increase in receptor expression.

We confirmed that changes in ERα expression resulted in ERα-dependent gene expression by analyzing changes in expression of ERα target genes compared with untreated controls (Fig. 4C). Genes that have previously been identified as being estrogen regulated in ovarian cancer (36) or known classical estrogen-regulated genes were investigated (37). Trastuzumab-treated and combination groups showed increases or decreases in expression of ERα target genes, appropriate to the expected activating or repressing
action of ERα. Examples of upregulated genes include TFF3, FBLN1, AGR2, and PDZK1 whereas downregulated genes include TGFBI, LCN2, VIM, AKAP12, PLAU, and CDH6 (Fig. 4C). qRT-PCR measurement of expression of selected examples of these genes (TFF3, TGFBI, and LCN2) confirmed these changes (Supplementary Fig. S3B), which were of greatest magnitude and most significant for the combination of trastuzumab and pertuzumab. Less effect was noted for trastuzumab alone and even less effect with pertuzumab alone. Again, these molecular changes are consistent with the changes in morphology. To exclude the possibility that this was a cell line–specific phenomenon, we measured ERα expression in the primary ovarian carcinoma xenografts treated with trastuzumab and pertuzumab. Individual tumors showed an increase in ERα expression in 4 of 5 cases (Fig. 2B), suggesting that ERα regulation by HER2-directed therapy could be exploited as a therapeutic strategy in a proportion of ovarian cancers.

**Estrogen receptor expression is lower in clear cell than in serous and endometrioid tumors**

Assessment of ERα expression by quantitative immunofluorescence in 122 ovarian tumors of mixed histologic types confirmed lower expression of ERα in clear cell ovarian carcinomas than in serous and endometrioid subtypes (Fig. 5A). This suggests that in ovarian cancer, wherein mixed histology is frequently observed, different populations of cells should be targeted with different agents to achieve therapeutic success.

**Combination therapy with an aromatase inhibitor and trastuzumab results in an increased rate of complete tumor response**

The response of SKOV3 xenografts to combination therapy with the aromatase inhibitor letrozole and trastuzumab was assessed to establish whether the clinically relevant combination of 2 targeted agents might be a useful therapeutic strategy in ovarian cancer. SKOV3 xenografts treated with a combination of trastuzumab and letrozole achieved complete tumor response in 44% (4 of 9) mice compared with 10% (1 of 10) complete response in mice treated with trastuzumab alone (Fig. 5B). None of the letrozole-only–treated tumors responded completely to therapy. These data suggest that combining endocrine inhibition with trastuzumab therapy might be an effective therapeutic strategy in HER2- and ERα-positive ovarian cancer.

**Discussion**

Although the therapeutic effects of endocrine agents and HER2-targeted antibodies, and their interactions, are relatively well-characterized for breast cancer, there is...
little information on the action or interaction of these agents in ovarian cancer, even though they have shown promise for some patients in the clinic (19, 20, 24). When combined, trastuzumab and pertuzumab show an enhanced antitumor activity in ovarian cancer xenografts, as they do in breast (38, 39) and non–small-cell lung cancer xenografts (39). Because there is cross-talk between pathways downstream of receptor tyrosine kinases [such as the mitogen-activated protein kinase (MAPK) pathway] and endocrine signaling pathways, understanding the molecular interactions between these agents is crucial to accurate selection of patients and maximizing clinical response rates. It is feasible that the combination of antibodies act to produce vertical inhibition of the pathway, further modifying phenotype. Alternatively, they may act separately to activate apoptosis in different populations of tumor cells allowing clonal selection. To help identify ovarian cancers that might be sensitive to combined HER2-directed therapy, HER receptor biomarkers may be of value. High HER2 expression is predictive in breast cancer for response to trastuzumab and consistent with this, the 2 responsive xenografts SKOV3 and HOX424 showed high expression of HER2 protein that did not apparently diminish on treatment in contrast to unresponsive xenografts that expressed reduced HER2 and wherein expression was reduced on treatment. HER3 has been proposed as a biomarker of pertuzumab response in ovarian cancer. Consistent with this, HER3 protein expression was increased by treatment with pertuzumab (20, 40).
molecular phenotype and therefore responsiveness to other therapies. ERα was upregulated in response to treatment with trastuzumab (alone or in combination with pertuzumab) but not pertuzumab alone. A similar phenomenon has been reported in breast cancer (35). This was not simply a magnitude effect, as the quantitative immunofluorescence is exquisitely sensitive. Although a decrease in ERα-negative clear cell areas was observed in trastuzumab-treated tumors, ERα was also upregulated after treatment, consistent with activation and repression of ERα target genes. This effect was not cell line dependent, with similar increases seen in primary ovarian cancer xenografts. Finally, combining trastuzumab with the aromatase inhibitor letrozole resulted in an increased response compared with either agent alone. The low expression of ERα in the clear cell areas of the xenografts is consistent with low ERα expression in clear cell carcinomas in surgical resection specimens both within our data and other published studies (25). It is intriguing that the ovarian cancer xenograft (HOX424) that showed best response to the antibody combination contained a clear cell element. Ovarian clear cell cancers are frequently platinum resistant (41, 42), so HER2-directed therapies may provide an option for high HER2-expressing clear cell cancers. Recently, it has been reported that 14% of ovarian clear cell cancers possess HER2 gene amplification and protein overexpression, and these represent a promising target group (43).

The morphology of the tumor may therefore be a useful surrogate for the underlying molecular phenotype, but these data also suggest that effective therapy in complex epithelial cancers requires assessment of the heterogeneity of the tumor at a phenotypic and molecular level and that different components of tumors may require therapy with different agents to achieve complete therapeutic response. In this case, clear cell areas seem to be more sensitive to the effects of trastuzumab, whereas residual disease seems to be sensitized to the effects of endocrine therapy through upregulation of ERα. Consideration of both spatial and temporal heterogeneity is a challenge in the clinical setting, as diagnostic biopsies are frequently small and may not be representative of the tumor as a whole and multiple
biopsies during the course of treatment may carry unacceptable clinical risk or be impossible; nevertheless, multiple tissue samples are often taken in cancer therapy in the neoadjuvant (i.e., systemic treatment before surgery) setting or in “window-of-opportunity” studies in clinical trials (44). Our data suggest that molecular analysis of tumor samples after therapy may be more informative for driving future treatment decisions than tests carried out on treatment-naive biopsies alone, as the cancer changes in response to therapy. Personalized cancer care may have to become more reactive to the changing disease in the future to improve patient outcomes.

In summary, our studies showed that trastuzumab and pertuzumab exhibited enhanced antitumor activity in ovarian cancer xenografts compared with either agent alone. In addition, HER2 antibody–targeted therapy had a spatial and temporal effect on tissue morphology and the expression of ERTs, which might be exploited for therapeutic benefit.

References

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