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SHORT COMMUNICATION

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In vitro effects of trastuzumab and vinorelbine in trastuzumab-resistant breast cancer cells

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Abstract *Purpose*: The majority of patients who initially respond to trastuzumab will progress within 1 year. Currently, patients who progress after trastuzumab-based therapy are often maintained on trastuzumab combined with a different chemotherapeutic agent, such as vinorelbine. However, evidence supporting the continued use of trastuzumab in these breast cancers is lacking. *Methods*: We created a preclinical model of trastuzumab resistance using the SKBR3 HER-2-overexpressing breast cancer cell line. Dose-response and cell cycle alterations in response to trastuzumab and/or vinorelbine were assessed. *Results*: In contrast to the parental SKBR3 cells, vinorelbine-mediated growth inhibition and apoptosis were not significantly enhanced by the addition of trastuzumab in the trastuzumab-resistant pools. *Conclusions*: These results suggest that the continued treatment of trastuzumab-resistant breast cancers with trastuzumab-containing regimens may not be effective. A randomized clinical trial of trastuzumab plus vinorelbine versus vinorelbine alone should be conducted in patients with HER-2-overexpressing breast cancer to determine the optimal duration of trastuzumab therapy upon progression.

Keywords Apoptosis · Dose-response · Herceptin Resistance · SKBR3

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Introduction

Trastuzumab (Herceptin; Genentech, San Francisco, Calif.) is a recombinant humanized monoclonal antibody directed against the extracellular domain of the HER-2 tyrosine kinase receptor, which is overexpressed in approximately 20–30% of invasive breast carcinomas [1]. Response rates to trastuzumab as a single agent range from 12% to 40% for a median duration of 9 months [2, 3]. Treatment regimens combining trastuzumab with the taxane paclitaxel [4, 5] or docetaxel [6] show increased response rates, time to progression and survival versus trastuzumab alone. However, many patients with HER-2-overexpressing tumors never respond to trastuzumab, and the majority of patients who do achieve an initial response will acquire resistance within 1 year [3].

Vinorelbine tartrate (Navelbine; GlaxoSmithKline, Philadelphia, Pa.) is a semisynthetic vinca alkaloid that inhibits cell growth by binding to tubulin and promoting apoptosis [7]. In vitro studies have demonstrated that vinorelbine and trastuzumab synergistically inhibit survival of HER-2-overexpressing breast cancer cells [8]. Treatment of HER-2-positive metastatic breast cancer patients with trastuzumab in combination with vinorelbine has achieved a 68% to 84% overall response rate [9, 10, 11]. These phase II trials included previously untreated patients as well as those with prior exposure to anthracyclines and/or taxanes. None of these studies included patients previously exposed to trastuzumab.

The optimal duration of trastuzumab therapy is not known. Patients who progress on trastuzumab are generally given a new chemotherapeutic agent such as vinorelbine but are still maintained on trastuzumab. While patients may demonstrate an increased antitumor response to this new regimen, it is unclear whether trastuzumab is still contributing or whether the new cytotoxic agent is solely responsible. We evaluated the growth-inhibitory effects of combined trastuzumab/vinorelbine in an in vitro trastuzumab-resistant model.

Materials and methods

Materials

Trastuzumab was dissolved in sterile water at 20 mg/ml. Vinorelbine tartrate was dissolved in DMSO at 10 mg/ml. The MTS CellTiter 96 Aqueous One Solution proliferation assay (Promega, Madison, Wis.) was used in accordance with the manufacturer's guidelines.

Cell culture

SKBR3 estrogen receptor-negative breast cancer cells were purchased from the American Type Culture Collection (ATCC, Manassas, Va.). Trastuzumab-resistant SKBR3 pools were developed by continuous exposure to trastuzumab (4 μg/ml for pool 1 and 8 μg/ml for pool 2) for 3 months, during which the medium was replaced every 4 days and cells were passaged when 70% confluency was reached. Cells regained morphology similar to that of the parental line after 3 months of trastuzumab exposure, and have since been maintained in 4 μg/ml trastuzumab. Trastuzumab resistance was confirmed by dose-response studies as described below. All cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Dose-response studies

SKBR3 cells and trastuzumab-resistant pools were seeded at 1×10^3 cells/well in 96-well dishes. After 24 h cells were treated in triplicate with twofold serial dilutions of trastuzumab, vinorelbine, or both drugs simultaneously at a fixed trastuzumab to vinorelbine ratio of 520.83 (ng/ml) to 1 (n*M*) using at least three doses above and three doses below the individual IC_{50} of each drug. After 5 days cells were exposed to the MTS reagent and optical density was measured in a microplate reader as directed by the manufacturer. All experiments were done in triplicate. Growth inhibition is expressed as the percentage of remaining viable cells in relation to untreated cultures set at 100% viability. DMSO alone did not affect cell viability. Combination indices (CI) were obtained using the commercial software package Calcusyn (Biosoft, Cambridge, UK) [12]. Statistically, drug synergy, addition, and antagonism are defined by CI values less than 1.0, equal to 1.0, or greater than 1.0, respectively. At least three doses above and three doses below the individual IC_{50} of each drug were tested in order to determine the median-effect using the computer software.

Cell cycle analysis

SKBR3 cells and trastuzumab-resistant pools were treated with 250 or 500 ng/ml of trastuzumab and/or vinorelbine at 0.48 or 0.96 n*M*. After 5 days of drug treatment, cells were fixed overnight in 70% ethanol and resuspended in propidium iodide (50 μg/ml) supplemented with RNase A (1 μg/ml). DNA content was measured using a FACScan cytometer (Becton Dickinson). All experiments were done in triplicate to determine standard deviations between individual experiments.

Results

The HER-2-overexpressing SKBR3 cells were treated with serial dilutions of trastuzumab and/or vinorelbine at a fixed ratio spanning the IC_{50} of each drug (IC_{50} trastuzumab 125 ng/ml, IC_{50} vinorelbine 1.5 n*M*). The viabilities of cells at the various drug concentrations are shown in Fig. 1A. Additionally, representative plots of the affected fraction of cells versus the CI value for the drug mixture are shown for parental and pool 1 cells in Fig. 1B. The dose-response assay demonstrated a CI value of 0.15, which is in agreement with the published CI value of 0.34 [8], indicating strong synergy between these agents in the SKBR3 parental cells. Trastuzumab-resistant pools were maintained in 4 μg/ml of trastuzumab at all times. Both pools remained 100% viable at concentrations of trastuzumab up to 32 μg/ml, a concentration at which all SKBR3 parental cells were killed (data not shown). The sensitivity of both pools to vinorelbine was very similar (IC_{50} pool 1 0.7 n*M*, IC_{50} pool 2 0.65 n*M*). In contrast to parental cells, neither of the trastuzumab-resistant pools demonstrated an increased dose-response to combined trastuzumab/vinorelbine versus vinorelbine alone. These results suggest that trastuzumab does not increase the cytostatic/cytotoxic effects of single-agent vinorelbine in breast cancer cells that have progressed on trastuzumab.

Flow cytometric cell cycle analysis was performed to determine if the results of the dose-response assays were a reflection of cytostatic or cytotoxic effects due to cell cycle arrest or apoptosis. Similar to other mitotic inhibitors, vinorelbine has been demonstrated to promote apoptosis in breast cancer cells [13]. The primary alteration observed in SKBR3 cells treated with a combination of 0.96 n*M* vinorelbine and 500 ng/ml trastuzumab was increased apoptosis. The percentage of sub-diploid SKBR3 parental cells doubled following treatment with vinorelbine (Fig. 2). Addition of trastuzumab with vinorelbine caused a further twofold increase in apoptotic cells for a greater than fourfold total rise in apoptosis relative to untreated cultures. A modest 1.5- to 2-fold rise in apoptosis was observed for the trastuzumab-resistant pools treated with vinorelbine alone. In contrast to the parental cells, neither pool demonstrated a significant increase in vinorelbine-mediated apoptosis when trastuzumab was added. Similar results were obtained with other dose combinations of trastuzumab and vinorelbine (data not shown).

Discussion

Trastuzumab offers clinical benefit to a subset of HER-2-overexpressing metastatic breast cancers [3, 4, 14]. However, the majority of these patients will demonstrate disease progression within 1 year. At that time, patients are generally maintained on trastuzumab with a second chemotherapeutic agent. Vinorelbine is synergistic with trastuzumab preclinically and improves clinical response rates to trastuzumab in trastuzumab-naive patients [10]. However, there is little clinical or scientific data addressing the continued use of trastuzumab in breast cancers that have progressed while on trastuzumab. A retrospective analysis of HER-2-overexpressing metastatic breast cancer patients who received a second trastuzumab-containing regimen

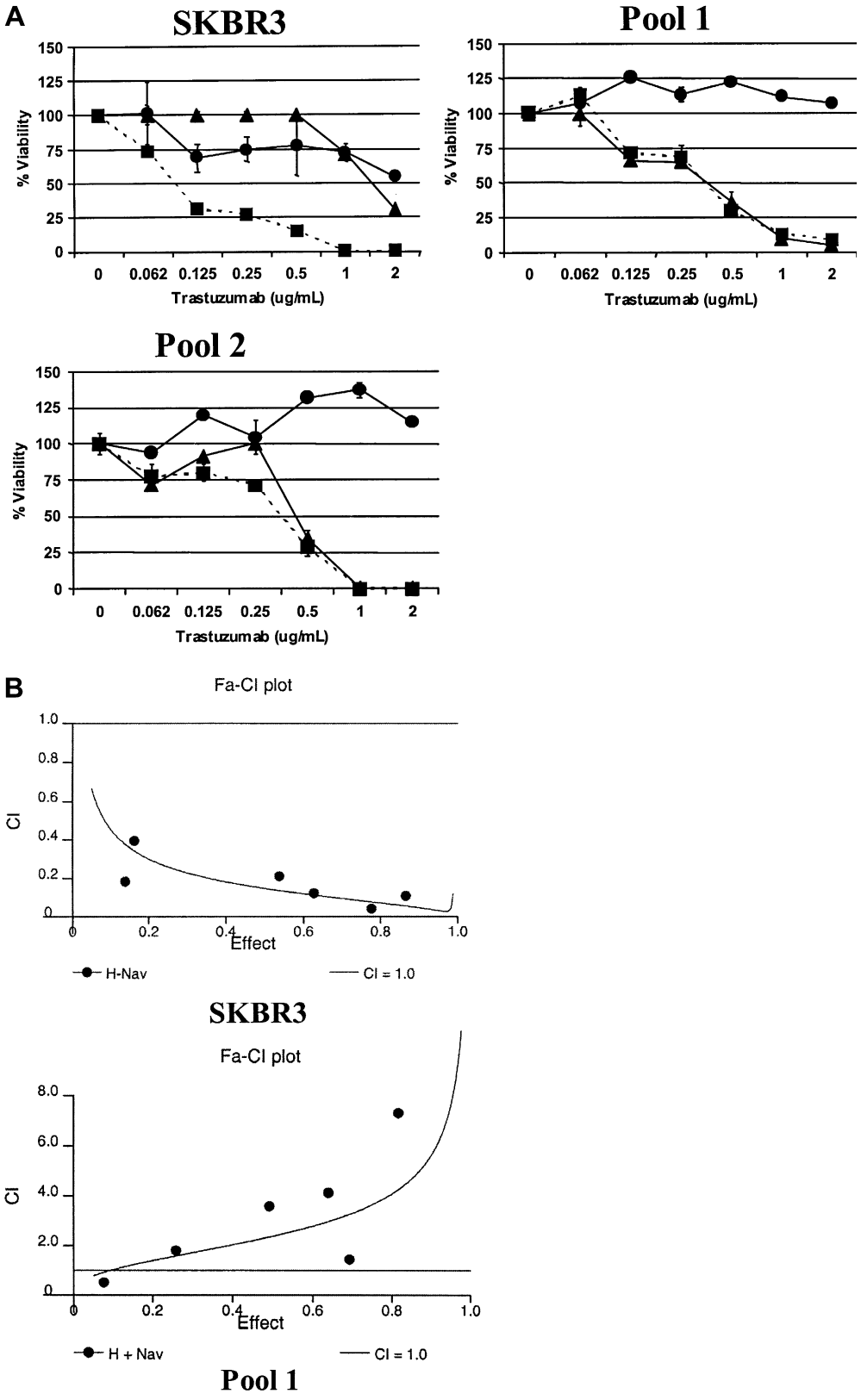

Fig. 1A, B Dose-response of SKBR3 parental and trastuzumab-resistant pools to trastuzumab. SKBR3 parental breast cancer cells and trastuzumab-resistant pools 1 and 2 were treated in triplicate with twofold serial dilutions of trastuzumab (●), vinorelbine (▲), or both drugs simultaneously (■ *with dotted line*) at a fixed trastuzumab to vinorelbine ratio of 0.520 (μg/ml) to 1 (n*M*). Concentrations of trastuzumab were 0.062, 0.125, 0.25, 0.5, 1 and 2 μg/ml and for navelbine were 0.12, 0.24, 0.48, 0.96, 1.92 and 3.84 n*M*. After 5 days cells were exposed to MTS reagent and optical density was measured in a microplate reader. **A** Growth inhibition is expressed as the percentage of viable cells relative to untreated cultures. **B** Plots of the fraction of cells affected by the drugs (*Fa*) versus the CI (*CI*) were created using the commercial software package Calcusyn (Biosoft, Cambridge, UK). At least three doses above and three doses below the individual IC_{50} of each drug were tested in order to determine the median effect using the computer software. Representative plots for SKBR3 and pool 1 cells are shown. Statistically, drug synergy, addition, and antagonism are defined by CI values less than 1.0, equal to 1.0, or greater than 1.0, respectively. SKBR3 cells show CI values below 1.0, indicating synergy, while CI values of pool 1 cells are above 1.0, indicating antagonism between the drugs

upon progressing on the first trastuzumab-based regimen suggested that some patients may continue to respond to trastuzumab when a new chemotherapy is given, while others do not [15]. Together, this retrospective analysis and our in vitro study strongly support the need for a randomized clinical trial evaluating the efficacy of a second trastuzumab-containing regimen.

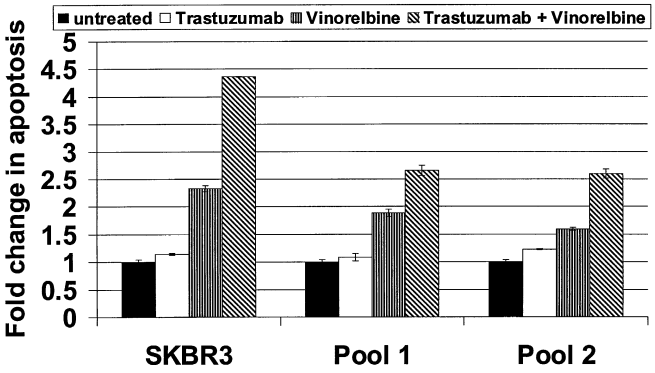

Fig. 2 Fold change in apoptosis in SKBR3 parental and trastuzumab-resistant pools treated with trastuzumab and/or vinorelbine. SKBR3 cells and trastuzumab-resistant pools were treated with 500 ng/ml of trastuzumab and/or 0.96 n*M* vinorelbine. After 5 days of drug treatment, cells were fixed, resuspended in propidium iodide, and analyzed for DNA content using a FACScan cytometer. Fold changes in sub-diploid content (sub-G_1) relative to untreated cells are shown. Standard deviation bars from three independent assays are shown for all experimental groups

We created an in vitro model of trastuzumab resistance using the established HER-2-overexpressing breast cancer cell line SKBR3, in which trastuzumab/vinorelbine synergy has previously been documented [8]. We confirmed synergy between these agents in our parental line and demonstrated that the response is primarily cytotoxic as illustrated by enhanced apoptosis. In contrast to the parental cells, trastuzumab-resistant SKBR3 derivatives did not display growth inhibition in response to trastuzumab. However, pools did demonstrate a slightly increased response to single-agent vinorelbine versus parental cells. Addition of trastuzumab to vinorelbine did not significantly increase cytotoxicity in either pool. These results suggest that vinorelbine alone induces the apoptosis observed in the trastuzumab-resistant cells exposed to trastuzumab/vinorelbine combinations, and argues that continuous treatment of resistant breast cancers with trastuzumab may be ineffective.

The molecular mechanisms guiding the development of trastuzumab resistance are currently unknown. Multiple mechanisms are likely to exist, and may include mutations in HER-2 that disrupt antibody binding, alterations in downstream signaling molecules, or compromised immune function in advanced cancer patients [16, 17, 18]. These mechanisms may vary from one breast tumor or patient to the next. Thus, it is possible that although we did not observe an added benefit from trastuzumab in our pools, other trastuzumab-resistant pools derived from other HER-2-overexpressing lines may demonstrate an enhanced response to combination trastuzumab/vinorelbine versus vinorelbine alone. Hence, elucidating the mechanisms by which these cancers develop resistance to trastuzumab is imperative. In addition, it is possible that biological mechanisms such as immune-mediated responses or angiogenesis may be necessary to achieve responses to second trastuzumab-containing regimens. As such mechanisms may not be fully appreciated in vitro, randomized clinical testing of second trastuzumab-containing regimens versus chemotherapy alone is critical.

Another hypothesis concerning treatment of trastuzumab-resistant breast cancers is that temporarily interrupting trastuzumab therapy and administering chemotherapy alone may eventually restore sensitivity to trastuzumab. Importantly, interruption of trastuzumab maintenance for 2 months (16 passages) did not restore sensitivity of our pools to trastuzumab. This would argue against a benefit from the continuous treatment of trastuzumab-resistant cancers with trastuzumab. However, it is possible that these pools may eventually regain sensitivity.

While the translation of in vitro data into clinical situations is associated with obvious problems, the results of this study support the need for a more detailed analysis of the contribution of continued trastuzumab therapy in patients who have progressed while on trastuzumab. A randomized multicenter trial was launched at UT M. D. Anderson Cancer Center to explore the efficacy of vinorelbine alone versus combination trastuzumab/vinorelbine in patients progressing on trastuzumab plus taxane. Unfortunately, this clinical trial was closed prematurely because of poor accrual. This was attributed in part to the strong belief that trastuzumab should be continued upon progression. Determining whether trastuzumab is still useful in trastuzumab-resistant cancers is important as patients could potentially avoid the toxicity, costs, and inconvenience of continued infusions of trastuzumab if there is limited benefit. A randomized clinical trial to test this hypothesis should be conducted in patients with HER-2-overexpressing metastatic breast cancer who are progressing on trastuzumab-based therapy.

References

1. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL (1987) Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235:177
2. Baselga J, Tripathy D, Mendelsohn J, Baughman S, Benz CC, Dantis L, Sklarin NT, Seidman AD, Hudis CA, Moore J, Rosen PP, Twaddell T, Henderson IC, Norton L (1996) Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. J Clin Oncol 14:737
3. Cobleigh MA, Vogel CL, Tripathy D, Robert NJ, Scholl S, Fehrenbacher L, Wolter JM, Paton V, Shak S, Lieberman G, Slamon DJ (1999) Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER-2 overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. J Clin Oncol 17:2639

4. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J, Norton L (2001) Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 344:783
5. Seidman AD, Fornier M, Esteva FJ, Tan L, Kaptain S, Bach A, Panageas KS, Arroyo C, Valero V, Currie V, Gilewski T, Theodoulou M, Moynahan ME, Moasser M, Sklarin N, Dickler M, D'Andrea G, Cristofanilli M, Rivera E, Hortobagyi GN, Norton L, Hudis C (2001) Weekly trastuzumab and paclitaxel therapy for metastatic breast cancer with analysis of efficacy by HER2 immunophenotype and gene amplification. J Clin Oncol 19:2587
6. Esteva FJ, Valero V, Booser D, Guerra LT, Murray JL, Pusztai L, Cristofanilli M, Arun B, Esmaeli B, Fritsche HA, Sneige N, Smith TL, Hortobagyi GN (2002) Phase II study of weekly docetaxel and trastuzumab for patients with HER-2-overexpressing metastatic breast cancer. J Clin Oncol 20:1800
7. Esteva FJ, Valero V, Pusztai L, Boehnke-Michaud L, Buzdar AU, Hortobagyi GN (2001) Chemotherapy of metastatic breast cancer: what to expect in 2001 and beyond. Oncologist 6:133
8. Pegram MD, Lopez A, Konecny G, Slamon DJ (2000) Trastuzumab and chemotherapeutics: drug interactions and synergies. Semin Oncol 27:5
9. Burstein HJ, Kuter I, Campos S.M, Gelman RS, Tribou L, Parker LM, Manola J, Younger J, Matulonis U, Bunnell CA, Partridge AH, Richardson PG, Clarke K, Shulman LN, Winer EP (2001) Clinical activity of trastuzumab and vinorelbine in women with HER-2 overexpressing metastatic breast cancer. J Clin Oncol 19:2722
10. Burstein HJ, Harris LN, Marcom PK, Lambert-Falls R, Havlin K, Overmoyer B, Friedlander RJ, Gargiulo J, Strenger R, Vogel CL, Ryan PD, Ellis MJ, Nunes RA, Bunnell CA, Campos SM, Hallor M, Gelman R, Winer EP (2003) Trastuzumab and vinorelbine as first-line therapy for HER2-overexpressing metastatic breast cancer: multicenter phase II trial with clinical outcomes, analysis of serum tumor markers as predictive factors, and cardiac surveillance algorithm. J Clin Oncol 21:2889
11. Jahanzeb M, Mortimer JE, Yunus F, Irwin DH, Speyer J, Koletsky AJ, Klein P, Sabir T, Kronish L (2002) Phase II trial of weekly vinorelbine and trastuzumab as first-line therapy in patients with HER2(+) metastatic breast cancer. Oncologist 7:410
12. Chou TC, Talalay P (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22:27
13. Hahm HA, Dunn VR, Butash KA, Deveraux WL, Woster PM, Casero RA Jr, Davidson NE (2001) Combination of standard cytotoxic agents with polyamine analogues in the treatment of breast cancer cell lines. Clin Cancer Res 7:391
14. Vogel CL, Cobleigh MA, Tripathy D, Gutheil JC, Harris LN, Fehrenbacher L, Slamon DJ, Murphy M, Novotny WF, Burchmore M, Shak S, Stewart SJ, Press M (2002) Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. J Clin Oncol 20:719
15. Mackey J, Gelmon KA, Verma S, Gertler SZ, Bangemann N, Klimo P, Schneeweib A, Soulieres D, Bell R (2002) Continued use of Herceptin after disease progression in women with HER2 positive metastatic breast cancer: results from a retrospective analysis of 105 cases. Proc Am Soc Clin Oncol 21:207
16. Lu YH, Zi XL, Zhao YH, Mascarenhas D, Pollak M (2001) Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (Herceptin). J Natl Cancer Inst 93:1852
17. Albanell J, Baselga J (2001) Unraveling resistance to trastuzumab (Herceptin): insulin-like growth factor-I receptor, a new suspect. J Natl Cancer Inst 93:1830
18. Yu DH (2001) Mechanisms of ErbB2-mediated paclitaxel resistance and trastuzumab-mediated paclitaxel sensitization in ErbB2-overexpressing breast cancers. Semin Oncol 28:12