Survivin Expression Is Regulated by Coexpression of Human Epidermal Growth Factor Receptor 2 and Epidermal Growth Factor Receptor via Phosphatidylinositol 3-Kinase/AKT Signaling Pathway in Breast Cancer Cells

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Abstract

Survivin, a member of the inhibitor of apoptosis protein family, is widely expressed in a variety of human cancer tissues. Survivin inhibits activation of caspases, and its overexpression can lead to resistance to apoptotic stimuli. In this study, survivin protein expression was assessed by immunohistochemical staining of 195 invasive breast cancer specimens. Overall, 79.5% of the tumors were positive for survivin. The expression of epidermal growth factor receptor (EGFR) family, human epidermal growth factor receptor 2 (HER2) and EGFR, was also examined in 53 cases, and consequently, it was indicated that survivin positivity might be correlated with the coexpression of HER2 and EGFR. To clarify the regulatory mechanism of survivin expression in breast cancer cells, the effect of HER2 and/or EGFR expression on the survivin levels was examined. It was revealed that the survivin protein level was up-regulated by the coexpression of HER2 and EGFR, leading to the increased resistance against etoposide-induced apoptosis in breast cancer cells. Conversely, survivin levels and apoptosis resistance were decreased when cells were treated with HER2-specific inhibitor, Herceptin. Although Herceptin could down-regulate both phosphatidylinositol 3-kinase (PI3K)/AKT signal and mitogen-activated protein/extracellular signal-related kinase (ERK) kinase 1 (MEK1)/ERK signal in HER2-positive breast cancer cells, PI3K-specific inhibitor but not MEK1-specific inhibitor could decrease the survivin levels. The present study clarified the regulatory mechanism of HER2 in the expression of survivin protein in breast cancer cells. (Cancer Res 2005; 65(23): 11018-25)

Introduction

Survivin was identified as a member of the inhibitor of apoptosis protein (IAP) family with single baculovirus IAP repeat domain (1). Survivin protein has a molecular weight of 16.5 kDa and is the smallest member of the mammalian IAP family. Survivin has the capability to inhibit caspase-3, caspase-7, and caspase-9 in cells, and its overexpression can lead to resistance to cell death caused by various apoptotic stimuli (2). Survivin is present during fetal development but undetectable in terminally differentiated normal adult tissues (3). Importantly, survivin is abundantly expressed in most cancer cells and tissues, including colorectal cancer, lung cancer, gastric cancer, bladder cancer, melanoma, hepatocellular carcinoma, and malignant lymphoma (4–11). There are a number of studies showing that the overexpression of survivin was associated with poor clinical prognosis or resistance to the chemotherapy in certain tumors. It has been reported that survivin was expressed in many cases of breast cancer as well (12–14). However, the molecular mechanism of the up-regulation of survivin in breast cancer cells has not been clarified yet.

Human epidermal growth factor receptor 2 (HER2, also known as erbB2) is frequently overexpressed in breast cancer tissues (15). HER2 and its relatives HER1 (epidermal growth factor receptor, EGFR), HER3, and HER4 belong to the EGFR family of receptor tyrosine kinases (16, 17). Several studies have shown an association of HER2 overexpression in human breast carcinomas with poor clinical prognosis and therapeutic response. EGFR and other EGFR members are also expressed in breast cancers, and there are reports showing an association between coexpression of distinct EGFR members and poor prognosis (17, 18). The molecular basis for this association is still unclear, although it has been suggested to involve increased proliferation, angiogenesis, and invasive potential. HER2 currently represents one of the most appropriate molecular targets for cancer-specific therapy (15). Indeed, trastuzumab (Herceptin), a monoclonal antibody (mAb) directed against the extracellular domain of HER2, is therapeutically effective in HER2-positive breast carcinomas.

The present study addressed the molecular mechanism of survivin expression in breast cancer cells and tissues. Based on the immunohistologic observation suggesting the positive correlation between survivin expression and the expression of EGFR members (EGFR and HER2), we assumed that the survivin levels might be regulated by coexpression of distinct EGFR members. Here, we present results that experimentally support this hypothesis. Gene transfer–mediated up-regulation of both HER2 and EGF led to an increase of survivin expression, and Herceptin-mediated HER2 suppression down-regulated the survivin expression. The HER2 signaling pathway involved in the regulation of survivin expression was determined by the use of specific signaling inhibitors. The present study clarified for the first time the significance and the regulatory mechanism of the EGFR members in survivin expression in breast cancer cells.

Materials and Methods

Tissue samples. This study was approved under our institutional guidelines for the use of human subjects in research. Patients and their families gave informed consent for the use of tissue specimens in research.

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Table 1. Survivin expression in breast cancer

Breast cancer	Survivin negative, *n* (%)	Survivin positive, *n* (%)	Total cases
Invasive ductal cancer	33 (20)	132 (80)	165
Invasive lobular cancer	7 (23.3)	23 (76.7)	30
Total	40 (20.5)	155 (79.5)	195

The breast cancer specimens used in this study were obtained from patients who underwent surgical resection at the Department of Surgery, Sapporo Medical University Hospital. Paraffin-embedded samples of 195 invasive breast cancers, collected over the past 10 years, were selected from the surgical pathology files of the Division of Clinical Pathology, Sapporo Medical University Hospital. The study comprised 165 invasive ductal carcinomas and 30 invasive lobular carcinomas.

Immunohistochemical staining. Immunohistochemical staining was done on formalin-fixed, paraffin-embedded sections. Four- to 5-μm-thick sections were cut, deparaffinized in xylene, and rehydrated in graded alcohol. Antigen retrieval was done by boiling for 20 minutes in a microwave oven (Micro MED T/T Mega, 800 W) in a preheated 0.01 mol/L concentration of sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by 3% hydrogen peroxide in ethanol for 5 minutes. Slides were incubated for 1 hour with the mAb: anti-HER2 oncoprotein CB11 (1:50 dilution, Novocastra Laboratories, Newcastle upon Tyne, United Kingdom), anti-EGFR EGFR113 (1:10 dilution, Novocastra Laboratories), or rabbit polyclonal antibody against survivin (1:100 dilution, NOVUS Biologicals, Littleton, CO). After the incubation, the slides were washed thrice with PBS and incubated for 30 minutes with MAX-PO (MULTI) secondary antibody mixture (Nichirei, Tokyo, Japan). After washing thrice with PBS, staining was done by incubation for 1 to 2 minutes with 3,3′-diaminobenzidine used as the chromogen, and counterstaining was done with Myer's hematoxylin.

Evaluation of immunohistochemistry results. Survivin immunoreactivity was evaluated semiquantitatively according to the percentage of cells showing distinct nuclear and/or diffuse cytoplasmic immunohistochemical reaction. Nuclear and/or cytoplasmic immunoreactivities were assessed in at least five high-power fields at ×400 magnification and assigned to one of the following categories: 0, <5%; 1, 5% to 20%; 2, >20%. Because tumors showed heterogeneous staining, the dominant pattern was used for scoring. A cutoff value of >20% was defined as a positive staining, and tumors with a score of 0 or 1 were considered negative.

The intensity of HER2 immunoreactivity to tumor cell membrane was scored according to DAKO (Carpinteria, CA) criteria as follows: 0, any detectable membrane signal in <10% of the tumor cells; 1, weak incomplete membrane stain in >10% of the tumor cells; 2 and 3, moderate to strong membrane positivity in >10% of the tumor cells (19). In the present study, tumors with a score of 0 or 1 were defined as negative, and those with a score of 2 or 3 were defined as positive.

EGFR immunoreactivity was evaluated according to the same criteria as used for survivin, except for in the case of membrane and/or cytoplasmic immunoreactivities to tumor cells.

Cell lines and culture conditions. The human embryonic kidney cell line 293T and human breast cancer cell lines (MCF7 and SKBR-3) were obtained from American Type Culture Collection (Manassas, VA). SKBR-3 cells contain ~10 copies of *c-erbB2* gene and have an overexpression of HER2 (20). Human breast cancer cell lines (HMC-1 and HMC-2) were established and characterized in our laboratory (21, 22). All of these cells were cultured in DMEM (Sigma-Aldrich, St. Louis, MO) with 10% heat-inactivated fetal bovine serum (Filtron, Brooklyn, Australia) at 37°C in humidified 5% CO_2 atmosphere.

Reagents. The humanized anti-HER2 mAb trastuzumab was purchased from Chugai pharmaceutical Co., (Tokyo, Japan). LY294002, a specific

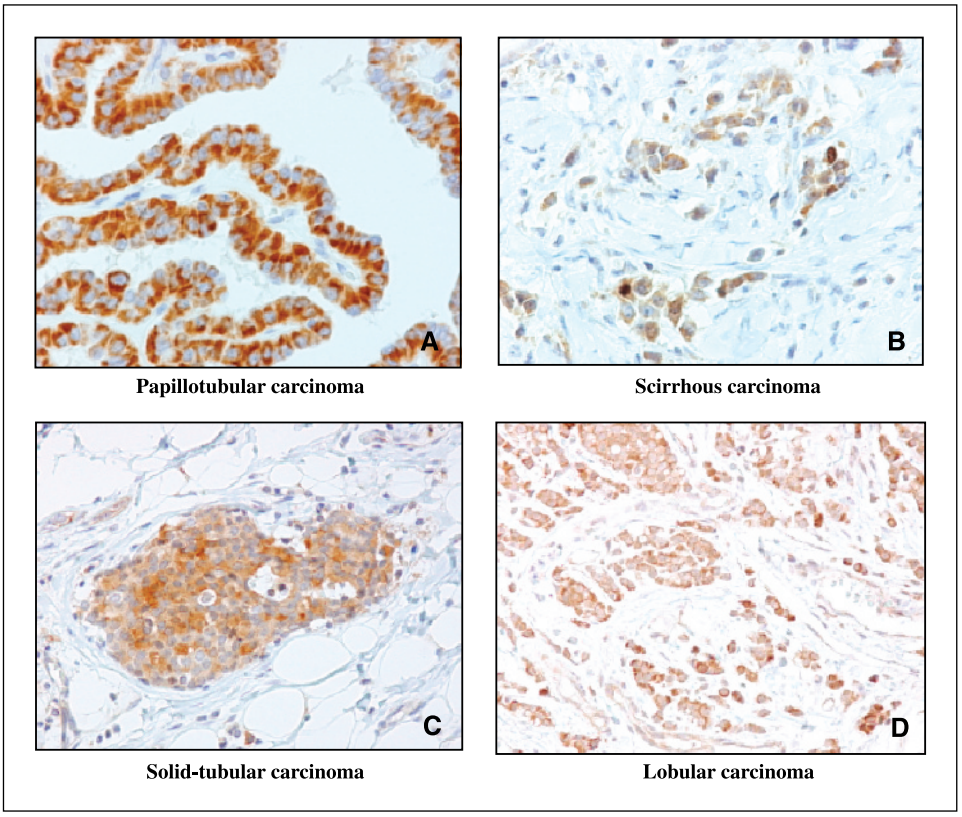

Figure 1. Immunohistochemical staining of survivin in paraffin-embedded tissue specimens of invasive breast cancer. Representative photographs of immunohistochemical staining of survivin in various histologic types of invasive breast cancer specimens: papillotubular carcinoma (*A*), scirrhous carcinoma (*B*), solid-tubular carcinoma (*C*), lobular carcinoma (*D*). Survivin was detectable in the cytosol and/or nucleus of tumor cells. Magnification, ×100.

Table 2. Relationship between survivin expression and HER-2/EGFR expression in breast cancer

	Survivin-positive cases, *n* (%)		
HER-2$^-$, EGFR$^-$	0/5 (0)		
HER-2$^-$, EGFR$^+$	3/5 (60)	3/10 (30)	
HER-2$^+$, EGFR$^-$	2/10 (20)		38/53 (71.7)
HER-2$^+$, EGFR$^+$	33/33 (100)	35/43 (81.4)	

inhibitor of the p110 catalytic subunit of phosphatidylinositol 3-kinase (PI3K) and PD98059, a specific inhibitor of mitogen-activated protein (MAP)/extracellular signal-related kinase (ERK) kinase 1 (MEK1) were purchased from Cell Signaling Technology, Inc. (Beverly, MA). The inhibitors were stored in aliquots at −20°C at a concentration of 50 mmol/L in DMSO. Etoposide (VP-16) was purchased from Sigma-Aldrich and was stored as 10 mg/mL stock in DMSO.

Plasmids and gene transfection. Complementary DNA encoding full-length HER2 or EGFR was kindly provided by Dr. Mark I. Greene (University of Pennsylvania). The cDNA was cloned into pcDNA3 mammalian expression vector (Invitrogen, San Diego, CA). The resulting expression plasmids pcDNA3-HER2 (pHER2) and pcDNA3-EGFR (pEGFR) were used for the transfection. Cells (1×10^6) were transfected with the plasmid by using LipofectAMINE 2000 (LF2000) reagent (Invitrogen) according to the manufacturer's protocol. For stable transfection, HMC-2 cells were transfected with pHER2 plasmid and replated in a selection medium containing 1.0 mg/mL Geneticin (Invitrogen) on the second day after the transfection. The culture medium was replaced twice weekly until colonies of G418-resistant clones arose. Stable pHER2-transfected cells were subcloned and designated as HMC-2-HER2 clone7, HMC-2-HER2 clone9, and HMC-2-HER2 clone10.

Western blotting. Cultured cells were washed in ice-cold PBS, homogenized in ice-cold radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 1.0% NP40, 0.5% deoxycholic acid, 0.1% SDS, 50 mmol/L Tris-HCl (pH 8), protease inhibitor cocktail (Complete, Roche Diagnostics, Basel, Switzerland)] for 30 minutes, and clarified by centrifugation at $12,000 \times g$ for 20 minutes at 4°C. The total protein concentrations of the supernatants were determined by using micro bicinchoninic acid assay reagents (Pierce, Rockford, IL). The lysates were boiled for 5 minutes with SDS sample buffer and then separated by 7.5% or 12% SDS-PAGE. The proteins were transferred electrophoretically to a polyvinylidene fluoride membrane (Immobilon-P, Millipore, Billerica, MA). The membranes were incubated with blocking buffer (5% nonfat dry milk in PBS) at room temperature and then incubated for 60 minutes with the following mAb or polyclonal antibody: mouse anti-human survivin mAb (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human c-erbB2 antibody (Oncogene Science, Uniondale, NY), mouse anti-human EGFR mAb (Novocastra Laboratories), rabbit anti-human AKT antibody (New England Biolabs, Beverly, MA), rabbit anti-human phosphorylated protein kinase B (pSer^{473}-AKT; Sigma-Aldrich), rabbit anti-human ERK1/2 antibody (Sigma-Aldrich), mouse anti-human phosphorylated ERK (pERK; pTyr^{204}-ERK) mAb M3682 (Sigma-Aldrich), or mouse anti-β-actin mAb AC-15 (Sigma-Aldrich). After washing thrice with wash buffer (0.1% Tween 20, PBS), the membrane was reacted with peroxidase-labeled secondary antibody (peroxidase-labeled goat anti-mouse IgG antibody or peroxidase-labeled goat anti-rabbit IgG antibody; KPL, Gaithersburg, MD) for 2 hours. Finally, the signal was visualized by using an enhanced chemiluminescence detection system (Amersham Life Science, Arlington Heights, IL) according to the manufacturer's protocol.

Flow cytometry. Approximately 2×10^6 cells were incubated with FITC-labeled anti-HER2 mAb antibody (Becton Dickinson, San Jose, CA) at room temperature for 30 minutes, washed twice with PBS, and resuspended in 1 mL of PBS. Flow cytometric detection of FITC-positive cells was done using a FACScan flow cytometer and CELLQuest software (Becton Dickinson). An average of 10,000 events were analyzed.

Apoptosis assay. HMC-2 wild type, HMC-2-HER2 clone7, HMC-2-HER2 clone9, or SKBR-3 cells were cultured in DMEM containing the indicated concentration of VP-16 in the presence or absence of 20 μg/mL Herceptin for 24 hours. After washing twice with ice-cold PBS, 2×10^6 cells were stained with Annexin V/FULUOS staining solution (Roche Diagnostics, Mannheim, Germany) containing FITC-labeled Annexin V and propidium iodide according to the manufacturer's protocol. After 15 minutes of incubation at room temperature, cells were resuspended in 1.0 mL incubation buffer and analyzed using a flow cytometer (FACScan and CELLQuest software, Becton Dickinson).

Results

Immunohistochemical detection of survivin, human epidermal growth factor receptor 2, and epidermal growth factor receptor in breast cancer tissues. Formalin-fixed, paraffin-embedded breast cancer specimens were analyzed for the expression of survivin, HER2, and EGFR by immunohistochemical staining. Of the 195 breast cancer specimens, survivin protein was

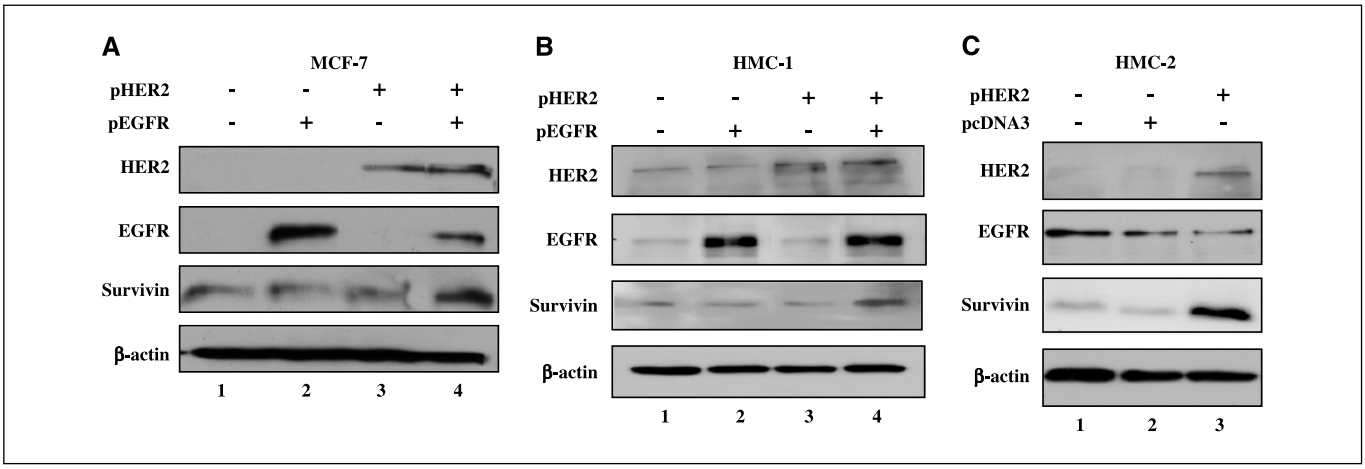

Figure 2. Coexpression of HER2 and EGFR up-regulates survivin protein level in breast cancer cells. Breast cancer cell lines MCF-7 (*A*), HMC-1 (*B*), and HMC-2 (*C*) were transfected with pHER2 and/or pEGFR expression plasmids. Forty-eight hours after transfection, cell lysates were collected and examined for HER2, EGFR, survivin, and β-actin protein levels by Western blotting. In the case of HMC-2 cells (*C*), pEGFR was not transfected because they were expressed as endogenous EGFR.

positively stained in the nuclei and/or cytoplasm of tumor cells in 155 cases (79.5%). There was no significant difference in the survivin positivity between invasive ductal carcinoma (80.0%) and invasive lobular carcinoma (76.7%; Table 1). In Fig. 1, the representative survivin staining of breast cancer specimens with four distinct histopathologic types is shown. Survivin staining was negative in the surrounding noncancerous mammary gland cells and stromal cells. To clarify the association of survivin expression with other breast cancer–related molecules, 53 specimens were examined for the expression of HER2 and EGFR by immunostaining. It was revealed that survivin staining was positive in 81.4% of HER2-positive cases, whereas it was negative in 70% of HER2-negative cases (Table 2). Interestingly, survivin staining was positive in all the EGFR-positive cases among the HER2-positive cases. In contrast, it was negative in all the cases of both HER2 and EGFR-negative breast cancer. It was indicated that survivin expression might be correlated with the expression of either or both HER2 and EGFR.

Coexpression of human epidermal growth factor receptor 2 and epidermal growth factor receptor up-regulates survivin protein level in breast cancer cells. To determine whether expression of HER2 or EGFR affects the expression of survivin in breast cancer cells, gene transfer–mediated expression of HER2 and/or EGFR was done using three breast cancer cell lines. MCF-7, HMC-1, and HMC-2 cells were transfected with pHER2 and/or pEGFR expression plasmids. Forty-eight hours after the transfection, cell lysates were collected and examined for survivin, HER2, and EGFR protein levels by Western blotting (Fig. 2). MCF-7 cells displayed neither HER2 nor EGFR expression (Fig. 2*A, lane 1*). When either HER2 or EGFR was expressed in MCF-7 cells, the survivin protein level did not change (Fig. 2*A, lanes 2* and *3*). However, it was markedly increased when both HER2 and EGFR were expressed (Fig. 2*A, lane 4*). A similar result was obtained from the transfection study using HMC-1 cells, which had low endogenous HER2 expression but did not express EGFR expression (Fig. 2*B*). Increases in levels of both HER2 and EGFR could lead to up-regulation of the survivin level, whereas an increase in either one only failed to produce an increase. As shown in Fig. 2*C*, HMC-2 cells expressed endogenous EGFR but not HER2 (Fig. 2*C, lane 1*). Therefore, cells were transfected with either pcDNA3 vector or pHER2. It was shown that transfection with pHER2 alone resulted in an increase of survivin level in HMC-2 cells (Fig. 2*C, lane 3*). These data imply that survivin protein level can be up-regulated by the coexpression of HER2 and EGFR, and that expression of HER2 without EGFR might be insufficient for the up-regulation.

Stable pHER2-transfectant clones of HMC-2 have increased survivin levels. Next, we established stable pHER2-transfectant clones by transfection, G418 selection, and subcloning of HMC-2 cells. The resulting HMC-2-HER2 clones (clone7, clone9, and clone10) were characterized by Western blotting for levels of HER2, EGFR, and survivin proteins. Although there seemed to be some clonal variations in the HER2 levels and EGFR levels, all three clones had higher levels of HER2 and EGFR compared with wild-type HMC-2 cells. As shown in Fig. 3, survivin levels were markedly increased in all of these HMC-2-HER2 clones. The data were consistent with the result of the transient transfection study (Fig. 2*C*).

HMC-2-HER2 clones are resistant against VP-16-induced apoptosis. To examine if the increased survivin levels in the HMC2-HER2 clones confer resistance to apoptosis on the cells, cells were cultured with VP-16 for 24 hours and analyzed for the apoptotic rates. A quantitative apoptosis analysis was done by using Annexin V/propidium iodide double staining assay. In this assay, Annexin V–positive and propidium iodide–negative cells represent early apoptotic cells, which were counted by flow cytometer. In the presence of 10 μmol/L VP-16, ~20% of wild-type HMC-2 cells fell into apoptosis, whereas apoptotic rates of HMC-HER2 clones (clone7 and clone9) were limited to only 8% to 10% (Fig. 4). These data indicate that the survivin protein induced by the overexpression of HER2 can exert an antiapoptotic effect on the HMC-2-HER2 cells. It may explain, at least in part, the molecular mechanism of the poorer prognosis in HER2-positive breast cancer patients.

Herceptin down-regulates survivin protein levels in human epidermal growth factor receptor 2–positive breast cancer cells. Herceptin is a humanized mAb reacting against the extracellular domain of HER2. It can reduce the cell surface levels of HER2, suppress intracellular signaling of HER2 activation, and induce apoptosis in certain breast cancer cells with *c-erbB2* gene amplification (15). We next examined if Herceptin can down-regulate the survivin levels in HER2-positive breast cancer cells. Flow cytometric analysis was done for HER2 expression on the cell surface. High levels, moderate levels, and low levels of HER2 were detected on the cell surface of SKBR-3, HMC2-HER2 clone9, and HMC-1, respectively (Fig. 5). SKBR-3 cells are reported to have 10 copies of *c-erbB2* gene amplification (20). In contrast, HER2 could not be detected on wild-type HMC-2 cells. These cell surface HER2 levels were almost compatible with the Western blotting data representing whole HER2 proteins in cells. When the cells were incubated with 20 μg/mL of Herceptin in the medium for 48 hours, survivin protein levels were decreased in all three HER2-positive cells but not in HER2-negative HMC-2 wild-type cells (Fig. 5). The data clearly show that Herceptin can down-regulate the survivin levels in HER2-positive breast cancer cells.

Herceptin reduces resistance to etoposide-induced apoptosis in human epidermal growth factor receptor 2–positive cancer cells. To know whether Herceptin-induced decrease in

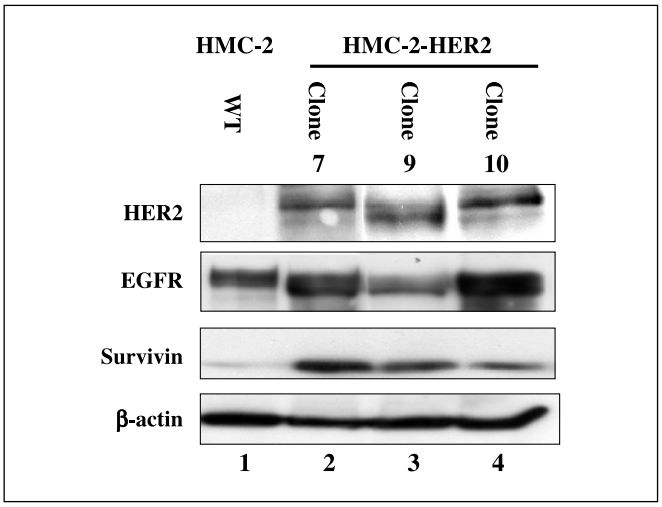

Figure 3. Western blotting analysis of stable pHER2-transfectant clones of HMC-2 cells. Stable pHER2-transfectant clones of HMC-2 cells were established by the transfection, G418 selection, and subcloning. The resulting HMC-2-HER2 clones (clone7, clone9, and clone10) were characterized by Western blotting for the levels of HER2, EGFR, survivin, and β-actin proteins.

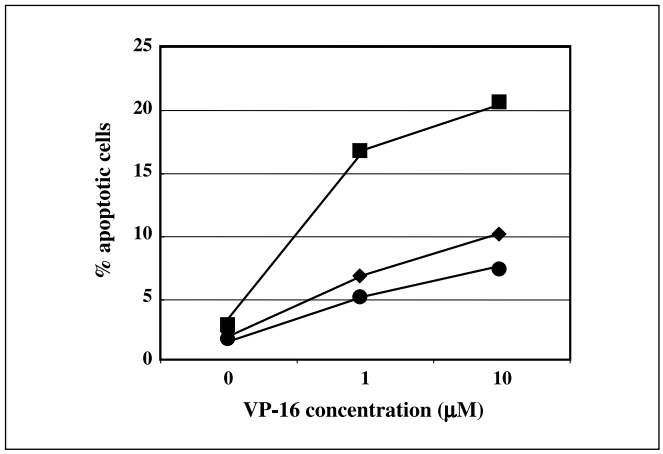

Figure 4. VP-16-induced apoptosis of HMC-2-HER2 clones. HMC-2 wild type (■), HMC-2-HER2 clone7 (♦), or HMC-2-HER2 clone9 (●) cells were cultured with the indicated concentrations of VP-16 for 24 hours and analyzed for their apoptotic rates. A quantitative apoptosis analysis was done by using Annexin V/propidium iodide double staining assay. Annexin V–positive and propidium iodide–negative cells represent early apoptotic cells, which were counted by flow cytometer.

survivin level can affect apoptosis resistance of HER2-positive tumor cells, VP-16-induced apoptosis assay was examined by using HMC-2-HER2 clone9 and SKBR-3 cells (Fig. 6). Cells were cultured for 24 hours in a medium containing 0, 1, 10, or 100 μmol/L VP-16 with or without 20 μg/mL of Herceptin followed by Annexin V/propidium iodide double staining assay. Annexin V–positive and propidium iodide–negative early apoptotic cells were counted by flow cytometer. In the absence of Herceptin, the apoptotic cell rate of HMC-2-HER2 clone9 cells was limited to ~10% after the stimulation with 10 μmol/L VP-16. However, it increased to 17% in the presence of Herceptin (Fig. 6*A*). It was noted that the rate was almost compatible with the apoptotic cell rate of wild-type HMC-2 cells stimulated with 10 μmol/L VP-16 (Fig. 4). A similar effect of Herceptin was observed in the examination using SKBR-3 cells, which had high levels of endogenous HER2 and EGFR (data not shown). Herceptin was capable of reducing the resistance against VP-16-induced apoptosis in endogenous HER2-positive SKBR-3 cells (Fig. 6*B*) as well as in the *c-erbB2* gene-transfected HMC-2 cells.

Herceptin down-regulates intracellular human epidermal growth factor receptor 2 signaling in SKBR-3 cells. To elucidate the mechanism by which HER2 regulates survivin expression, intracellular signaling molecules associated with HER2 activation were examined. HER2 is a receptor type tyrosine kinase that has distinct intracellular signaling pathways, including MAP kinase (MAPK) pathways and PI3K/AKT pathway, which can be probed by the specific anti-phosphoprotein antibodies. Because SKBR-3 cells have moderate levels of pERK and phosphorylated AKT (pAKT) constitutively, the phosphoprotein levels in SKBR-3 cells were examined for the effect of Herceptin by using anti-pERK ($pSer^{473}$) antibody and anti-pAKT ($pTyr^{204}$) antibody. SKBR-3 cells were incubated with varying concentrations of Herceptin (0, 4, 10, and 20 μg/mL) for 48 hours, and the cell lysates were subjected to Western blotting. Total protein levels of AKT and ERK did not change in the presence of Herceptin. However, levels of pAKT and pERK were decreased in the presence of 10 or 20 μg/mL of Herceptin (Fig. 7), indicating that Herceptin was capable of inhibiting both MEK1/ERK pathway and PI3K/AKT pathway of intracellular HER2 signal. The same concentration of Herceptin treatment resulted in down-regulation of survivin expression in SKBR-3 cells (Fig. 7). The data implied that survivin level might be regulated by MEK1/ERK pathway and/or PI3K/AKT pathway of HER2 signal.

Survivin expression is regulated by phosphatidylinositol 3-kinase/AKT pathway of intracellular human epidermal growth factor receptor 2 signals. To determine further the HER2 signaling pathway involved in the regulation of survivin protein level, the effects of specific signal inhibitors on the survivin expression were examined. SKBR-3 cells were incubated with either PI3K inhibitor LY294002 or MEK1 inhibitor PD98059 for the indicated hours, and then levels of phosphoproteins and survivin were analyzed by Western blotting. The survivin level was decreased after 6 hours of incubation with the PI3K inhibitor but not with the MEK1 inhibitor, whereas each inhibitor was able to reduce the specific downstream phosphoprotein levels, pAKT and pERK, respectively (Fig. 8*A* and *B*). It was

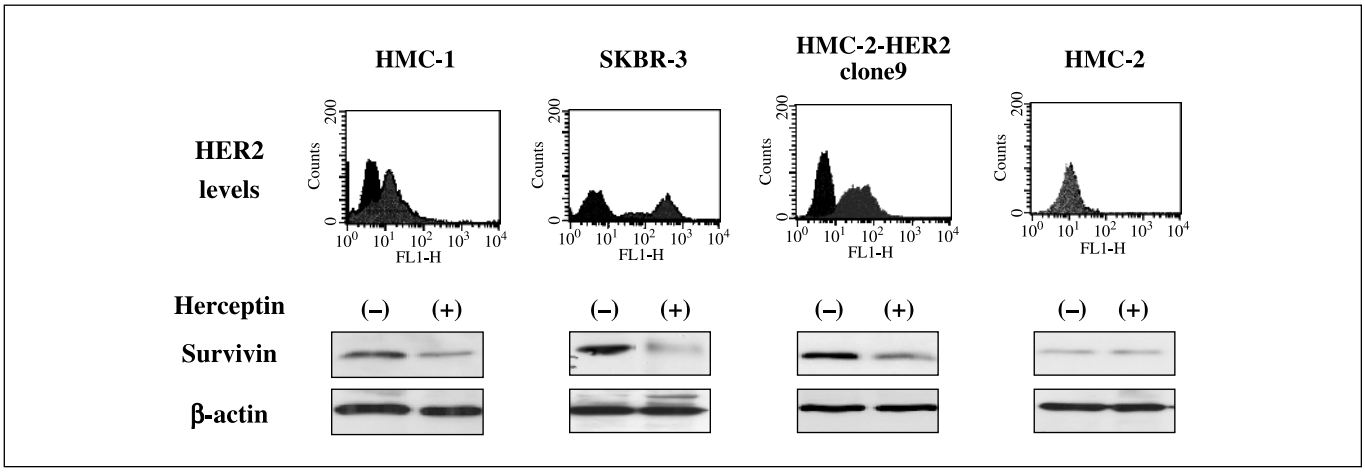

Figure 5. Flow cytometric analysis for HER2 expression and Western blotting analysis for survivin levels in the presence or absence of Herceptin. Breast cancer cell lines HMC-1, SKBR-3, HMC-2-HER2 clone9, and HMC-2 wild-type cells were analyzed for the cell surface levels of HER2 by flow cytometer (*top*). Cells were stained with FITC-labeled anti-HER2 antibody (*gray*) or FITC-labeled mouse IgG1 (*black*). *Bottom,* cells were incubated with or without 20 μg/mL Herceptin for 48 hours, and the lysates were analyzed for the survivin and β-actin protein levels by Western blotting.

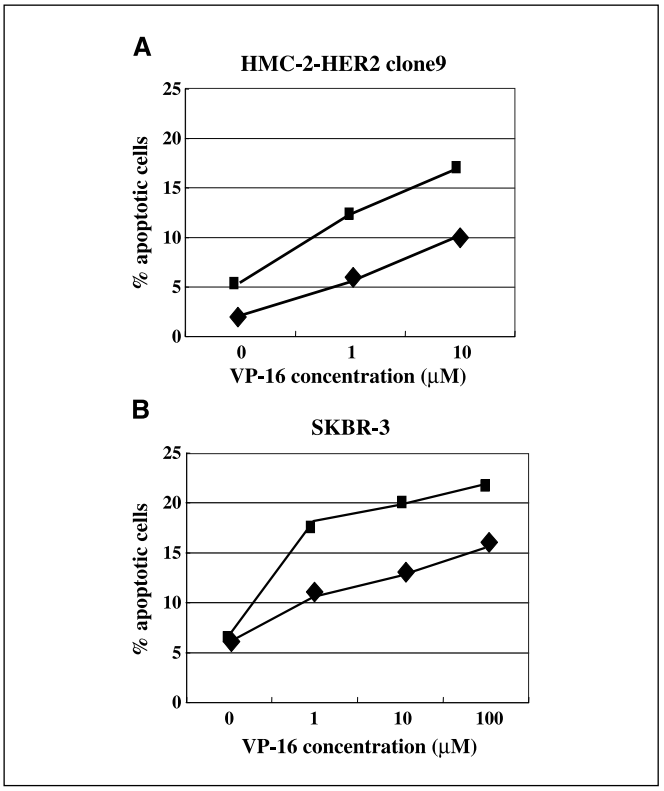

Figure 6. VP-16-induced apoptosis of HER2-positive cancer cells in the presence or absence of Herceptin. HMC-2-HER2 clone9 (*A*) and SKBR-3 (*B*) were cultured for 24 hours in a medium containing 0, 1, 10, or 100 μmol/L VP-16 with (■) or without (♦) 20 μg/mL Herceptin followed by the Annexin V/propidium iodide double staining assay. Annexin V–positive and propidium iodide–negative apoptotic cells were counted by flow cytometer.

indicated that it takes >2 hours for the survivin protein level to decrease in the presence of the PI3K inhibitor. Collectively, it was clarified that survivin protein level could be regulated, at least in part, by the PI3K/AKT pathway of intracellular HER2 signals in breast cancer cells.

Discussion

Survivin was originally isolated as a member of the IAP family and has been shown to be capable of suppressing the activity of caspases (1, 2). In addition, it has been shown that survivin plays an important role in the mitotic process and cell proliferation (23, 24). Among IAP family members, survivin and livin are unique in their tissue expression profiles because those two members are abundantly expressed in certain tumor tissues but not in normal adult tissues (3, 25, 26). There are a number of reports concerning the correlation between survivin expression in cancer tissues and poor prognosis of the patients (4–11), suggesting that the protein has prognostic significance (27). It is also involved in the resistance to chemotherapy observed in certain tumors (28, 29). Overexpression of survivin rendered tumor cells resistant to chemotherapeutic reagents, whereas reduction of its expression increased the sensitivity (29–35). Therefore, survivin is considered as an ideal molecular target for cancer therapy.

It has been reported that survivin is expressed in ~60% to 80% of breast cancers (12–14). Our results were almost compatible with previous studies and showed that there was no difference in the survivin positivity between invasive ductal carcinoma and invasive lobular carcinoma. In addition to the survivin expression in a variety of histologic types of tumor tissues (9, 26, 36–38), it shows a clear cell cycle–dependent expression at mitosis. This is largely controlled at the transcriptional level and involves cell cycle–dependent element/cell cycle gene homology region boxes that are located in the proximal survivin promoter (39, 40). Several regulatory factors have been reported thus far, including p53 (41, 42), progesterone (43), IFN-responsive factor-1 (44), and retinoblastoma protein/E2F complex (45) as negative regulatory factors and nuclear factor-κB (NF-κB), Sp-1, T-cell factors, and Ha-ras as positive regulatory factors (32–34). In addition to the transcriptional regulation, survivin protein levels are regulated by the ubiquitin/proteasome-mediated protein degradation system (46, 47).

To clarify the regulatory mechanism of survivin expression in breast cancer, several breast cancer–related molecules, such as HER2, EGFR, E-cadherin, β-catenin, and sex hormone receptors, were examined by immunohistochemical staining. Consequently, it was indicated that the survivin protein positivity detected by immunohistochemical staining might be correlated with HER2 and EGFR expression but not with E-cadherin, β-catenin, or hormone receptors (data not shown). The positive correlation between survivin and HER2 expression has been reported in histopathologic examinations by other groups (35). However, the present study highlighted for the first time the significance of coexpression of HER2 and EGFR for the up-regulation of survivin protein.

The EGFR family consists of four members: EGFR (HER1), HER2, HER3, and HER4, which share structural homology consisting of an extracellular domain and a cytoplasmic signal transduction domain with tyrosine kinase activity (16). Many kinds of tumor cells express multiple EGFR members, which interact to form an

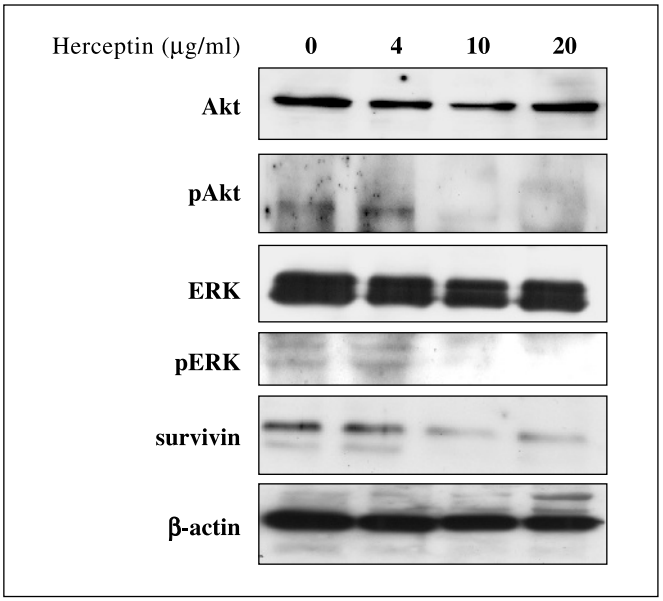

Figure 7. Effect of Herceptin on the protein levels of signaling molecules and survivin in SKBR-3 cells. SKBR-3 cells were incubated with varying concentrations of Herceptin (0, 4, 10, or 20 μg/mL) for 48 hours, and the cell lysates were subjected to Western blotting. Phosphoprotein levels were examined by using anti-pERK ($pSer^{473}$) antibody and anti-pAKT ($pTyr^{204}$) antibody.

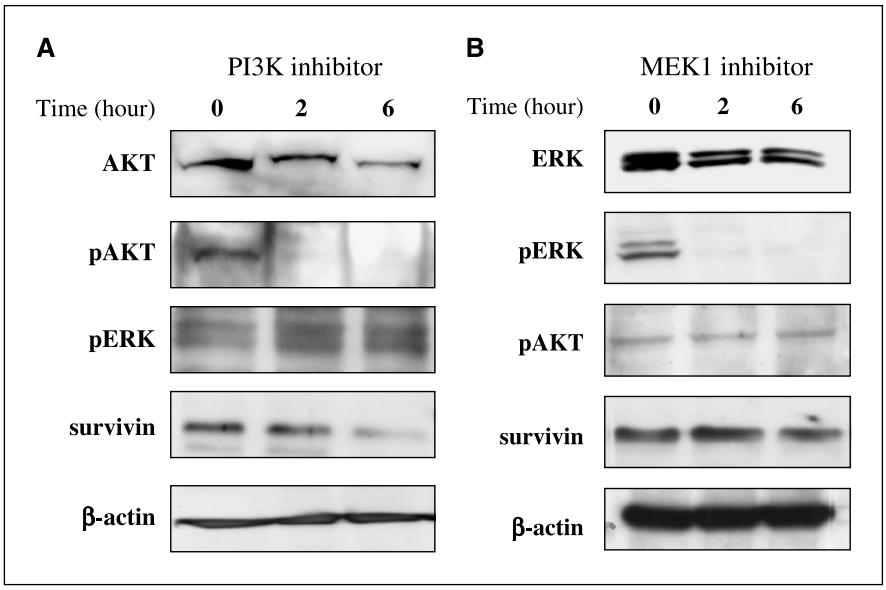

Figure 8. Effect of PI3K inhibitor or MEK1 inhibitor on the protein levels of signaling molecules and survivin in SKBR-3 cells. SKBR-3 cells were incubated with either PI3K inhibitor LY294002 (*A*) or MEK1 inhibitor PD98059 (*B*) for the indicated hours, and levels of phosphoproteins and survivin were analyzed by Western blotting.

array of homodimers and heterodimers. HER2 is predominantly activated by forming a heterodimer with other EGFR members rather than forming a homodimer (18, 48, 49). For example, coexpression of HER2 and EGFR was shown to synergistically transform NIH3T3 fibroblasts (50). In breast cancer, HER2 overexpression in conjunction with expression of EGFR and/or HER3 has been shown to be associated with a poor prognosis (17). In the present immunostaining study, ~20% of HER2-positive and EGFR-negative cases were survivin positive. It is likely that such cases have HER3 or HER4 expression instead of EGFR.

Heterodimerization of HER2 can result in the activation of intracellular signaling cascades, such as PI3K/AKT pathway and MAPK pathways (16, 51, 52). For example, PI3K is activated by the EGFR family tyrosine kinases followed by phosphorylation and activation of the downstream protein kinase AKT (53). MEK1 is one of the MAPK kinase family members, which is activated by RAF-1, and phosphorylates the downstream protein kinases ERK1/2 (54). The involvement of these two signaling pathways in HER2 signaling was confirmed by the treatment of SKBR-3 cells with Herceptin (Fig. 7). The importance of the PI3K/AKT pathway in the cell survival signal of growth factor receptors has been reported (54–56), and our results are consistent with this. Because NF-κB can be activated in the downstream of AKT, it is likely that survivin level is regulated by this transcriptional factor.

In conclusion, the present study clarified the regulatory mechanism of HER2 in survivin expression in breast cancer cells. Up-regulation of survivin protein by HER2 and EGFR signals may explain, at least in part, the molecular basis for the poor prognosis and chemoresistance of HER2-positive breast cancer. In addition, our study provides a significant insight into the molecular mechanism of the synergistic antitumor effect of Herceptin in combination with chemotherapeutic reagents.

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Survivin Expression Is Regulated by Coexpression of Human Epidermal Growth Factor Receptor 2 and Epidermal Growth Factor Receptor via Phosphatidylinositol 3-Kinase/AKT Signaling Pathway in Breast Cancer Cells

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