RESEARCH COMMUNICATION

HER-2 Expression Correlates with Survivin in Primary Invasive Ductal Breast Cancers

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Abstract

Background: The expression of HER-2 and Survivin is correlated with poor prognosis of breast cancer, but a possible interaction of these two proteins remains to be established. Aim: The aim of this study is to determine the possible relationship between HER-2 and Survivin in primary invasive ductal breast cancer. Methods: Eighty-six patients diagnosed with primary invasive ductal breast cancer were enrolled in the study. Cancerous breast tissue biopsies were analyzed for the expression levels of HER-2, survivin, ERK1/2, p-ERK1/2, AKT, and p-AKT proteins by Western blot. The levels of HER-2 and survivin mRNA were also assessed by real-time quantitative reverse transcription – polymerase chain reaction (RT-PCR). Finally, the correlation of disease state and protein or mRNA expression was analyzed using the bivariate Pearson's correlation method (two-tailed). Results: Western blot analysis showed a significant correlation in protein levels among HER-2, p-AKT, and survivin in the primary invasive ductal breast cancer tissues (all $p<0.01$). However, the levels of HER-2 protein and Survivin mRNA were not correlated ($p=0.154$). Conclusions: HER-2 expression significantly correlates with Survivin at the protein level in primary invasive ductal breast cancer tissues, and the correlated changes might act via p-AKT, rather than at the level of transcriptional regulation.

Keywords: HER-2 - p-AKT - survivin - breast cancer

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Introduction

Breast carcinoma is the most common malignancy in women worldwide, and is the second leading cause of cancer deaths in women (Anim et al., 2005; Debled et al., 2009). Despite the historically low incidence of breast cancer in Japan, the breast cancer death rate has approximately tripled over the past 30 years (Kasami et al., 2008). Breast cancer is a heterogeneous disease encompassing several different phenotypes with consistently different biological characteristics (Rody et al., 2004; Hussein et al., 2008). The cancer-specific protein Survivin is a multifunctional protein implicated in the control of cell proliferation, inhibition of apoptosis, and promotion of angiogenesis (Tanaka et al., 2000; Nassar et al., 2008). Recent studies have reported that Survivin expression is associated with adverse outcome in patients with breast cancer (Brennan et al., 2008; Nassar et al., 2008). In addition, expression of the human epidermal growth factor receptor-2 (HER-2) is associated with tumor progression and poor prognosis in breast cancer (Zhou et al., 2001a; b). Using qualitative immunohistochemistry analysis, some studies have identified a correlation between the expression of HER-2 and Survivin in the tumor tissues (Hiroko et al., 2005; Ryan et al., 2006; Wenle et al., 2006).

Survivin is known to be positively regulated though the PI3-K/AKT signaling pathway (Papapetropoulos et al., 2000; Carter et al., 2001; McCubrey et al., 2006). In addition, the anti-apoptotic effects of HER2 were shown to be mediated by signaling through the PI3-K/AKT signaling pathway (Ryan et al., 2006). Thus, it is possible that HER2, by acting through the PI3-K/AKT pathway, may block apoptosis by up-regulating expression of Survivin. However, the underlying mechanism responsible for the correlated changes in their expression levels has not been established.

Therefore, we obtained 86 mastectomy specimens from patients with primary invasive ductal breast cancer. The expressions of HER-2 and Survivin in breast cancer tissues were examined by Western blot and real time RT-PCR to study the relationship between the expression of HER-2 and Survivin.

Materials and Methods

Patients and tissue specimens

Eighty-six patients who underwent mastectomy operation at the First Hospital of the China Medical University during November 2008 to April 2009 were enrolled in this study. All the patients were histologically confirmed to have primary invasive

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ductal breast carcinomas. The mean age of the enrolled patients was 50.83 years old (range from 30 to 81). By immunohistochemical analsis, there were thirty-six HER-2 negative (HER-2 -), twenty-two HER-2 positive (HER-2+), twenty-five HER-2 double positive (HER-2++), and three HER-2 triple positive (HER-2+++) patients. Typical strongly positive expression of HER-2 is shown in Figure 1. After FISH analysis, there were 6 cases with HER-2 gene amplification out of 25 HER-2++ patients. Typical expression of HER-2 gene amplification is shown in Figure 2. Only specimens larger than 1 centimeter were selected for the study. Fresh tissues were obtained within 15 minutes from the interruption of blood supply. Tissues were snap frozen and stored at -70°C until processing for RNA and protein extraction.

The present study was conducted in accordance with international guidelines and approved by the institutional ethics committee. Patients and their families were informed of the study's objectives, and signed consent was obtained from all the participants.

Antibodies

Polyclonal mouse anti-human HER-2 and monoclonal mouse anti-human Survivin antibodies were purchased from Santa Cruz Biotechnology (CA, USA). Monoclonal rabbit anti-human extracellular signal-related kinase1/2 (ERK1/2), phosphorylated ERK1/2 (P- ERK1/2), protein kinase B (AKT), phosphorylated AKT (P-AKT), and β-actin were purchased from Cell Signaling Technology, Inc. (MA, USA). All other reagents for antibody reactions were purchased from TaKaRa (Dalian, China).

Primers design

The gene sequences used were obtained from Genbank and the primer pairs were designed using the Primer Premier 5.0 software (Premier Co., CA, USA). Primers were synthesized by SBS Genetech Co., Ltd (Beijing, China). The oligonucleotide primer sequences were as follows: HER-2 forward primer 5'-AGG GAA ACC TGG AAC TCA CC-3' and reverse primer 5'-GCA CAA TCC GCA GCC TCT-3', yielding a 138-bp product; Survivin forward primer: 5'-CTG CGG AGA AAG TGC G-3' and reverse primer: 5'-GGG AAT AAA CCC TGG AAG-3', yielding a 110-bp product. The mRNA level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference. GAPDH forward primer: 5´-AAG GCT GTG GGC AAG G-3´ and reverse primer: 5´-TGG AGG AGT GGG TGT CG-3´, yielding a 238-bp product.

Western blot analysis

All 86 specimens were processed for analysis by Western blot. The protein levels of Her-2, Survivin, ERK1/2, p-ERK1/2, AKT, and p-AKT were determined using the specific polyclonal or monoclonal antibodies described above. β-actin was used as an internal control. Western blot was performed as described previously (Xiong et al., 2007). Briefly, protein (50 μg) was electrophoresed by SDS-PAGE. After electrophoretic transfer, the membranes were incubated with the specific antibody. The bound antibody was visualized by chemiluminescence. Optical density (OD) values from each protein were evaluated by ScanImage software. Each sample was detected by Western blot three times using the same conditions, and the average values were used for data analysis.

Real time RT-PCR analysis

Total RNA was extracted from 86 breast cancer specimens using Trizol reagent (TaKaRa). Real time RT-PCR analysis analysis was performed as described previously (Xiong et al., 2007). Briefly, total RNA (500 ng) was reverse transcribed into cDNA using the TaKaRa PCR Thermal Cycler Dice. HER-2, Survivin, and GAPDH mRNA levels were detected by real-time PCR using AB7500 from Applied Biosystem. The expression ratios of HER-2 mRNA and Survivin mRNA were determined by the $2^{-\triangle\triangle C_T}$ (Livak) method (Livak and Schmittgen, 2001). In addition, each sample was subjected to three times real time RT-PCR testes using the same conditions, and the average values were used for data analysis.

Statistical analysis

All data were analyzed with SPSS software (Version 13.0; IL, USA). Correlation analysis was performed by the Pearson Correlation method (two-tailed). A p-value of less than 0.05 was considered statistically significant.

Results

Representative Western blot findings are shown in Figure 1, FISH staining in Figure 2 and immunohistochemistry in Figure 3. Our data showed that those cancer specimens which overexpressed HER-2 protein also had high expression levels of Survivin protein. A significant correlation was observed among the expression levels of HER-2, p-AKT, and Survivin at the protein level in studied breast cancer tissues (r=0.789, p=0.000; r=0.773, p=0.000; r=0.780, p=0.000, respectively; Tables 1 and 2). Furthermore, significant correlation was identified between the expression levels of HER-2 and P- ERK1/2.

The expression of HER-2 and Survivin mRNA in 86 primary breast cancer samples was studied by real time RT-PCR. There was no significant correlation detected between the HER-2 protein and Survivin mRNA levels (p>0.05) (Table 3).

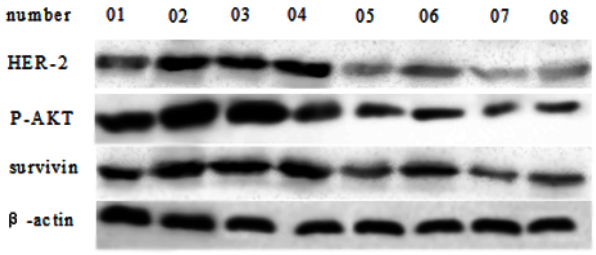

Figure 1. Representative Expression of HER-2, P-AKT, Survivin, and β-actin in Primary Invasive Ductal Breast Cancer Tissues Detected by Western Blot Analysis (Case Subjects 1-8).

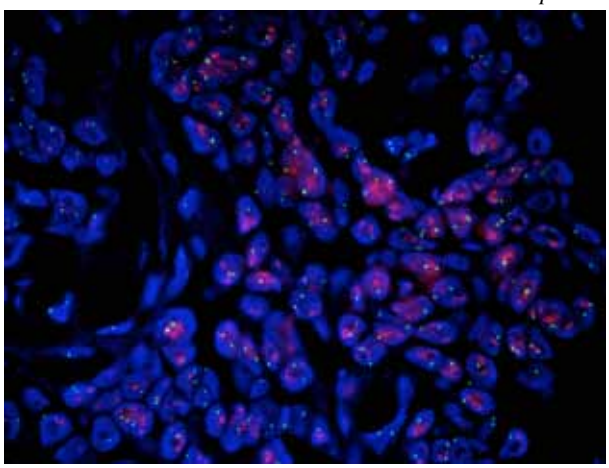

Figure 2. Representative FISH Staining for HER-2 in Primary Invasive Ductal Breast Cancer Tissue. The Vysis Path Vysion HER-2/DNA Probe Kit Uses Two Different Probes. One is a Locus Specific Identifier (LSI) HER-2/neu Labeled in Spectrum Red and the other is a Chromosome Enumerator Probe (CEP) 17 Labeled in Spectrum Green. Signal Enumeration was Conducted at×400 Magnification with the Appropriate Filter

Table 1. Correlations between HER-2 and Other Proteins in Primary Invasive Breast Cancers

Proteins	r*	p value
Survivin	0.753	0.001
ERK1/2	0.429	0.025
p-ERK1/2	0.581	0.011
AKT	0.421	0.017
p-AKT	0.775	0.001

*Pearson correlation coefficient

Table 2. Correlations between Survivin and Other Proteins in Primary Invasive Breast Cancers

Proteins	r*	p
ERK1/2	0.426	0.020
p-ERK1/2	0.413	0.024
AKT	0.528	0.015
p-AKT	0.762	0.001

*Pearson correlation coefficient

Table 3. Correlation between HER-2 Protein and Survivin mRNA in Primary Invasive Breast Cancers

Parameters	r*	p
HER-2 protein Survivin mRNA	0.314	0.154

*Pearson correlation coefficient

Discussion

This study investigates the expression levels of HER-2, Survivin, ERK1/2, P- ERK1/2, AKT, and P-AKT in primary breast cancer tissues. Our data showed a positive correlation between the HER-2 and Survivin protein levels suggesting the presence of a common regulatory mechanism for HER-2 and Survivin in primary breast cancer.

At present, the relationship between HER-2 and Survivin remains controversial. Using qualitative immunohistochemical analysis, some studies have

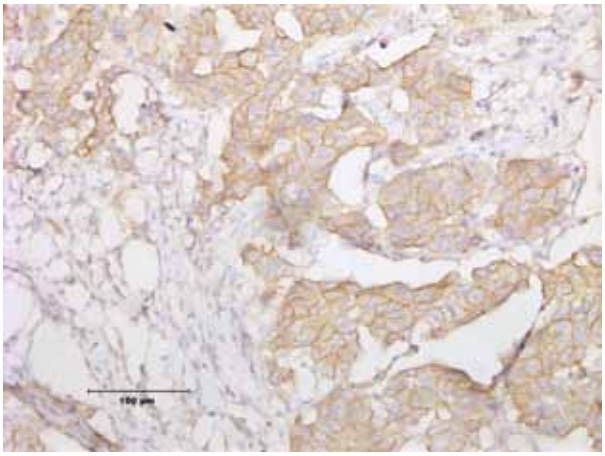

Figure 3. Representative Immunohistochemistry Staining for Strongly Positive HER-2 Protein in Primary Invasive Ductal Breast Cancer Tissue. The Intensity of HER-2 Immunoreactivity to Tumor Cell Membrane was scored at×400 Magnification

reported a positive correlation between the level of HER-2 and Survivin in tumor tissues (Zhao et al., 2000). On the other hand, Ryan et al., (2006) failed to find a significant association between the level of HER-2 and Survivin using ELISA-based assays. Since the half-life of the Survivin protein is only 30 minutes in the absence of new protein synthesis, the way human tissue was handled and processed might have an impact on the results obtained from different investigators (Zhao et al., 2000). Garcia et al., (2001) proposed that tissues used for detecting activated phosphorylation status of signal transduction proteins must be snap frozen in liquid nitrogen or fixed in 10% neutral-buffered formalin within 15 minutes after the interruption of blood supply in order to reduce antigen loss. In the current study, we collected specimens strictly following this described method.

There have been reports of significant correlation between the level of HER-2 and P-AKT protein in breast cancer cell lines; however, no data has been obtained using primary breast cancer tissues (Siddiqa et al. 2008; Bacus et al., 2002). To our knowledge, the current study might be the first to report significant correlation in protein levels between HER-2 and P-AKT in primary breast cancer tissues. In some studies, it has been shown that Survivin can be positively regulated though the PI3-K/AKT signaling pathway (Papapetropoulos et al., 2000; Carter et al., 2001; Fukuda and Pelus, 2002; McCubrey et al., 2006). In the present study, it was also observed that the expression of P-AKT significantly positively related to Survivin in primary invasive ductal breast cancer which provided a strong evidence for the outcomes in breast cancer cell lines mentioned above. Hence, our data suggest that the protein level of HER-2 was significantly correlated with that of Survivin in primary invasive ductal breast cancer tissues, and p-AKT might be involved in the correlation.

Besides the correlation between P-AKT and Survivin, the study also revealed a significant relationship between HER-2 and P-ERK1/2, and the data were consistent with the findings reported by Yarden et al., (2001). However, we detected no correlation between the level of Survivin and

P- ERK1/2 in our study, which was also in agreement with the data reported by others (Nassar et al., 2008; Hiroko et al. 2005). Thus, the correlated expression between HER-2 and Survivin was unlikely to be mediated via P- ERK1/2.

Since there was no significant correlation between the protein level of HER-2 and mRNA level of Survivin, the correlated expression at the protein level did not take place at the transcriptional level.

In conclusion, the protein level of HER-2 was significantly correlated with that of Survivin in primary invasive ductal breast cancer tissues, and p-AKT might be the common regulator to control the above mentioned correlation. In addition, the possible relevance among the factors may occur in the post-translational level. More study is needed to further confirm this hypothesis.

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References

Anim JT, John B, Abdulsathar SS, et al (2005). Relationship between the expression of various markers and prognostic factors in breast cancer. *Acta Histochem*, **107**, 87-93.

Bacus SS, Altomare DA, Lyass L, et al (2002). AKT2 is frequently up-regulated in HER-2/neu-positive breast cancers and may contribute to tumor aggressiveness by enhancing cell survival. *Oncogene*, **21**, 3532-40.

Brennan DJ, Rexhepaj E, O'Brien SL, et al (2008). Altered cytoplasmic-to-nuclear ratio of Survivin is a prognostic indicator in breast cancer. *Clin Cancer Res*, **14**, 2681-9.

Carter BZ, Milella M, Altieri DC, et al (2001). Cytokine-regulated expression of survivin in myeloid leukemia. *Blood*, **97**, 2784-90.

Debled M, Madranges N, Trainaud A, et al (2009). First-line capecitabine monotherapy for slowly progressing metastatic breast cancer: do we need aggressive treatment? *Oncology*, **77**, 318-27.

Fukuda S, Pelus LM (2002). Elevation of Survivin levels by hematopoietic growth factors occurs in quiescent $CD34^+$ hematopoietic stem and progenitor cells before cell cycle entry. *Cell Cycle*, **1**, 322-6.

Garcia R, Bowman TL, Niu G, et al (2001). Constitutive activation of Stat3 by the Src and Janus kinase tyrosine kinases participates in growth regulation of human breast carcinoma cells. *Oncogene*, **20**, 2499-513.

Hiroko A, Toshihiko T, Kenjiro K, et al (2005). 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. *Cancer Res*, **65**, 11018-25.

Hussein MR, Abd-Elwahed SR, Abdulwahed AR (2008). Alterations of estrogen receptors, progesterone receptors and c-erbB2 oncogene protein expression in ductal carcinomas of the breast. *Cell Biol Int*, **32**, 698-707.

Kasami M, Uematsu T, Honda M, et al (2008). Comparison of estrogen receptor, progesterone receptor and Her-2 status in breast cancer pre- and post-neoadjuvant chemotherapy. *Breast*, **17**, 523-7.

Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, **25**, 402-8.

McCubrey JA, Steelman LS, Abrams SL, et al (2006). Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. *Adv Enzyme Regul*, **46**, 249-79.

Nassar A, Lawson D, Cotsonis G, et al (2008). Survivin and caspase-3 expression in breast cancer: correlation with prognostic parameters, proliferation, angiogenesis, and outcome. *Appl Immunohistochem Mol Morphol*, **16**, 113-20.

Nassar A, Sexton D, Cotsonis G, et al (2008). Survivin expression in breast carcinoma: correlation with apoptosis and prognosis. *Appl Immunohistochem Mol Morphol*, **16**, 221-6.

Papapetropoulos A, Fulton D, Mahboubi K, et al (2000). Angiopoietin-1 inhibits endothelial cell apoptosis via the Akt/Survivin pathway. *J Biol Chem*, **275**, 9102-5.

Rody A, Diallo R, Poremba C, et al (2004). Estrogen receptor alpha and beta, progesterone receptor, pS2 and HER-2/neu expression delineate different subgroups in ductal carcinoma in situ of the breast. *Oncol Rep*, **12**, 695-9.

Ryan BM, Konecny GE, Kahlert S, et al (2006). Survivin expression in breast cancer predicts clinical outcome and is associated with HER2, VEGF, urokinase plasminogen activator and PAI-1. *Ann Oncol*, **17**, 597-604.

Siddiqa A, Long LM, Li LX, et al (2008). Expression of HER-2 in MCF-7 breast cancer cells modulates anti-apoptotic proteins Survivin and Bcl-2 via the extracellular signal-related kinase (ERK) and phosphoinostitide-3 kinase (PI3K) signaling pathways. *BMC Cancer*, **8**, 129-36.

Tanaka K, Iwamoto S, Gon G, et al (2000). Expression of Survivin and its relationship to loss of apoptosis in breast carcinomas. *Clin Cancer Res*, **6**, 127-34.

Wenle X, John B, Jay S, et al (2006). Regulation of Survivin by ErbB2 signaling: therapeutic implications for ErbB2-overexpressing breast cancers. *Cancer Res*, **66**, 1640-7.

Xiong H, Yu S, Zhuang L, et al (2007). Changes of Survivin mRNA and protein expression during paclitaxel treatment in breast cancer cells. *J Huazhong Univ Sci Technolog Med Sci*, **27**, 65-7.

Yarden Y, Sliwkowski MX (2001). Untangling the ErbB Signaling Network. *Nat Rev Mol Cell Biol*, **2**, 127-37.

Zhao J, Tenev T, Martins LM, et al (2000). The ubiquitin-proteasome pathway regulates Survivin degradation in a cell cycle-dependent manner. *J Cell Sci*, **113**, 4363-71.

Zhou BP, Liao Y, Xia W, et al (2001b). Cytoplasmic localization of p21 by Akt-induced phos-phorylation in HER-2/neu overexpressing cells. *Nat Cell Biol*, **3**, 245-52

Zhou BP, Liao Y, Xia W, et al (2001a). HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. *Nat Cell Biol*, **3**, 973-82.