Report

Increased phosphorylation of Akt in triple-negative breast cancers

Shinobu Umemura,[1,4] Sei Yoshida,[3] Yoshikazu Ohta,[3] Kenichiro Naito,[3] R. Yoshiyuki Osamura[1] and Yutaka Tokuda[2]

Departments of [1]Pathology and [2]Surgery, Tokai University School of Medicine, Isehara, Kanagawa 259-1193; [3]Pharmacology Research Laboratories II, Takeda Pharmaceutical Company, Osaka 532-8686, Japan

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Cells from breast cancers lacking hormone receptors (estrogen receptor [ER], progesterone receptor [PgR]) and human epidermal growth factor receptor (HER) 2 strongly express the cell proliferation marker Ki-67. However, the mechanisms of and stimulus signals involved in cell proliferation of this type of breast cancer are not well understood. The aim of the present study was to examine the characteristics of signal transduction in triple-negative (ER-, PgR-, and HER2-negative) breast cancers. For 44 tumor samples, western blotting analysis was conducted to examine the phosphorylation of HER2, external signal-regulated kinase (ERK)1 and -2 and Akt, and the immunohistochemical phenotypes of the samples with respect to ER and HER2 were also assessed. Phosphorylation of HER2 was detected in 4 of 15 immunohistochemically HER2-positive tumor samples (26.7%). ERK1/2 was more highly phosphorylated in triple-negative breast cancers. Phosphorylation of Akt kinase was significantly higher in triple-negative breast cancers. Triple-negative breast cancers are characterized by increased phosphorylation of Akt kinase. In the present study, we found for the first time that there is a population with a significantly activated Akt pathway in this type of breast cancer. (*Cancer Sci* 2007; 98: 1889–1892)

In mammary glands, estrogen plays a major role in duct elongation and branching, and accelerates the proliferation of epithelial cells, whereas progesterone contributes to lobule formation. In non-neoplastic mammary glands, estradiol is thought to act as a paracrine mitogen because no co expression of estrogen receptor (ER)α and cell proliferation markers (Ki-67 or proliferating cell nuclear antigen [PCNA]) has been detected. A recent study by Dimitrakakis *et al*. demonstrated that ERα and the estrogen-induced proteins MYC, cyclin D1, and stromal cell-derived factor-1 are co-expressed within the nuclei of monkey mammary glands.[1] Responsiveness to estrogen is retained after carcinogenesis in approximately 70% of all breast cancers. Two different mechanisms have been proposed to explain the mitogenic effects of estrogens in breast cancer tissue:[2] genomic action via estrogen-responsive elements located in the promoter regions of c-*fos*[3] and c-*myc*,[4] which have mitogenic activity involving G_1-phase progression; or indirect action on *cyclin D1* gene transcription.[5] Induction of cyclin D1 is strictly regulated in a hormone-dependent manner, and cAMP response elements in the promoter region of the *cyclin D1* gene require the activation function (AF)-1 and AF-2 domains of ERα. Other non-genomic actions of estrogen via mitogen-activated protein kinase (MAPK)–external signal-regulated kinase (ERK) and phosphatidylinositol 3 kinase (PI3K)–Akt have also been proposed.[2]

Human epidermal growth factor receptor (HER)2 is a tyrosine kinase receptor, the biological and clinical role of which has been investigated thoroughly in breast cancer. No specific ligands for HER2 have been found, but dimers containing HER2 (e.g. homodimers of HER2 or HER2–HER3 heterodimers) can strongly activate intracellular signaling for cell proliferation, cell survival, motility, and adhesion.[6] Two major signaling pathways for the HER family are the Raf–MEK–ERK pathway and the PI3K–phosphoinositide-dependent kinase (PDK1)–Akt pathway.[7] These signal-transduction pathways regulate the expression of genes in many ways to promote cell-cycle progression by inhibition of p27[8,9] and activation of cyclin D1,[10] and to inhibit apoptosis by phosphorylation of Bad and caspase 9.[11]

Thus, the mechanisms of cell proliferation have been thoroughly investigated for hormone receptor-positive and HER2-positive breast cancers. In contrast, in breast cancers lacking hormone receptors and HER2, the signaling pathways responsible for cell proliferation are not well characterized. Cell proliferation in triple-negative breast cancer cells must be regulated in an ER or HER2 signaling-independent manner. The present study was conducted to examine the phosphorylation of HER2, ERK1/2, and Akt kinases, which are key kinases in two major signaling pathways, in order to obtain data to support further investigations into the molecular events contributing to cell proliferation.

Materials and Methods

Patients and tumor samples. Forty-four samples of breast cancer tissue were obtained from patients who underwent surgery at Tokai University Hospital (Isehara, Japan). All patients gave their informed consent to participate in this study. The 44 samples comprised 39 invasive carcinoma samples and five *in situ* ductal carcinoma samples. Tissue samples were obtained from the main tumor mass, avoiding massive necrosis or intermingling non-neoplastic breast tissue. Samples were snap frozen in liquid nitrogen and stored at –80°C for western blotting. The samples were also processed to formalin-fixed and paraffin-embedded sections for immunohistochemical (IHC) study. None of the patients received any preoperative adjuvant hormones or chemotherapy. Clinical and IHC data for the breast cancers are presented in Table 1. The study design was approved by the institutional ethics committee and the patients were informed about the privacy policy of the study from surgeons before the surgery.

Immunohistochemistry. Paraffin sections (4 μm thick) were examined immunohistochemically for ER and HER2. ER was stained using an automated process (Benchmark; Ventana Japan, Yokohama, Japan), and HER2 was stained using a detection kit (DAKO HercepTest; DakoCytomation) in accordance with the respective manufacturers' instructions. IHC slides were evaluated using positive controls for each antibody (as recommended by the manufacturers of the antibodies), and negative controls were incubated in normal serum rather than primary antibodies. Samples

[4]To whom correspondence should be addressed.
E-mail: umemura@is.icc.u-tokai.ac.jp

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Table 1. Pathological characteristics of cases in the present study

Pathological characteristics		*n*
Estrogen receptor	Positive	33
	Negative	11
Progesterone receptor	Positive	29
	Negative	15
Human epidermal growth factor receptor 2	Score 0	15
	Score 1	14
	Score 2	8
	Score 3	7
Histological grade	Grade I	11
	Grade II	11
	Grade III	17

were deemed to be positive for ER if more than 10% of the cancer cells immunoreacted. HER2 immunoreactivity was assigned a score of 0, 1+, 2+, or 3+, using the recommended standard criteria.[12] Scores of 2+ and 3+ were interpreted as positive, and scores of 0 and 1+ were deemed to be negative.

Protein extraction and immunoblotting. Frozen tumor tissue samples were broken up and homogenized in RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecylsulfate [SDS]). Soluble extracts (5 μg protein) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (0.45-μm pore size; Millipore, Bedford, MA, USA). The membrane was washed with 0.5% Tween-20 in Tris-buffered saline (pH 7.4) (TBS-T), blocked in 3% bovine serum albumin in TBS-T, and incubated with the appropriate primary and secondary antibodies. Protein-specific and phosphorylation-specific polyclonal antibodies against HER2, ERK1/2, and Akt (Cell Signaling Technology, Beverly, MA, USA) were used. Protein bands were detected using an enhanced chemiluminescence detection method (ECL plus; GE Healthcare Biosciences, Piscataway, NJ, USA) and measured and digitized using the LAS-1000 Plus Lumino-Image Analyzer (Fuji Photo Film, Kanagawa, Japan). The values for phosphorylated and total proteins were normalized against the intrinsic actin level (clone AC-15; Sigma-Aldrich, St Louis, MO, USA), and analyzed statistically using the *t*-test and Mann–Whitney test.

Immunohistochemistry for phosphorylated Akt. Formalin-fixed and paraffin-embedded sections were evaluated by immunohistochemistry for phosphorylated Akt (pAkt). The primary antibody used was a polyclonal antibody for pAkt (ser473, #9277; Cell Signaling Technology, Danvers, MA, USA). After deparaffinization, antigen retrieval was carried out by heating in 10 mM sodium citrate buffer (pH 6.0). Sections were incubated with primary antibodies overnight at 4°C. The reaction was visualized using VECTASTAIN Elite ABC standard kit (VECTOR Laboratories, Burlingame, CA, USA). IHC evaluation was carried out fundamentally according to the principle of HSCORE[13] with modifications. Briefly, the proportion of positive cells (percentage) with different intensity (0, negative; 1, weak; 2, moderate; 3, strong) was estimated semiquantitatively at high magnification. The subtotal score obtained by multiplication of percentage (%) and intensity (0, 1, 2, 3) was obtained for three areas per case, and the average score calculated was given as the IHC score for pAkt. The correlation between IHC score and the results of immunoblotting was analyzed by linear regression analysis.

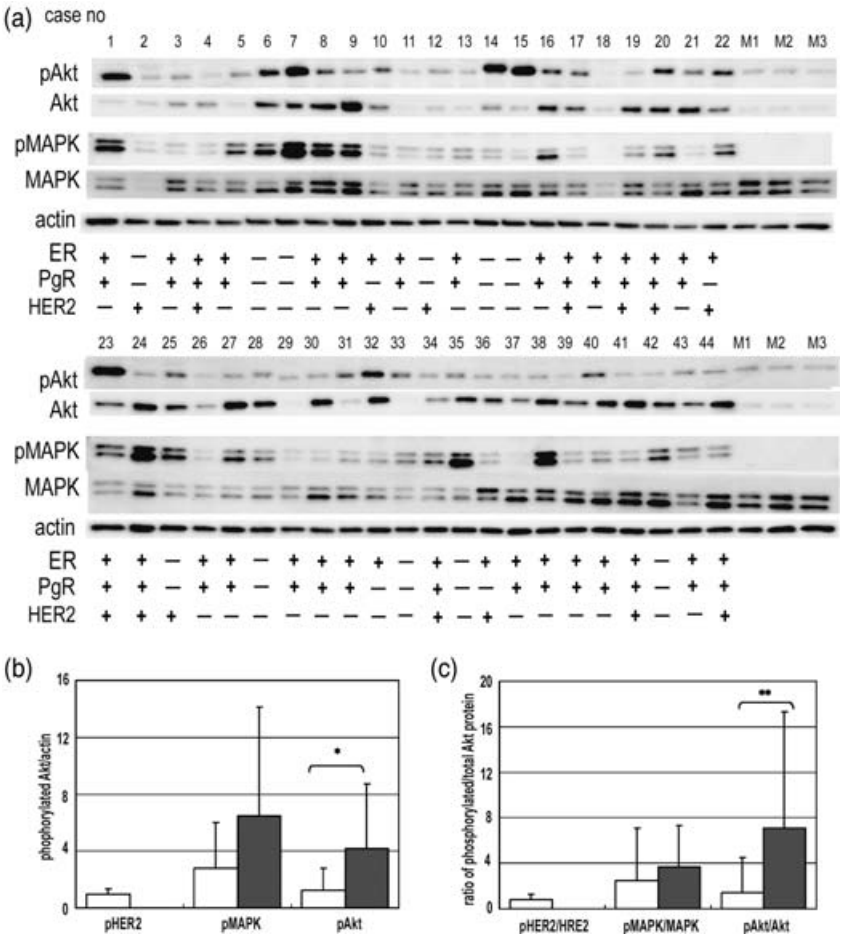

Fig. 1. Immunoblotting analysis for human epidermal growth factor receptor (HER) 2, external signal-regulated kinase (ERK)1/2, and Akt kinases. (a) Protein extracts from 44 tumor samples were separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis and immunoblotted using antibodies for phosphorylated and total HER2, ERK1/2, and Akt. M1, M2, and M3 are markers for protein extracts in graded concentrations. (b) Phosphorylated HER2, ERK1/2, and Akt were compared between ER-, PgR-, and HER2-negative cancers (■), and other types of breast cancers, including ER^+ and $HER2^-$, ER^+ and $HER2^+$, and ER^- and $HER2^+$ cancers (□). Asterisks indicate *P*-value (0.0023 for the *t*-test and 0.06 for the Mann–Whitney test). Error bars represent SD. (c) The ratios of phosphorylated and total HER2, ERK1/2, and Akt were also compared between triple-negative breast cancers (■) and others (□). Asterisks indicate *P*-value (0.006 for the *t*-test).

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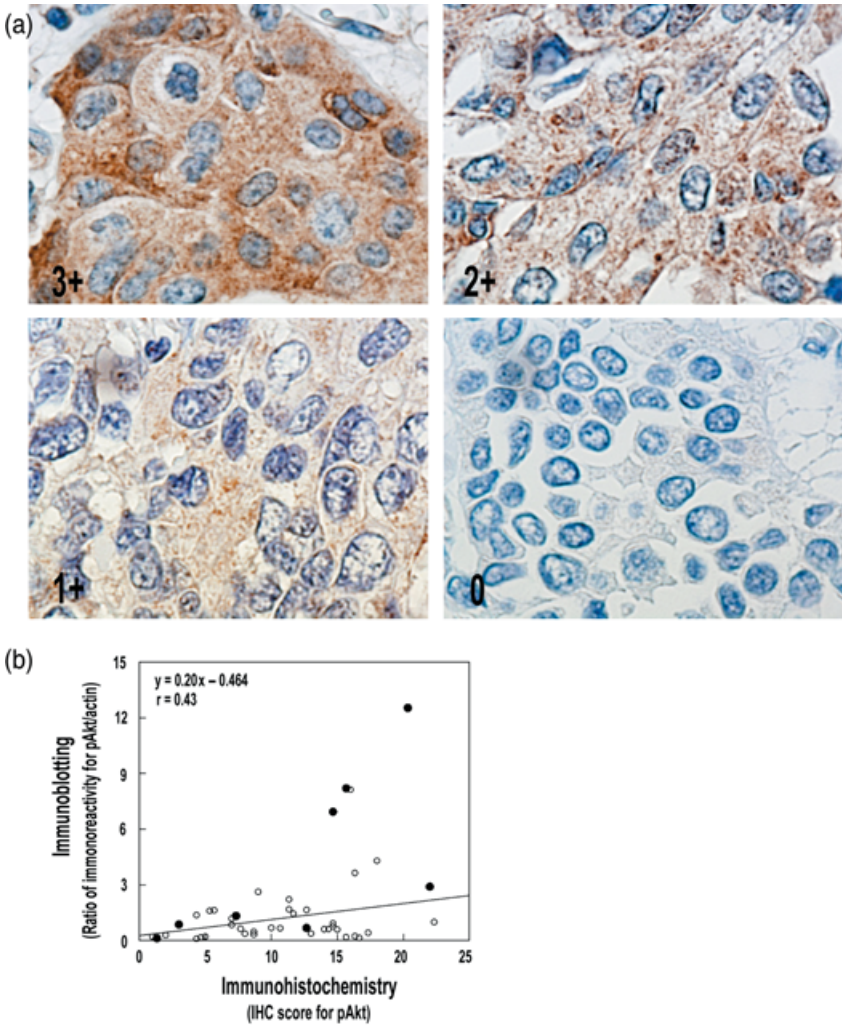

Fig. 2. Immunohistochemical analysis for phosphorylated Akt. Immunohistochemical results were evaluated by a combination of immunohistochemical intensity and population. The population of carcinoma-positive cells with strong (3+), medium (2+), or weak (1+) intensity, or negative cells, were estimated. (b) The results of immunoblotting and immunohistochemical analysis were in good correlation. Solid circles indicate ER^-, and PgR^- and human epidermal growth factor receptor (HER)2^-, and open circles indicate other types of breast cancers, including ER^+ and $HER2^-$, ER^+ and $HER2^+$, and ER and $HER2^+$.

Results and Discussion

Western blotting analysis demonstrated that HER2, ERK1/2, and Akt were phosphorylated to various degrees (Fig. 1a). There were eight cases of triple-negative breast cancers (i.e. cases 6, 7, 14, 15, 28, 33, 35, and 42). Phosphorylation of HER2 was detected in four of the 15 HER2-positive breast cancers (4/15, 26.7%). Thor *et al*. studied HER2 phosphorylation in 816 primary breast cancers using IHC methods and found that HER2 was phosphorylated in 12% (37/307) of HER2-positive breast cancers, and in 4.5% of all breast cancers.[14] It might be expected that overexpressed HER2 protein is phosphorylated constitutively, thus stimulating transmission of growth signals to the nuclei. However, our results support the findings of previous studies that demonstrate that overexpressed HER2 protein is not always phosphorylated, but rather, may be phosphorylated in a limited number of tumors.[15]

The phosphorylated MAPK (pMAPK) level and ratio of pMAPK : MAPK was slightly higher in triple-negative breast cancers than in the other groups, but the difference was not statistically significant (Fig. 1b). In triple-negative breast cancers, the pAkt level and the ratio of pAkt : Akt were significantly greater compared with the other groups (Fig. 1b,c). Phosphorylation of Akt was also analyzed by immunohistochemical methods. Immunoreactivity for pAkt was variable from cell to cell; therefore, we evaluated the result by a combination of staining intensity (Fig. 2a) and percentage. The results of immunoblotting and immunohistochemistry were in good correlation (Fig. 2b). Triple-negative breast cancers were presented as solid circles that demonstrated a population with highly phosphorylated Akt.

Triple-negative breast cancers are speculated to consist of several groups with different phenotypes, including so-called basal-like type, apocrine carcinoma, metaplastic carcinoma, carcinoma with myoepithelial differentiation, and others. Accumulating genotypic knowledge has necessitated alterations in the phenotypic categories, but we have to learn how genotypic and phenotypic findings are correlated and how these categories are biologically and clinically significant. For example, a genetic profiling study proposed the category of 'basal-like type',[16,17] but we have to know which immunohistochemical panel is sufficient and adequate to identify this group[18,19] and whether carcinoma with metaplasia (squamous, spindle, matrix-producing, and myoepithelial differentiation) should be differentiated or not.[20,21] We also have to know how 'molecular apocrine carcinoma', proposed by genetic study,[22] is correlated with histological type and whether this category is clinically significant.

Akt has a number of roles in intracellular events, and the biological significance of increased phosphorylation of Akt in triple-negative breast cancer has not been clarified so far. It is worth further investigating how pAkt is related to triple-negative cancers with activated integrin-mediated signaling, with androgen receptor-related breast cancers, or others.

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