been fully elucidated; however, several molecular and cellular effects have been reported in experimental and preclinical models. The proposed mechanisms of trastuzumab actions are as follows: (1) diminished receptor signaling, (2) G1 arrest by modulation of the cyclin-dependent kinase (cdk) inhibitor p27, (3) the induction of apoptosis, (4) the inhibition of angiogenesis, (5) immune mechanisms such as antibody-dependent cell-mediated cytotoxicity (ADCC), (6) the inhibition of HER2 ECD cleavage and (7) the inhibition of DNA repair.[14–16]

Preclinical studies

In several preclinical investigations, the antiproliferative activity of trastuzumab has been revealed in both in vitro and in vivo studies. Trastuzumab inhibited the cell proliferation of SK-BR3, a breast cancer cell line with HER2 overexpression.[17] In addition, trastuzumab has been revealed to have both synergistic and additive interactions with conventional chemotherapeutic agents, including platinum analogs, taxanes, anthracyclines, vinorelbine, and cyclophosphamide.[17,18] Trastuzumab was also able to induce strong dose-dependent growth suppression of naturally HER2-overexpressing BT-474 breast cancer xenografts. Treatment with anti-HER2 antibody of well-established BT-474 breast cancer xenografts naturally overexpressing HER2 in athymic mice resulted in dose-dependent antitumor activity. In combination studies, treatment with anti-HER2 antibody and either paclitaxel or doxorubicin resulted in a greater growth inhibition than that observed with either agent alone. The combination of anti-HER2 antibody and paclitaxel resulted in the highest tumor growth inhibition, while also demonstrating a significantly superior complete tumor regression rate in comparison to either paclitaxel or anti-HER2 antibody alone.[19] These preclinical data, showing the synergistic and additive interactions of trastuzumab with several chemotherapeutic agents, thus suggested rational combinations to be further evaluated in clinical trials. In fact, many of these drug combinations have since been shown to demonstrate significant antitumor efficacy in the clinical setting.

Clinical studies

Trastuzumab as a single agent

Trastuzumab was first employed for metastatic breast cancer (MBC). The activity and safety of trastuzumab as a single agent were investigated in two phase II clinical trials in women with HER2-overexpressing MBC who had progressed after one or two chemotherapeutic regimens.[20,21] The objective response rates (ORRs) were 11.6% and 15% in these two studies.[20,21] Cardiac dysfunction was the most common adverse event, occurring in 5% of treated patients, many of whom had received doxorubicin prior to trastuzumab.

In a study that was conducted to investigate the efficacy and safety of trastuzumab as a single agent in the first-line treatment of HER2-overexpressing MBC, the response rate was 26%.[8] In this randomized phase II trial of first-line treatment, the patients were randomly assigned to one of two dose levels of trastuzumab (4 mg/kg initially followed by 2 mg/kg weekly, or 8 mg/kg to start, followed by 4 mg/kg weekly). The overall RR was 35% for women with 3+ immunohistochemical (IHC) staining, nearly double that reported for the previously treated patients. On the other hand, the overall RR was zero for those with 2+ IHC staining.[8] The RRs in the patients with and without *HER2* gene amplification determined by fluorescence in situ hybridization (FISH) analysis were 34% and 7%. There was no clear dose-response relationship for response, survival, or adverse events. This study concluded that single-agent trastuzumab was active and well-tolerated as a first-line treatment for women with MBC with HER2 3+ overexpression, determined by either IHC or gene amplification by FISH.[8] From these clinical trials, it has been shown that trastuzumab administered as a single agent is both active and well tolerated.[8,20,21]

Trastuzumab in combination with chemotherapy

The results of a pivotal randomized phase III trial indicated that trastuzumab significantly enhanced the activity of first-line chemotherapy, thus providing a survival advantage to women with HER2-overexpressing breast cancer.[9] The addition of trastuzumab to any of the chemotherapy regimens was associated with a significantly longer time to disease progression (TTP; 7.4 months versus 4.6 months with chemotherapy alone), a higher RR (50% versus 32%), and a longer median overall survival (OS; 25.1 months versus 20.3 months).[22] As a result, numerous combinations of trastuzumab with cytotoxic agents have been evaluated in clinical studies.

Trastuzumab plus anthracyclines

The benefit of adding trastuzumab to anthracycline-based chemotherapy was tested in the abovementioned pivotal multicenter trial of 469 women with previously untreated, HER2-overexpressing MBC.[22] Women who were exposed to adjuvant anthracyclines (n = 188) were randomly assigned to paclitaxel with or without trastuzumab, while those who were anthracycline-naive (n = 281) were randomly assigned to doxorubicin plus cyclophosphamide (AC) or epirubicin plus cyclophosphamide (EC), with or without trastuzumab. The addition of trastuzumab to AC or EC was associated with a significantly longer TTP (7.8 versus 6.1 months with AC or EC alone), a higher RR (56% versus 42%), and a longer median OS (26.8 versus 21.4 months).[22] However, cardiotoxicity was more common with combined treatment, especially with AC plus trastuzumab. Cardiac dysfunction developed in 27% of the AC-plus-trastuzumab group, compared to 8%, 13%, and 1% in the groups receiving AC alone, paclitaxel plus trastuzumab,

and paclitaxel alone, respectively.[22] These results led to the recommendation that concomitant anthracyclines and trastuzumab be avoided and the concurrent administration of anthracyclines and trastuzumab should still be limited to clinical trials.

Trastuzumab and taxanes

Combination therapy of taxanes and trastuzumab has revealed good efficacy and favorable toxicity. In the abovementioned trial,[22] patients with doxorubicin-refractory, HER2-positive MBC were randomly assigned to paclitaxel administered every 3 weeks with or without trastuzumab. Compared to paclitaxel alone, the RR with combination therapy was significantly higher (38% versus 16%) and the TTP was significantly longer (6.9 versus 3.0 months). Weekly paclitaxel and trastuzumab is a particularly well-tolerated combination.[23] In a trial of 95 women who were unselected for HER2 status, the RR for HER2-overexpressing tumors ranged from 67% to 81%, depending upon the specific type of assay used.[23] On the other hand, the RRs were 41% to 46% in patients with HER2-normal expression.[23] Treatment was associated with grade 3/4 neutropenia in 6%, and 3 patients had severe cardiac complications. A large multicenter cooperative group trial is now underway comparing weekly versus every-3-week paclitaxel plus trastuzumab. Similar high RRs (50% to 76%) and a favorable toxicity profile have been reported with combinations of docetaxel plus trastuzumab.[24–27] The superiority of trastuzumab plus docetaxel compared to docetaxel alone was suggested in a phase II trial, in which 186 patients with previously untreated MBC were randomly assigned to docetaxel with or without trastuzumab.[25] The addition of trastuzumab to docetaxel resulted in significantly better RR (61% versus 34%), OS (31 versus 23 months), and TTP (11.7 versus 6 months), and there was little difference in toxicity between the groups. Good efficacy of weekly docetaxel plus trastuzumab in HER2-overexpressing MBC has also been reported.[24,27] From these trials, the combination of a taxane and trastuzumab is currently considered to be the best first-line option for women with HER2-overexpressing MBC, although so far no trial has compared trastuzumab plus a taxane versus trastuzumab alone.

Trastuzumab and platinum compounds

Based on preclinical studies, the synergistic interaction of platinum compounds and trastuzumab was expected.[17,18] There have been encouraging reports with combinations of trastuzumab and either cisplatin or carboplatin, with and without a taxane.[28–31]

Trastuzumab and vinorelbine

Vinorelbine used as a single agent has demonstrated good efficacy both as first-line (41%-50%) and as second-line (25%-40%) chemotherapy for MBC.[32–36] Synergistic activity of the combination of trastuzumab and vinorelbine has been shown in in vitro studies.[18,37] Recent clinical studies of the combination of trastuzumab and vinorelbine in untreated or heavily pretreated patients with HER2-positive MBC have shown high objective RRs.[34–36,38] Vinorelbine did not increase the cardiac toxicity of trastuzumab. In addition, RRs in excess of 60% were noted in women who received vinorelbine plus trastuzumab as a second- or third-line regimen for MBC.

The results of combination therapies with trastuzumab and various cytotoxic agents are shown in Table 1.

Duration of trastuzumab-based treatment

Whether trastuzumab should be continued with an alternative cytotoxic agent at the time of progression on a trastuzumab-containing regimen is an important clinical question. No randomized trial has yet been conducted to answer this question. There have been some retrospective studies to assess whether trastuzumab-based treatment beyond disease progression shows any evidence of efficacy.[39–41] Eighty patients with HER2-overexpressing MBC received trastuzumab, either as monotherapy or in combination with chemotherapy, beyond disease progression.[39] In total, 32 responses (40%) were observed, most of them during the second- or third-line f treatment. The median survival from disease progression after trastuzumab administration was 22.2 months.[39] Another retrospective study revealed a similar objective RR for second-line therapy in comparison to first-line therapy, and some patients responded to second-line who had not responded to first-line therapy. Cardiac events were reported in 22 patients, but none were fatal, and most patients were able to continue receiving trastuzumab.[40] The similar result also showed the efficacy and safety of sequential trastuzumab-based treatment beyond disease progression.[41] An extension study of the pivotal phase III trial discussed above was conducted to determine the safety of continuing trastuzumab beyond disease progression.[42] Patients were offered either chemotherapy plus trastuzumab or trastuzumab alone, at progression after chemotherapy plus trastuzumab therapy. Although not designed to evaluate efficacy, the RR was 11% among those assigned to trastuzumab plus chemotherapy, and the median response duration was 6.7 months.[42] The authors noted that the novel and targeted activity of trastuzumab (including direct antiproliferative activity, synergistic interaction with a number of standard chemotherapy agents, and antiangiogenic activity) suggested the efficacy of the continuation of trastuzumab-based therapy.[42] It is difficult to define which patients should continue to be treated with trastuzumab; however, continuing trastuzumab therapy combined with chemotherapy after disease progression on a first-line trastuzumab-containing regimen seems feasible and safe for HER2-overexpressing MBC. In the absence of any evidence showing a clear benefit, the theoretical benefits of continuing trastuzumab must be weighed against the high cost of the therapy, and the potential for adverse effects.

Table 1. Trastuzumab in combination with various cytotoxic agents

Reference	*n*	Regimen	ORR (%)	TTP (months)	OS (months)
Trastuzumab + taxanes					
Meden[79]	12	T + D (qw)	50	NR	NR
Esteva[24]	30	T + D (qw)	63	9	NR
Soidman[23]	95	T + P (qw)	57	9[a]	NR
Montemurro[26]	42	T + D (q3w)	67	8	NR
Tedesco[27]	26	T + D (qw)	50	12.4	22.1
Gori[80]	25	T + P (qw)	56	8.6	NR
Raff[81]	17	T + D (qw)	59	8.5	NR
Marty[25]	92	T + D (q3w)	61	11.7	31.2
Trastuzumab + platinum salt					
Pegram[82]	37	T + CDDP (q4w)	24	8.4	NR
Burrie[83]	31	T + P + CBDCA (qw)	84	14.2	32.2
Pegram[29]	62	T + D + CBDCA (q3w)	58	12.7	NR
	62	T + D + CDDP (q3w)	79	9.9	NR
Perez[31]	43	T + P + CBDCA (q3w)	65	9.9	27.6
	48	T + P + CBDCA (qw)	81	13.8	38.4
Trastuzumab + vinorelbine					
Jahanzeb[36]	40	T + V (qw)	78	18	NR
Burstein[34]	54	T + V (qw)	68	NR	NR
Papaldo[38]	35	T + V (qw)	51	9	27
Trastuzumab + gemcitabine					
O'Shaunessy[84]	61	T + G (qw)	38	5.8	14.7
Fountzilas[85]	40	T + P + G (qw)	53	13.7	NR
Stemmler[30]	20	T + G + CDDP (qw)	40	10.2	18.8

ORR, objective response rate; TTP, time to disease progression; OS, overall survival; w, week(s); T, trastuzumab; D, docetaxel; P, paclitaxel; CDDP, cisolutin; CBDCA, carboplatin; V, vinorelbine; G, gemcitabine; NR, not reported

[a] Response duration

Adjuvant therapy

At the annual meeting of the American Society of Clinical Oncology (ASCO) in 2005, the sensational results of adjuvant trastuzumab were first reported and were published within a short time after that meeting. Emerging data support a benefit of trastuzumab in the adjuvant setting, as well as in the metastatic setting.

The National Surgical Adjuvant Breast and Bowel Project (NSABP)-B31 trial compared four cycles of cyclophosphamide and doxorubicin (AC) followed by paclitaxel every 3 weeks (group 1) with the same regimen plus 52 weeks of trastuzumab starting on day 1 of paclitaxel therapy (group 2). The North Central Cancer Treatment Group (NCCTG) N9831 trial compared three regimens: AC followed by paclitaxel given weekly for 12 weeks (group A), the same regimen followed by 52 weeks of trastuzumab after paclitaxel (group B), and the same regimen plus 52 weeks of trastuzumab initiated concomitantly with paclitaxel (group C). Investigators from the NSABP and the North American Intergroup have reported a combined analysis of the control arms from both trials (NSABP group 1 plus N9831 group A) versus the sequential trastuzumab groups from each trial (NSABP group 2 plus N9831 group C).[43] This pooled analysis was not part of the original treatment designs, but it was conducted with the approval of the National Cancer Institute. The absolute difference in disease-free survival between the trastuzumab group and the control group was 12% at 3 years. Trastuzumab therapy was thus associated with a 33% reduction in the risk of death (3-year OS, 91% versus 87%; hazard ratio [HR], 0.67).[43] The 3-year cumulative incidence of class III or IV congestive heart failure or death from cardiac causes in the trastuzumab group was 4.1% in the NSABP-B31 trial and 2.9% in the N9831 trial.[43] The question of whether concurrent trastuzumab (with paclitaxel and thereafter) is better than sequential trastuzumab (i.e., starting after the completion of paclitaxel) could not be answered conclusively.

Remarkably similar results were reported in an interim analysis of the large international, multicenter HERceptin Adjuvant (HERA) trial.[44] This trial compared one or two year trastuzumab given every 3 weeks with observation in patients with HER2-positive and either node-negative or node-positive breast cancer who had completed locoregional therapy and at least four cycles of neoadjuvant or adjuvant chemotherapy. A planned interim analysis of data for 1694 patients in a 1-year trastuzumab group and 1693 patients in a control group has been reported.[44] Events (recurrences, new cancer [breast or other], death) were half as common in the treatment group as in the control group (HR, 0.54; 95% confidence interval [CI], 0.43–0.67; $P < 0.0001$), although there was no improvement in the OS in the treatment group. Severe cardiotoxicity developed in 0.5% of the women who were treated with trastuzumab. These findings are similar to both the postoperative outcomes reported by Romond and colleagues [43] and to earlier comparisons of first-line chemotherapy with or without trastuzumab in the treatment of HER2-positive cancers.[9,25] A Breast Cancer International Research Group (BCIRG) trial (BCIRG 006) is now evaluating the role of taxane with or without trastuzumab following an anthracycline-

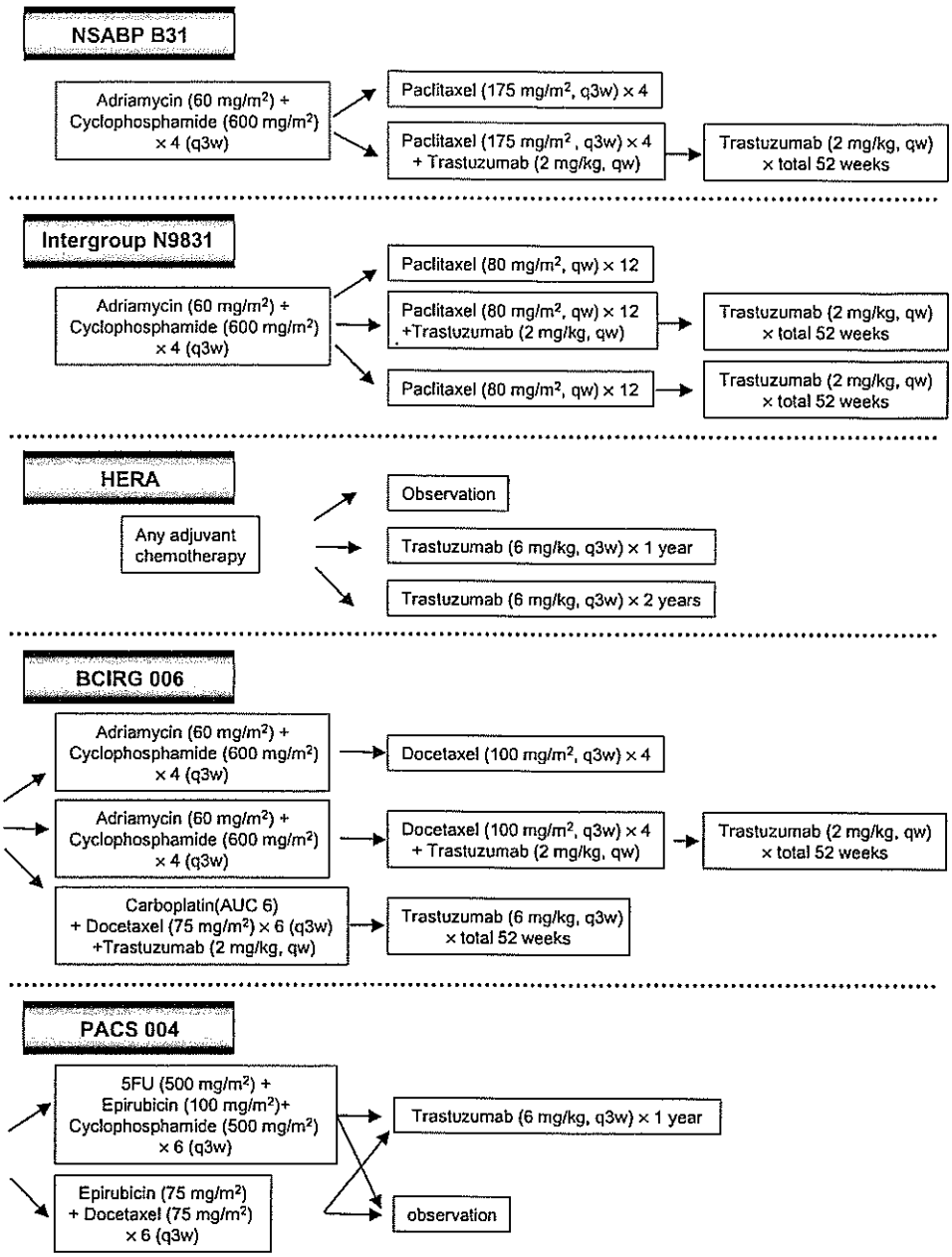

Fig. 1. Schematic drawing of ongoing adjuvant trials containing trastuzumab. *w*, week(s); *5-FU*, 5-fluoruracil

containing regimen (AC followed by docetaxel with or without trastuzumab). The third experimental arm of this study is a non-anthracycline regimen (carboplatin plus docetaxel and trastuzumab; TCH). This study includes HER2-positive, node-positive or high-risk node-negative breast cancer. In a preliminary report from the 2005 San Antonio Breast Cancer Symposium, with a 23-month median follow-up, disease-free survival (DFS) was significantly better in both the trastuzumab-containing arms in comparison to AC followed by docetaxel (HRs for DFS, 0.49 and 0.61 for AC/docetaxel/trastuzumab and TCH, respectively.[45] There was no significant difference between the two trastuzumab-containing arms. In addition, there appeared to be fewer severe cardiac adverse events with TCH in comparison to either the anthracycline group (symptomatic cardiac events in 1.2% treated with TCH versus 2.3% and 1.2% for those treated with AC plus docetaxel with and without trastuzumab, respectively; the absolute left ventricular ejection fraction (LVEF) declined by more than 15% and was below the lower limit of normal in 0.4% of patients treated with TCH versus 2.4% and 0.6% for those treated with AC plus docetaxel with and without trastuzumab, respectively). Figure 1 summarizes the design of ongoing adjuvant trials containing trastuzumab.

The preliminary data from these trials suggest that the addition of trastuzumab to adjuvant chemotherapy benefits

women with HER2-overexpressing breast cancer, both in terms of disease recurrence and in terms of survival. However, a number of unresolved issues still remain. Should adjuvant trastuzumab and chemotherapy be administered concurrently or sequentially? What is the optimal chemotherapy regimen in this setting? Further data will help us to resolve these issues.

Trastuzumab as primary systemic therapy

Several phase II and phase III studies have evaluated primary systemic therapy (PST) with trastuzumab in combination with cytotoxic agents.[46–50] These combinations have achieved pathologic CR (pCR) rates of 12% to 65% and clinical CR (cCR) rates of 30% to 86%. These results compare favorably with those of primary systemic therapy using standard combinations in patients with unselected (HER2-positive or -negative) breast cancer. A randomized trial to determine whether the addition of trastuzumab to chemotherapy in the neoadjuvant setting could increase the pCR rate was performed in 42 patients with HER2-positive disease with operable breast cancer.[47] The patients were randomly assigned to either four cycles of paclitaxel followed by four cycles of fluorouracil, epirubicin, and cyclophosphamide (FEC) or to the same chemotherapy with simultaneous weekly trastuzumab for 24 weeks. The planned sample size was 164 patients; however, after 34 patients had completed therapy, the trial's Data Monitoring Committee stopped the trial because of the clear superiority of trastuzumab plus chemotherapy. The pCR rates were 25% and 66.7% for chemotherapy (n = 16) and trastuzumab plus chemotherapy (n = 18), respectively (P = 0.02). Although no clinical congestive heart failure was observed, long-term cardiac toxicity monitoring remains an open question. To establish the efficacy and safety of combinations of trastuzumab and cytotoxic agents, including anthracycline, further clinical studies are needed in larger groups of patients, with longer observation periods. In the neoadjuvant setting, other trials of the use of trastuzumab alone, or in various combinations with chemotherapy, are now ongoing. The findings of primary systemic therapies containing trastuzumab are shown in Table 2.

Adverse effects of trastuzumab

Infusion-related reactions and cardiotoxicity and are the two main safety concerns with the use of trastuzumab.[51] Infusion-related reactions, consisting of fever and chills, were the most common adverse effects, occurring in up to 40% of patients. In most cases, the symptoms were mild to moderate; however, 74 of 25000 patients (0.3%) were reported to have experienced a serious infusion-related reaction.[51] The majority of reactions occurred during or shortly after the first infusion and were characterized by respiratory symptoms. Most patients were successfully treated; 33 of the 74 patients noted above continued trastuzumab therapy with no recurrence of infusion reactions.[51]

Cardiac dysfunction has been the most significant serious adverse effect observed in the large multicenter trials.

Cardiomyopathy associated with trastuzumab is manifested as an asymptomatic decrease in LVEF. The risk of cardiac dysfunction with trastuzumab alone ranges from 2% to 7%;[52,53] however, the risk is particularly increased in patients treated concurrently with cytotoxic agents, especially anthracyclines.[53,54] The majority of clinicians consider that concurrent doxorubicin and trastuzumab are contraindicated in the setting of MBC. However, the superior efficacy of anthracycline plus trastuzumab combinations in metastatic disease has prompted several clinical trials studying combination therapy in the adjuvant setting. In these trials, trastuzumab has been administered sequentially after doxorubicin, and either concurrently with or sequentially after taxanes.[43] In a preliminary study, combined treatment with trastuzumab, AC, and paclitaxel was associated with a small but real increase in the risk of cardiac events.[43] Despite close monitoring and aggressive management, the early follow-up of these trials suggests that approximately 2% to 3% of all treated women will develop severe cardiac toxicity. It is possible that the incidence and severity of cardiac dysfunction will increase with longer follow-up.

Whether trastuzumab alone has cardiac toxicity is less clear, although there is indirect evidence supporting such an effect. In contrast to anthracycline-related cardiotoxicity, trastuzumab-associated toxicity does not appear to be dose-related, and it usually responds to standard medical treatment or the discontinuation of trastuzumab.[52,53] Guidelines and recommendations for management have been pro-

Table 2. Trastuzumab in primary systemic therapy

Reference	n	Regimen	ORR (%)	cCR (%)	pCR (%)
Burstein[46]	40	Trastuzumab (weekly) × 12 + paclitaxel (175 mg/m^2, 3-weekly)	75	30	18
Van Pelt[49]	22	Trastuzumab (weekly) × 12 + docetaxel (100 mg/m^2, 3-weekly)	77	41	NR
Wenzel[50]	14	Trastuzumab (weekly) × 12 + docetaxel (30 mg/m^2, weekly) + epirubicin (35 mg/m^2, weekly)	86	NR	7
Buzdar[47]	23	Trastuzumab (weekly) × 24 + FEC75 (3-weekly) followed by paclitaxel (225 mg/m^2, 3-weekly)	95.6	86.9	65.2
	19	FEC75 (3-weekly) followed by paclitaxel (225 mg/m^2, 3-weekly)	94.7	47.4	26.3
Coudert[48]	33	Trastuzumab (weekly) × 18 + docetaxel (100 mg/m^2, 3-weekly)	96	73	47

ORR, objective response rate; cCR, clinical complete response; pCR, pathological complete response; NR, not reported; FEC; fluoruracil + epirubicin + cyclophosphamide

posed for the formal assessment of cardiac function in patients receiving trastuzumab.[52]

The mechanism of cardiac dysfunction associated with trastuzumab is not clearly understood, although several hypotheses have been proposed. These include the modification of anthracycline-induced cardiotoxicity, immune-mediated destruction of cardiomyocytes, the effects on HER2 signaling pathways that are required for the maintenance of normal cardiac contractility, and the dependence on HER2 for myocyte survival, which is then impaired during trastuzumab treatment.[55] There is increasing experimental evidence supporting a direct toxic effect of HER2 blockade on the heart. HER2 signaling appears to play an important role in embryonic cardiac development and cardioprotection, at least in rodents.[56,57] In a conditional knockout mouse model, the ventricular-restricted deletion of HER2 expression eventually resulted in dilated cardiomyopathy, and cardiomyocytes from these mice showed enhanced susceptibility to anthracycline-induced cell death.[58] These data suggest that trastuzumab-related cardiotoxicity is not immune-mediated or due to effects outside the heart, and it does not result solely from the modification of anthracycline-induced cardiac toxicity.

Evaluation of HER2 status

Accurate HER2 testing is essential for optimal patient selection for trastuzumab. There are many methods to measure the activity of HER2, e.g., by DNA, RNA, and the protein level. For *HER2* gene amplification, FISH, chromogenic in situ hybridization (CISH), or differential polymerase chain reaction (PCR) are used. Northern blotting or reverse transcription polymerase chain reaction (RT-PCR) is used for analysis of the overexpression of HER2 mRNA. Overexpression of HER2 protein is evaluated by Western blotting, enzyme-linked immunosorbent assay (ELISA), or IHC. Clinically, IHC for HER2 overexpression and FISH for *HER2* gene amplification are widely used. There are currently four assays approved by the United States Food and Drug Administration (FDA) for the clinical testing of HER2 overexpression (by IHC) or *HER2* gene amplification (by FISH). Two are IHC assays (Herceptest; DAKO, Carpinteria, CA, USA, and the CB11 assay; Ventana Medical Systems, Tucson, AZ, USA); the other two are FISH assays (PathVysion HER-2 DNA probe kit; Vysis, Downers Grove, IL, USA, and INFORM HER-2/neu Test; Ventana Medical Systems).

A correlation between the extent of HER2 expression determined by IHC and the clinical response to trastuzumab has been confirmed.[8,59] Women whose breast tumors stain strongly (3+) for HER2 are most likely to respond to trastuzumab, while few of those with 2+ staining also benefit from the drug. On the other hand, a number of studies suggest that the identification of HER2 positivity by FISH rather than by IHC permits better selection of women with both metastatic and early-stage breast cancer who benefit from treatment with trastuzumab.[59–61] However, FISH is expensive and not widely available. Most of the discordance between FISH and IHC findings has been observed in the group with 2+ staining by IHC.[62,63] In one of the studies noted above,[59] trastuzumab was active in women whose tumors were 2+ IHC and FISH-positive, but not in those who were 2+ IHC and FISH-negative. In contrast, negative IHC results are highly concordant with FISH; the false-negative rate for IHC in one report was very low.[63,64] From these results, it appears that the most efficient testing algorithm for HER2 determination is achieved by using IHC as the method of choice, with FISH performed for cancers with indeterminate results (2+ score).[62–64]

Herceptin resistance

Not all HER2-overexpressing breast cancers respond to trastuzumab-based treatment. In addition, almost all tumors eventually become resistant to trastuzumab.[8,9] The molecular mechanisms accounting for trastuzumab resistance in patients are currently unknown. However, elucidating the molecular mechanisms by which tumors escape trastuzumab-based cytotoxicity is critical to improving the prognosis of breast cancer patients whose tumors overexpress HER2. Several molecular mechanisms contributing to trastuzumab resistance have been proposed.

The insulin-like growth factor (IGF) mitogenic signaling pathway is considered to be a therapeutic target in breast cancer, as its ligands and receptors are frequently overexpressed and implicated in promoting mitogenic, metastatic, and antiapoptotic phenotypes.[65] Overexpression of the insulin-like growth factor-I receptor (IGF-IR) has been reported to be associated with trastuzumab resistance.[66] Increased coexpression and interaction of HER2 with epidermal growth factor receptor (EGFR) family members [67,68] and heterodimerization of IGF-IR with HER2[69] have also been shown to contribute to trastuzumab resistance in breast cancer cells, and erbB kinase inhibitor has been shown to counteract the ability of erbB ligands to promote trastuzumab resistance.[70]

Constitutive Akt cell signaling was shown to inhibit the cell-cycle arrest and apoptosis mediated by trastuzumab and to contribute to trastuzumab resistance.[71,72] Decreased expression of the phosphatase and tensin homologue (PTEN) has been shown to be another very important and interesting mechanism of trastuzumab resistance;[73] the authors of this study also suggested that phosphoinositide-3-OH kinase (PI3K) inhibitors should be explored as potential therapies in trastuzumab-resistant tumors possessing low PTEN levels.[73]

$p27^{kip1}$ has been shown to play an important role in trastuzumab-induced G1 cell-cycle arrest and tumor growth inhibition, through post-translational regulation.[74] Trastuzumab increases the half-life of $p27^{kip1}$ by decreasing the cyclin E/cdk2-mediated phosphorylation of $p27^{kip1}$ and by blocking subsequent ubiquitin-dependent degradation.[74] The downregulation of $p27^{kip1}$ has been shown to be associated with trastuzumab resistance in breast cancer cells.[75]

The blocking of trastuzumab binding by MUC4, a cell surface mucin, has also been implicated in trastuzumab resistance. It has been shown that the overexpression of rat MUC4 reduces the binding of trastuzumab to HER2-expressing tumor cells.[76,77]

These data regarding trastuzumab resistance have been obtained from preclinical studies. One study using clinical materials showed that IGF-IR expression could not predict trastuzumab resistance,[78] and this result suggests the difficulty of directly translating the preclinical data to a clinical setting. It is necessary to confirm these observations in a clinical setting with further studies.

Conclusion

Molecular-targeted therapy is a great challenge in cancer treatment and it is a very interesting area of basic and clinical research. Trastuzumab, a recombinant humanized monoclonal antibody directed against the extracellular domain of the HER2 receptor tyrosine kinase, is one of the first molecular targeted drugs to be clinically applied, and it is currently one of the most successful molecular targeted therapies. Trastuzumab-based therapy has been shown to have both prognostic and survival advantages for patients with MBC with HER2 overexpression. In addition, trastuzumab therapy has also been suggested to be an extremely promising application in the adjuvant setting. However, the optimal duration of trastuzumab treatment is still unknown, and the mechanisms of its action and resistance have not yet been fully elucidated. To maximize the benefits and minimize the adverse effects of trastuzumab treatment, it is necessary to resolve these problems.

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Akt is frequently activated in HER2/neu-positive breast cancers and associated with poor prognosis among hormone-treated patients

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Akt/PKB is a serine/threonine kinase that plays an important role in survival when cells are exposed to different apoptotic stimuli. Aberrant activation of Akt/PKB in breast carcinoma is associated with poor prognosis and resistance to endocrine therapy and chemotherapy. The Akt signaling pathway currently attracts considerable attention as a new target for effective therapeutic strategies. We therefore investigated the relationship between activation of Akt and clinicopathologic variables including hormone receptor and HER2/neu status. Breast cancer tissues obtained from 252 patients were utilized for this study. We evaluated Akt activation by immunohistochemical assessment of the expression of phosphorylated Akt (pAkt) at Ser-473. Eighty-four cases (33.3%) were diagnosed as positive for pAkt expression. pAkt was significantly associated with HER2/neu overexpression ($p < 0.0001$). There was an inverse correlation between pAkt and PR expression ($p = 0.0321$); however, there was no association between pAkt and ER expression. Survival analysis showed that pAkt positivity was associated with poor disease-free survival in cases with postoperative hormone therapy; however, there was no association in cases without hormone therapy. Our results indicate that Akt activation induced poor prognosis in patients who received adjuvant hormone therapy. This finding suggests that inhibition of the Akt signaling pathway may increase the efficacy of hormone therapy and improve the prognosis of patients who receive adjuvant hormone therapy.
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Key words: Akt; HER2/neu; breast cancer; endocrine therapy

Akt, also known as PKB, is a serine/threonine protein kinase and has emerged as a crucial regulator of widely divergent cellular processes, including apoptosis, proliferation, differentiation and metabolism. Disruption of normal Akt/PKB signaling occurs frequently in several human cancers, and the enzyme appears to play an important role in cancer progression and cell survival.[1] Akt is activated by a variety of stimuli, through growth factor receptors, in a PI-3 kinase–dependent manner. The mechanisms by which Akt promotes cell survival include phosphorylation of the proapoptotic proteins BAD, caspase-9, Forkhead transcription factors and IκB kinase α. These reduce the binding of BAD to Bcl-x_L, inhibit caspase-9 protease activity and Fas ligand gene transcription and activate the nuclear factor-κB cascades.[1]

ErbB2 (HER2/neu) is a member of the type I subclass of receptor tyrosine kinases, which has been associated with several types of human cancer. Numerous studies have demonstrated that *erbB2* is amplified and overexpressed in 20–30% of primary breast cancers and generally associated with poor prognosis.[2–6] In addition, HER2/neu overexpression is associated with resistance to chemotherapy and endocrine therapy.[7–9]

One of the major signaling pathways utilized by the erbB families is the PI-3K/Akt pathway, as well as the ras-/mitogen-activated protein kinase pathway. The ligand of erbB2 has not been identified; however, erbB2-containing heterodimers are potent activators of multiple signaling pathways involved in proliferation, invasion and survival.[10] Studies in breast cancer cells, primary breast tumors and transgenic mice all indicate that when erbB2 is overexpressed, it is constitutively associated with erbB3.[11] These ErbB2/erbB3 dimers strongly activate the PI3K-PKB/Akt pathway. This is supported by previous evidence that tumor cells overexpressing HER2/neu exhibit constitutive PKB/Akt activity.[12] Experimental studies have demonstrated that the malignant phenotypes of breast carcinomas with HER2/neu overexpression are partially due to PI-3K /Akt signaling.[8,9,13] In addition, constitutive and inducible Akt activity promotes resistance to chemotherapy, trastuzumab or tamoxifen in breast cancer cells.[14] These findings suggest that Akt activation could be used as a predictive marker for sensitivity to various therapies.

Activation of Akt/PKB has been positively associated with HER2/neu overexpression in breast carcinoma obtained from human material[15,16] and with a worse outcome among endocrine-treated breast cancer patients.[15,17] Among premenopausal patients treated with tamoxifen and/or goserelin, those with activated Akt were more prone to relapse with distant metastasis.[17] However, among postmenopausal patients, those negative for Akt showed significant benefit from tamoxifen.[15] These results support the findings from basic research that activated Akt promotes resistance to tamoxifen in breast cancer cells.[14]

In the present study, we investigated the incidence of Akt activation in breast carcinomas and correlated it with HER2/neu overexpression, other clinicopathologic variables and survival in 252 breast carcinomas in Japanese women. Akt/PKB activation was elevated significantly in cases with HER2 overexpression and associated with poorer prognosis in patients who received adjuvant hormone therapies.

Material and methods

Patient population and tumor specimens

A total of 252 primary human breast carcinoma specimens were obtained from patients who underwent surgery at the Department of Surgery and Science, Kyushu University Hospital, from 1991 to 2002. Informed consent was obtained from each patient prior to tissue acquisition. Clinical data were obtained from medical records. Resected tissues were routinely processed for histopathologic analyses by histopathologic specialists at our hospital. Histopathologic diagnosis was determined according to the criteria of the Japanese Breast Cancer Society.[18]

Antibodies

MAbs 6F11 and 1A6 (Ventana, Tucson, AR) were used for ER and PR staining. For HER2/neu evaluation, MAb CB11 (Ventana) was used. pAkt was detected using polyclonal antibodies against phosphorylated Ser-473 (Cell Signaling Technology, Beverly, MA).

Abbreviations: DAB, 3,3′-diaminobenzidine; DFS, disease-free survival; ER, estrogen receptor; LH-RH, luteinizing hormone–releasing hormone; MAb, monoclonal antibody; MPA, medroxyprogesterone acetate; pAkt, phosphorylated Akt; PI-3K, phosphatidylinositol-3 kinase; PI-3 kinase, phosphoinositide-3-OH kinase; PKB, protein kinase B; PKC, protein kinase C; PR, progesterone receptor.

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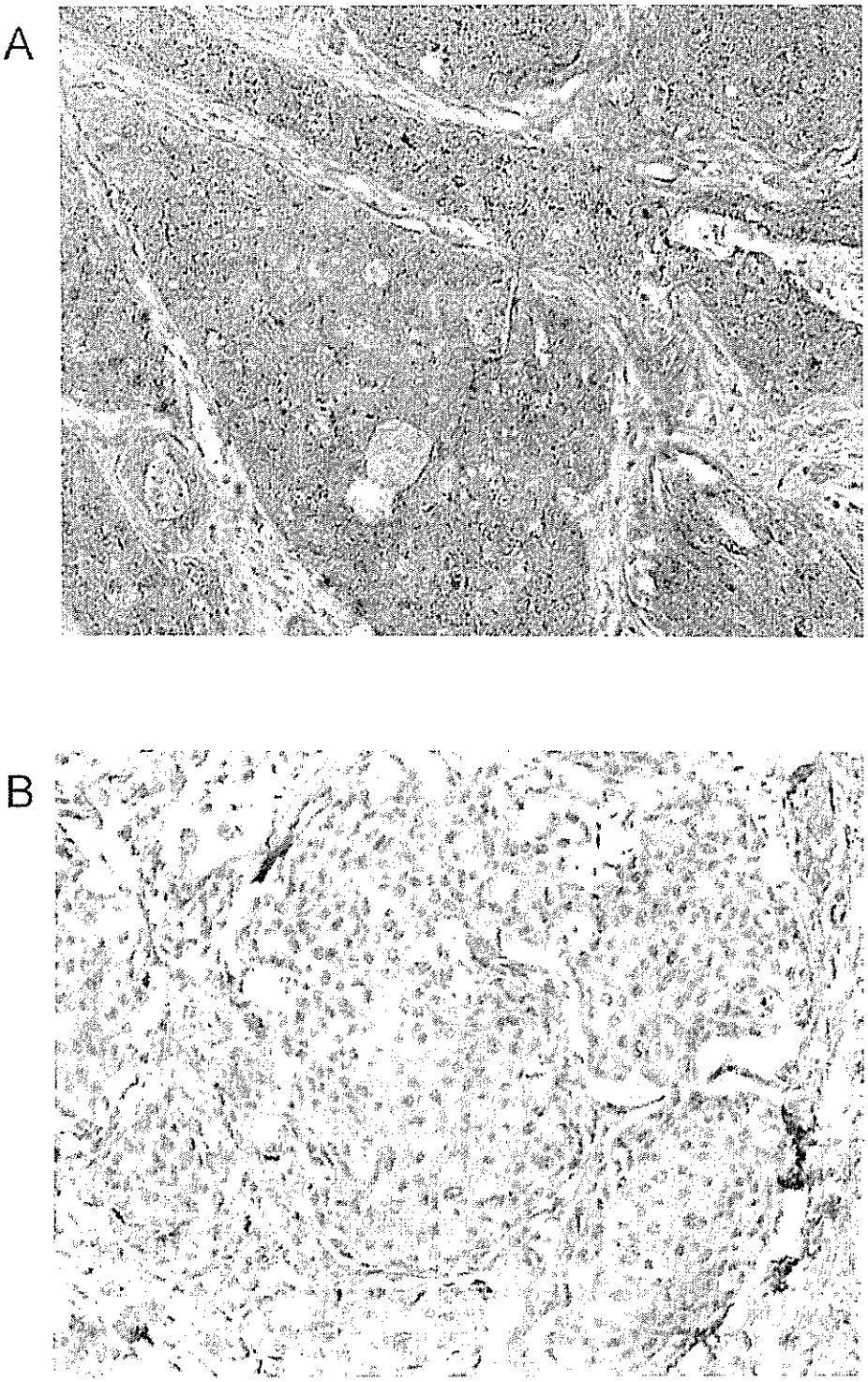

FIGURE 1 – Immunostaining of pAkt. Representative immunohistochemical staining of pAkt in breast carcinoma. pAkt was detected in the cytoplasm of tumor cells. (*a*) Positive immunostaining for pAkt. (*b*) Negative immunostaining for pAkt.

Immunohistochemistry and evaluation

Tissue samples were fixed by immersion in buffered formalin and embedded in paraffin. Sections (4 μ) were placed onto charged slides and dried at 60°C for 1 hr. Sections were deparaffinized and hydrated in water. Immunostaining of these paraffin sections was performed using the Ventana Discovery automated staining instru-

TABLE I – CORRELATIONS OF Akt ACTIVATION WITH HER2/neu EXPRESSION

pAkt	*n*	HER2/neu		*p* value
		Negative (%)	Positive (%)	
Negative	168	140 (83.3)	28 (16.7)	< 0.0001
Positive	84	48 (57.1)	36 (42.9)	

TABLE II – CORRELATIONS OF Akt ACTIVATION WITH HORMONE RECEPTOR EXPRESSION

pAkt	*n*	ER		*p* value	PR		*p* value
		Negative (%)	Positive (%)		Negative (%)	Positive (%)	
Negative	168	55 (32.7)	113 (67.3)	0.1632	82 (48.8)	86 (51.2)	0.0321
Positive	84	35 (41.7)	49 (58.3)		53 (63.1)	31 (36.9)	

TABLE III – CORRELATION BETWEEN pAkt EXPRESSION AND CLINICOPATHOLOGIC VARIABLES

Variables	pAkt		*p* value
	Negative (n = 168)	Positive (n = 84)	
Age (years)	54.7 ± 12.6	54.1 ± 11.0	N.S.
Tumor size (cm)	3.2 ± 2.0	3.2 ± 2.1	N.S.
Clinical stage			
0 (%)	1 (50)	1 (50)	N.S.
I (%)	38 (66.7)	23 (33.3)	
IIA (%)	60 (72.3)	33 (27.7)	
IIB (%)	45 (66.2)	23 (33.8)	
IIIA (%)	18 (56.3)	14 (43.7)	
IIIB (%)	6 (60.0)	4 (40.0)	
Axillary lymph node metastasis			
Negative (%)	112 (72.7)	42 (27.3)	0.0081
Positive (%)	53 (56.4)	41 (43.6)	
Pathologic classification			
Noninvasive ductal carcinoma (%)	4 (80.0)	1 (20.0)	N.S.
Papillotubular carcinoma (%)	43 (60.6)	28 (39.4)	
Solid tubular carcinoma (%)	35 (71.4)	14 (28.6)	
Scirrhous carcinoma (%)	65 (63.7)	37 (36.3)	
Mucinous carcinoma (%)	9 (90.0)	1 (10.0)	
Others (%)	9 (75.0)	3 (25.0)	

N.S., not significant.

ment (Ventana), and hematoxylin (Ventana) was employed as a nuclear counterstain. Immunostaining was visualized with a streptavidin peroxidase reaction using DAB as the chromogen (Ventana). A negative control reaction with no primary antibody was always performed alongside the reaction-containing sample.

Immunostaining was evaluated without knowledge of the clinical and pathologic parameters. ER and PR were recorded as positive if 10% or more of the nuclei in the invasive component of the tumor were stained.[19] HER2/neu was scored by widely accepted criteria that assessed the intensity and completeness of membrane staining.[20,21] The intensity of membrane staining was evaluated according to the following criteria: 0, none or up to 10% membrane staining; 1+, partial and/or faint membrane staining present in >10% of tumor cells; 2+, weak to moderate, complete membrane staining present in >10% of tumor cells; and 3+, strong, complete membrane staining present in >10% of tumor cells. Scores 0 and 1+ were considered normal (*i.e.*, negative for overexpression), and 2+ and 3+ were considered positive for HER2/neu overexpression. A specimen was considered positive for pAkt if 10% or more of the cytoplasm in the invasive component of the tumor stained positive for pAkt.

Statistical analysis

Associations between categorical variables were assessed by means of χ^2 tests. DFS was determined from the date of surgery to the date of relapse or last follow-up. DFS was estimated using the Kaplan-Meier method. The 2-sided log-rank test was used to test the association between variables and survival. The cut-off for significance was set at $p < 0.05$. Rates of recurrence in relation to expression of pAkt and other variables were estimated and tested using Cox's proportional hazards model.

Results

Expression of pAkt in primary breast cancer tissues

Phosphorylation of threonine-308 and serine-473 is required for activation of Akt1, and the phospho-Ser-473 Akt antibody recognizes only the phoshorylated/active form of Akt.[22,23] According to the manufacturer's information, the phospho-Akt (Ser-473) antibody used in our study detects Akt1 only when phosphorylated at serine-473 and Akt2 and Akt3 only when phosphorylated at equivalent sites. It does not detect Akt phosphorylated at other sites or related kinases such as PKC and p70 S6 kinase. pAkt was observed in the tumor membrane and cytoplasm, which is consistent with a previous report[23] (Fig. 1). A specimen was considered positive for pAkt if 10% or more of the cytoplasm in the invasive component of the tumor stained positive for pAkt. Representative positive and negative cases are shown in Figure 1. Eighty-four cases (33.3%) were diagnosed as positive for pAkt expression. We examined the correlation between pAkt expression and HER2/neu status. pAkt was expressed in significantly more of the HER2/neu-positive cases ($p < 0.0001$) (Table I). No significant correlation was observed between pAkt and ER expression; however, an inverse correlation was observed between pAkt and PR expression ($p = 0.032$) (Table II). Correlations between pAkt expression and other clinicopathologic variables are shown in Table III. Phosphorylation of Akt is associated with lymph node metastases ($p = 0.008$). No significant correlation was observed between pAkt expression and other clinicopathologic variables, such as age, tumor size, clinical stage and pathologic classification (Table III).

Prognostic value of phosphorylation of Akt in breast cancers

Because it has been suggested that high Akt activity in breast carcinoma is associated with resistance to hormone therapies and chemotherapy[14] and with poor prognosis,[15–17] we investigated whether pAkt might be associated with poor prognosis in our data set. We performed univariate survival analysis to show the association of DFS with pAkt in 240 patients whose clinical courses were available. There was no difference between pAkt-positive and -negative groups in terms of DFS for all cases (Fig. 2*a*). Of these 240 patients, 107 received postoperative hormone therapy, while the remaining 133 were not treated with hormone therapy. Interestingly, in the analysis of cases with postoperative hormone therapy, pAkt positivity was significantly associated with a higher risk of recurrence ($p = 0.0161$) (Fig. 2*b*). The hormone therapies received by these patients were as follows: selective estrogen receptor

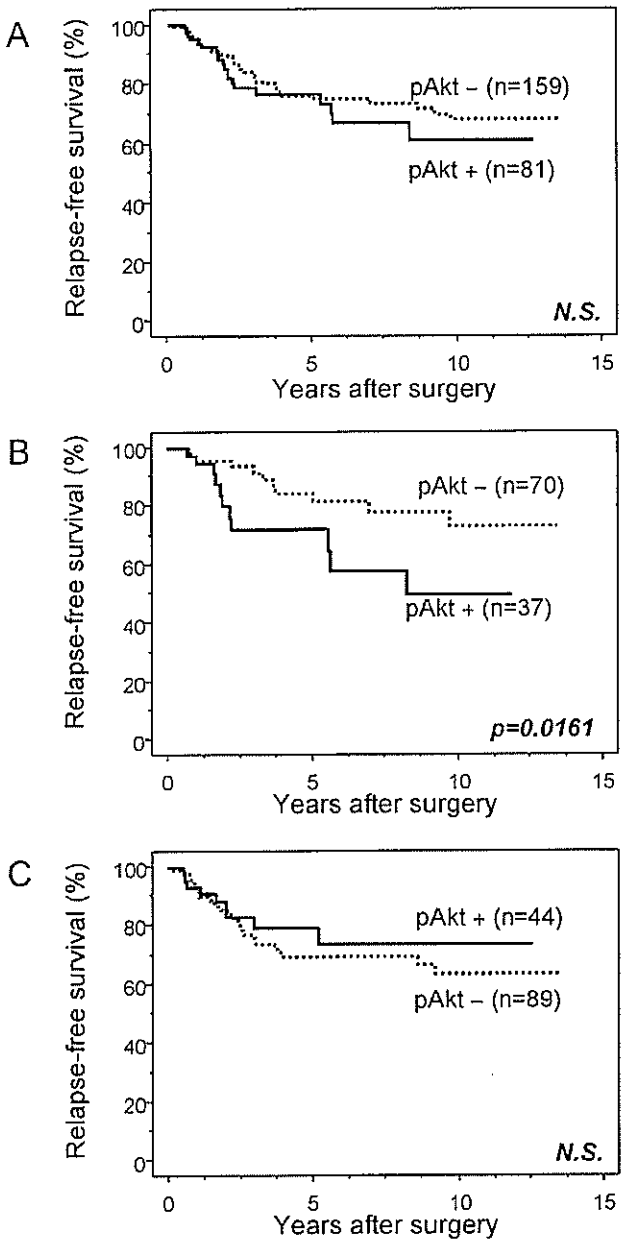

FIGURE 2 – Kaplan-Meier DFS curves of breast cancer patients. There was no difference between pAkt-positive and -negative groups in terms of DFS for all cases (*a*). In the analysis of cases with postoperative hormone therapy, pAkt positivity was significantly associated with a higher risk for recurrence (*b*). pAkt positivity had no prognostic value in cases without postoperative hormone therapy (*c*).

modulator (SERM) (tamoxifen or toremifene) in 81 patients, aromatase inhibitor (anastrozole or fadrozole) in 11 patients, LH-RH agonist (goserelin) in 8 patients, LH-RH agonist (goserelin) + tamoxifen in 5 patients and MPA in one patient. However, pAkt positivity had no prognostic value in cases without postoperative hormone therapy (Fig. 2*c*). Multivariate analysis of pAkt and traditional prognostic factors failed to indicate that pAkt was an independent prognostic factor (Table IV). However, in cases with postoperative hormone therapy, there was a tendency for higher risk in the pAkt-positive group compared to the pAkt-negative group ($p = 0.10$) (Table V).

TABLE IV – MULTIVARIATE ANALYSIS OF RECURRENCE IN ALL PATIENTS INCLUDING THE VARIABLES LYMPH NODE STATUS, HORMONE RECEPTOR STATUS AND pAkt

Variables	Rate ratio (95% CI)	Significance
Nodal status		
Negative	1	$p < 0.0001$
Positive	4.12 (2.2–7.6)	
ER		
Negative	1	$p = 0.95$
Positive	1.02 (0.5–2.0)	
PR		
Negative	1	$p = 0.95$
Positive	0.98 (0.5–1.9)	
pAkt		
Negative	1	$p = 0.40$
Positive	1.28 (0.7–2.3)	

CI, confidence interval.

TABLE V – MULTIVARIATE ANALYSIS OF RECURRENCE IN PATIENTS WHO RECEIVED POSTOPERATIVE HORMONE THERAPY INCLUDING THE VARIABLES LYMPH NODE STATUS, HORMONE RECEPTOR STATUS AND pAkt

Variables	Rate ratio (95% CI)	Significance
Nodal status		
Negative	1	$p = 0.0021$
Positive	4.98 (2.2–7.6)	
ER		
Negative	1	$p = 0.54$
Positive	0.7 (0.3–2.0)	
PR		
Negative	1	$p = 0.59$
Positive	0.77 (0.2–2.2)	
pAkt		
Negative	1	$p = 0.10$
Positive	2.08 (0.9–5.0)	

CI, confidence interval.

Discussion

Akt/PKB is a serine/threonine kinase and a downstream effector of PI-3K.[1] The major functions of the PI-3K/Akt signal pathway are to promote growth factor–mediated cell growth, proliferation, migration and survival.[1] This pathway has been intensively investigated in various malignancies. Because activation of the PI-3K/Akt pathway induces resistance to endocrine therapy and chemotherapy, inhibition of this pathway is now considered a promising strategy to improve the effect of therapies for various kinds of cancer (reviewed in Thompson and Thompson[24]).

erbB2/HER2/neu is a receptor tyrosine kinase, which has been most studied in breast cancer. Overexpression of erbB2/HER2/neu occurs in approximately 30% of human breast cancers and is generally associated with poor prognosis[3] and with resistance to systemic and local radiation therapies.[7]

Cell lines that overexpress HER2/neu exhibit high levels of Akt1.[25] In addition, a significant association has been demonstrated between the expression of HER2/neu and pAkt in 20 adenocarcinomas. Previous studies have shown that erbB2, when overexpressed, is constitutively associated with erbB3. Since erbB3 possesses 7 tyrosine residues that could be phosphorylated and act as binding sites for the SH2 domains of the p85 regulatory subunit of PI-3K, erbB2–erbB3 dimers strongly activate the PI-3K–PKB/Akt pathway. This provides a strong basis for studies that have demonstrated that tumor cells overexpressing erbB2 display constitutive PKB/Akt activity. These data implicate HER2/neu overexpression in activation of the Akt/PKB pathway and that the PKB/Akt pathway may play a major role in stimulating proliferation and survival in HER2/neu-overexpressing cells.

In the present study, we found that pAkt expression correlated significantly with HER2/neu overexpression. This finding was consistent with many *in vitro* studies using established cell lines

and breast cancer tissues.[15,16] We examined 252 breast cancer cases, which is considered sufficient power to draw a reliable conclusion.

Although we found no significant correlation between pAkt and ER expression, we found an inverse correlation between pAkt and PR expression ($p = 0.0321$). A recent study demonstrated that PR expression was reduced *via* the PI-3K/Akt pathway,[26] and this finding may support our results.

Because it has been shown that patients with high pAkt expression have a poor prognosis compared to other patients,[16] we first investigated the prognosis of patients analyzed in terms of pAkt expression. There was no difference in DFS among all patients. Then, we divided the patients into 2 groups, those who did and those who did not receive postoperative endocrine therapy. Interestingly, in the analysis of patients who received postoperative endocrine therapy, pAkt positivity was significantly associated with higher risk of recurrence ($p = 0.0161$) (Fig. 2*b*). Multivariate analysis, including pAkt and traditional prognostic factors, failed to indicate that pAkt was an independent prognostic factor in all cases (Table IV). However, in cases with postoperative hormone therapy, there was a tendency for higher risk in the pAkt-positive group compared to the pAkt-negative group ($p = 0.10$) (Table V). So far, there have been a few reports indicating the correlation between Akt activity and the effect of endocrine therapy using human material. Perez-Tenorio *et al.*[17] revealed that pAkt-positive patients were more prone to relapse with distant metastasis. These patients were premenopausal and treated with tamoxifen and/or goserelin. However, in the study of postmenopausal breast cancer patients, the benefit of tamoxifen was analyzed in ER-positive patients.[15] Patients with a negative Akt status showed significant benefit from tamoxifen, whereas there was no significant benefit from tamoxifen in patients with positive Akt status.[15] In the present study, we did not divide the patients according to menopausal status because of the paucity of patients. We found that DFS was worse only in patients who received postoperative endocrine therapy. They were administered a variety of agents; however, this result was consistent with the previous report. Activated Akt induces chemoresistance[13,14] in *in vitro* analyses; thus, it was expected that pAkt would be associated with poor prognosis in patients who received chemotherapy. However, we could not find any difference in patients who did or did not receive chemotherapy (data not shown). One possible reason for this observation is that many of these patients were treated with oral fluoropyrimidines. There is no evidence that oral fluoropyrimidines have sufficient efficacy as adjuvant chemotherapy. Thus, in the future, it would be interesting to study the association between pAkt and chemotherapy in chemotherapy regimens proven to have sufficient efficacy.

In the present study, pAkt was associated with positive nodal status, although there was no significant correlation between pAkt and tumor size, clinical stage and histopathologic classification. This suggests that pAkt may induce a more malignant phenotype *via* its role in antiapoptosis and proliferation.

In this study, we demonstrated that Akt/PKB activation was significantly elevated in cases of primary breast carcinoma with HER2/neu overexpression. Moreover, it is likely that evaluation of pAkt status, in addition to the status of hormone receptors and HER2/neu, will be useful in the prediction of the efficacy of postoperative endocrine therapy for breast cancer. However, to elucidate the significance of Akt/PKB activation in clinical outcome, we must utilize well-designed, prospective studies. The data obtained from such studies will likely provide very useful information about treatment for breast cancer patients.

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Review Article

Activation of PI3K/Akt signaling and hormone resistance in breast cancer

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Akt is a serine/threonine kinase that has been demonstrated to play an important role in survival when cells are exposed to different apoptotic stimuli. Recent studies show that aberrant activation of Akt in breast carcinoma is associated with a poor prognosis and resistance to endocrine therapy and chemotherapy. The Akt signaling pathway is currently attracting considerable attention as a new target for effective therapeutic strategies. We investigated the incidence of Akt activation in 252 primary breast carcinomas and relationships among the activation of Akt, HER2 overexpression, hormone receptor expression, and alteration of the PTEN gene. Eighty-four cases (33.3 %) were positive for pAkt expression. pAkt was significantly associated with HER2 overexpression ($p<0.0001$) and LOH at the PTEN gene locus ($p<0.01$). There was an inverse correlation between pAkt and PR ($p<0.05$). We also retrospectively examined the relationship between Akt activation and the efficacy of endocrine therapy for metastatic breast cancer. Of these 36 metastatic breast cancer cases, 12 cases (33.4%) were considered to show positive pAkt expression. In the pAkt-positive patients, endocrine therapy demonstrated worse efficacy than in pAkt-negative patients ($p<0.01$). In addition, the clinical benefit was the smallest in the patients positive both for HER2 and pAkt ($p<0.01$). The clinical benefit rate of estrogen deprivation therapy with AI or LH-RH agonist was significantly lower in the pAkt-positive patients than that in the pAkt-negative ones ($p<0.05$), and there was a tendency for the clinical benefit of SERM to be smaller in the pAkt-positive patients ($p=0.09$). These findings therefore suggest that Akt activation induces endocrine resistance in metastatic breast cancer, irrespective of the kind of endocrine agents that were administered. Our findings indicate that the activation of Akt in the downstream pathway of HER2 plays an important role in resistance to endocrine therapy for breast cancer. Our findings suggest that pAkt may be a useful predictor of resistance to endocrine therapy for breast cancer, while also suggesting that the inhibition of Akt may increase the efficacy of endocrine therapy.

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Key words: Akt, phosphorylated Akt, HER2, metastatic breast cancer, hormone therapy

Introduction

Endocrine therapy for breast cancer was first introduced more than 100 years ago, however, it is still the most effective systemic treatment for patients with hormone receptor positive breast cancer.

Tamoxifen is the most widely used selective estrogen receptor modulator (SERM) and it has been regarded as the gold standard endocrine therapy for hormone receptor-positive breast cancer for a long time[1]. Aromatase inhibitors (AIs) are new drugs, which are used for postmenopausal breast cancer, and they have demonstrated great efficacy in patients with hormone-sensitive breast cancer[2]. This increase in the use of the endocrine agents has resulted in the development of more strategies for the treatment of breast cancer. However, the major clinical problem in endocrine therapy is tumor resistance, either de novo or acquired during the treatment.

Major clinical trials have shown that the ER status is the strongest and the most reliable pre-

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dictor of the response to endocrine therapy[3]. Progesterone receptor (PR) is an estrogen -regulated gene and the presence of PR is an indicator of a functional ER protein and a higher likelihood of a positive response to endocrine therapy[4]. However, about 30% of both ER- and PR- positive tumors remain resistant to endocrine therapy and there are limitations in predicting the efficacy of endocrine therapy based on hormone receptor expression alone. These findings imply that factors other than ER and PR are involved in resistance to endocrine therapy. Recently, crosstalk between the signal transduction pathways and ER signaling has been a focus of research to determine breast cancer etiology and progression[5]. This crosstalk, which occurs at multiple levels, has recently been shown to be associated with endocrine resistance[6-8]. Estrogen-activated membrane ER either directly or indirectly activates membrane tyrosine kinase receptors and this interaction leads to the activation of key secondary signaling messengers and downstream kinase pathways such as ERK/MAPK and PI3K/Akt. These kinases can phosphorylate ER at key positions and in turn both activate nuclear ER transcriptional activity and promote ER-dependent transcription[8].

Akt, which is also known as protein kinase B (PKB), is a serine/threonine protein kinase that is activated by a variety of stimuli through growth factor receptors in a phosphoinositide-3-OH kinase (PI3K)-dependent manner[9]. The disruption of normal Akt signaling occurs frequently in several human cancers, and this enzyme appears to play an important role in cancer progression and cell survival[9]. The mechanisms by which Akt promotes cell survival include phosphorylation of the pro-apoptotic proteins BAD, caspase-9, forkhead transcription factors and IκB kinase α[9]. In addition, the mammalian target of rapamycin (mTOR), a downstream effector of the PI3K/Akt signaling pathway, activates p70S6 kinase and 4E-bining protein-1, and regulates the G1-S transition of the cell cycle.

Akt is activated by a variety of stimuli through growth factor receptors such as HER2 and EGFR, in a PI3K-dependent manner. Another major mechanism of Akt activation is a loss of the function of a novel tumor suppressor gene, phosphatase and tensin homolog deleted on chromosome 10 (PTEN)[10]. The fundamental *in vivo* role of PTEN appears to be inhibition of the PI3K-dependent activation of Akt. Breast cancer cell lines with a constitutively activated PI3K/Akt pathway due to HER2 overexpression and/or loss of the PTEN suppressor gene have been shown to be resistant to HER2-, EGFR-targeted therapies and to endocrine therapy with tamoxifen[11]. Recently, the activation of Akt has been shown to be associated with a worse outcome among endocrine-treated breast cancer patients[12, 13]. In addition, it has been revealed that breast cancer cell lines with activated Akt are especially sensitive to mTOR antagonism[14]. Therefore, the PI3K/Akt signaling pathway currently attracts considerable attention as a new target for effective therapeutic strategies.

In the present study, we investigated the incidence of Akt activation in 252 primary breast carcinomas and relationships among the activation of Akt and HER2 overexpression, hormone receptor expression, and alteration of the PTEN gene. In addition, the relationship between Akt activation and the efficacy of endocrine therapy for metastatic breast cancer was investigated. Here we demonstrate Akt activation was elevated significantly in cases with HER2 overexpression and /or LOH of the PTEN gene. Moreover, Akt activation was found to be significantly associated with resistance to endocrine therapy for metastatic breast cancer. Our results suggest that: (1) Akt activation induces resistance to endocrine therapy, (2) Akt activation thus appears to be useful as a predictive marker of endocrine therapy, and (3) the inhibition of the Akt signaling pathway may improve the efficacy of endocrine therapy for breast cancer.

Materials and Methods

Patient population and tumor specimens

A total of 252 primary human breast carcinoma specimens were used for the investigation of the incidence of Akt activation and the relationships among the Akt activation and other biomarkers in primary breast carcinomas. They were obtained from patients who underwent surgery at the Department of Surgery and Science, Kyushu University Hospital, from 1991 to 2002. Among them, primary human breast carcinoma specimens and corresponding normal tissues or peripheral blood were obtained from 138 patients and utilized for the analysis of the PTEN gene. Informed consent was obtained from each patient prior to tissue acquisition. Clinical data were obtained from med-

ical records. The histopathological diagnosis was determined according to the criteria of the Japanese Breast Cancer Society[15] by histopathological specialists at our hospital. Genomic DNA was extracted as previously described[16].

To investigate the relationship between Akt activation and the efficacy of endocrine therapy for metastatic breast cancer, a total of 36 patients with metastatic breast carcinoma were utilized in this study. All 36 patients had been treated with endocrine therapy at the Department of Surgery and Science, Kyushu University Hospital, or the Department of Breast Oncology, National Kyushu Cancer Hospital from 2002 to 2004. Primary human breast carcinoma specimens were obtained and subjected to pathological and immunohistochemical analyses. Ten patients were premenopausal and the others were postmenopausal. For adjuvant therapy, 13 patients had received chemotherapy 9 patients had received endocrine therapy and 9 patients had had both. The metastatic sites and the number of the patients were as follows; bone 12, lung 11, lymph node 10, soft tissue 6, pleura 2, and liver 1.

Assessment of efficacy

After initiating each endocrine therapy, the patients were assessed monthly to evaluate their clinical response. The response categories were defined according to World Health Organization criteria as complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD). Both CR and PR were regarded as an objective clinical response. When CR, PR or SD of longer than 6 months were obtained, the results were thus considered to demonstrate a clinical benefit.

Antibodies, immunohistochemistry and evaluation

Monoclonal antibodies 6F11 and 1A6 (Ventana Medical Instruments, Tucson, AZ, USA) were used for ER and PR staining. For HER2 evaluation the monoclonal antibody CB11 (Ventana Medical Instruments) was used. Phosophorylated-Akt was detected using polyclonal antibodies against phosphorylated Ser 473 (Cell Signaling Technology, Beverly, MA, USA).

Immunohistochemistry and evaluation

Tissue samples were fixed by immersion in buffered formalin and embedded in paraffin. Immunostaining of these paraffin sections was performed using the Ventana Discovery™ automated staining instrument (Ventana Medical Instruments), and hematoxylin (Ventana Medical Instruments) was employed as a nuclear counterstain. Immunostaining was visualized with a streptoavidin peroxidase reaction using DAB as the chromogen (Ventana Medical Instruments). A negative control reaction with no primary antibody was always performed alongside the reaction-containing sample.

Immunostaining was evaluated without knowledge of the clinical and pathological parameters. ER and PR were recorded as positive if 10% or more of the nuclei in the invasive component of the tumor were stained[17]. HER2 was scored by widely accepted criteria that assessed the intensity and completeness of membrane staining[18, 19]. A specimen was considered positive for pAkt if 10% or more of the cytoplasm in the invasive component of the tumor stained positive for pAkt[13].

LOH analysis

LOH at the PTEN locus was analyzed in this study with our new system for microsatellite analysis, termed high-resolution fluorescent microsatellite analysis (HRFMA)[20]. We used two microsatellite markers, D10S1765 and D10S1173[20]. The sequences of the primers for PCR analysis of D10S1765 were as follows: forward, 5'-CAATGGAACCAAATGTGGTC, and reverse, 5'-AGTCCGATAATGCCAGGATG. The sequences of the primers for PCR analysis of D10S1173 were as follows: forward, 5'-CATGCCAAGACTGAAACTCC, and reverse, 5'-AAACCCCAATGCCATAATGG. PCR reactions were performed as previously described[20]. The data were processed by the ABI software GeneScan. The cases showing heterozygosity were informative. The results were analyzed by comparing the peak value of the tumor DNA with that of normal control DNA. A reduction of more than 30% in the peak value in tumor DNA in comparison to normal control DNA in a minimum of one marker was judged to indicate LOH[20].

Statistical analysis

Associations between categorical variables were assessed by means of χ^2 tests. A p-value of less than 0.05 was considered to indicate a statistically significant difference.

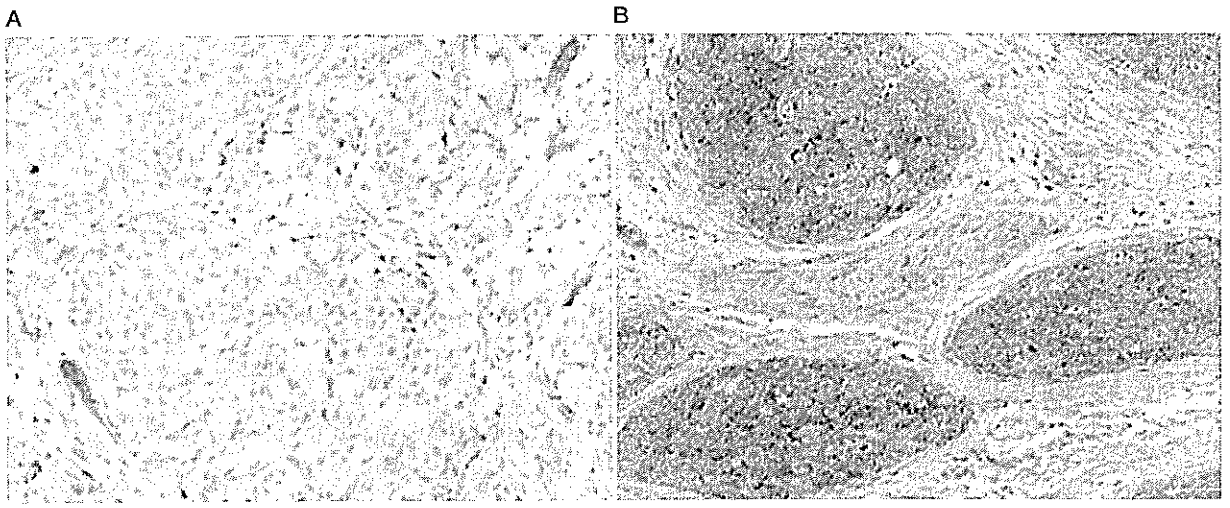

Fig 1. Immunostaining of phosphorylated-Akt (pAkt).
Representative immunohistochemistry staining of pAkt in breast carcinoma. pAkt was detected in the cytoplasm of tumor cells. (A) Negative immunostaining for pAkt. (B) Positive immunostaining for pAkt.

Results

1. Incidence of Akt activation and relationships among Akt activation and other biomarkers in primary breast carcinomas

Expression of pAkt in primary breast cancer tissues

The status of Akt activation was analyzed by the expression of phosophorylated-Akt (pAkt). A specimen was regarded as positive for pAkt when 10% or more of the cytoplasm of the tumor cells stained positively. Representative positive and negative cases are shown in Figure 1. Eighty-four cases (33.3 %) were positive for pAkt expression. We examined the relationship between pAkt expression and HER2 status. pAkt was significantly more expressed in the HER2 positive cases ($p<0.0001$) (Fig. 2). No significant correlation was observed between pAkt and ER expression, however PR expression was significantly lower in pAkt-positive cases ($p<0.05$) (data not shown).

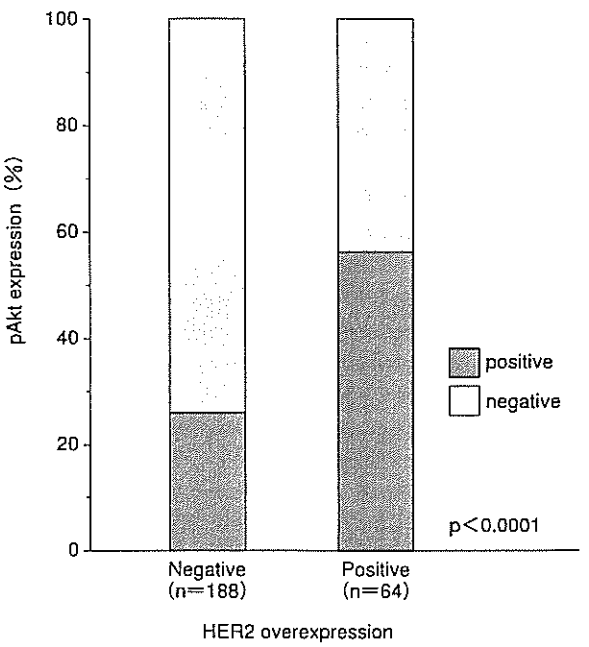

Fig 2. Relationship between pAkt and HER2 expression.
pAkt was significantly more expressed in the HER2-positive cases ($p<0.0001$). Thus, Akt activation was significantly correlated with HER2 overexpression.

LOH at the PTEN gene locus in breast carcinoma and association of PTEN LOH with Akt activation

The 138 breast tumors were analyzed for LOH at the PTEN gene locus, and the results from 131 cases (94.9%) were considered informative. Allelic loss of at least one marker was observed in 31 (23.7 %) of these 131 informative cases. Of these 131 cases, 48 cases (36.6 %) were positive for pAkt expression. pAkt expression was positive in 19 cases (61.3%) of patients with PTEN LOH. On the other hand, pAkt expression was positive in only 29 cases (29.0%) of patients with PTEN LOH. PTEN LOH was significantly associated with pAkt expression, which indicates activated Akt ($p<0.01$). Interestingly, pAkt was significantly more expressed when PTEN LOH and HER2 overexpression coexisted ($p<0.001$) (Table 1).

Table 1. Association between coexistence of PTEN LOH and HER2 overexpression and Akt activation

Factors	n	pAkt		p-value
		negative (n=83)	positive (n=48)	
PTEN				
ROH	100	71 (71.0)	29 (29.0)	p<0.01
LOH	31	12 (38.7)	19 (61.3)	
PTEN LOH/HER2				
ROH / negative	80	62 (77.5)	18 (22.5)	p<0.001
ROH / positive	20	9 (45.0)	11 (55.0)	
LOH / negative	14	6 (42.9)	26 (57.1)	
LOH / positive	17	6 (35.3)	11 (64.7)	

pAkt: phosphorylated Akt (%)

2. The relationship between Akt activation and the efficacy of endocrine therapy for metastatic breast cancer

Expression of hormone receptors and HER2 in breast carcinoma tissue specimens

In this study, 36 primary breast carcinoma specimens, obtained from the patients with metastatic breast cancer, were evaluated. ER and PR were positive in 33 cases (91.7%) and 26 cases (74.3%), respectively. Both ER and PR were positive in 23 cases (65.7%). HER2 positivity was 8.3% (3 cases) for HER2 2+ and was also 8.3% (3 cases) for HER2 3+.

Expression of pAkt in primary breast cancer tissue specimens and the relationship between Akt activation and the HER2 status

Of these 36 cases, 12 cases (33.3%) were regarded as positive for pAkt expression. In the 6 HER2 2+ or 3+ cases, pAkt expression was positive in 5 cases (83.3%). pAkt expression positively correlated with HER2 overexpression ($p<0.01$) even though the positivity of HER2 overexpression was low.

Response to endocrine therapies

The endocrine therapies received by these patients included the following; aromatase inhibitors (anastrozole or exemestane) in 23 patients, selective estrogen receptor modulator (SERM) (tamoxifen or tremifen) in 15 patients, LH-RH agonist (Goselerin) in 7 patients, and MPA (medroxyprogesterone acetate) in 1 patient. Objective response was observed in 16 cases (34.8%), while clinical benefit was observed in 27 (58.7%) (5 CR, 11 PR and 11 long SD).

Relationship between the clinical benefits and hormone receptor expression

Regarding hormone receptor expression, the clinical benefit rate was highest in the ER- and PR-positive group, however, no statistical differences were seen in the clinical benefit in terms of hormone receptor expression (data not shown).

Relationships between the clinical benefits and pAkt status

We investigated the relationship between pAkt status and clinical efficacy. In the pAkt-positive patients, endocrine therapy had significantly worse efficacy than in the pAkt-negative patients ($p<0.01$) (Fig. 3). In addition, the clinical benefit was the smallest in patients positive for both HER2 and pAkt ($p<0.01$) (Fig. 3).

Relationships between the clinical benefits and the status of pAkt by endocrine agents

We next evaluated whether the kind of endocrine therapy influenced the association between akt activation and clinical efficacy. We divided the endocrine therapies into estrogen deprivation therapy such as aromatase inhibitors (AIs) or LH-RH agonist and SERM. In pAkt-positive patients, the clinical benefit rate of estrogen deprivation therapy was significantly lower than that in pAkt-negative patients ($p<0.05$) (Fig. 4). The clinical benefit of SERM also tended to be smaller in the pAkt-positive patients ($p=0.09$) (Fig. 4). These findings suggest that Akt activation induces endocrine resistance irrespective of the kind of endocrine agents that were given for metastatic breast cancer.