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Alternative tyrosine phosphorylation of signaling kinases according to hormone receptor status in breast cancer overexpressing the insulin-like growth factor receptor type 1

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(Received February 2, 2006/Revised March 20, 2006/Accepted March 21, 2006/Online Publication May 9, 2006)

The insulin-like growth factor receptor type 1 (IGF1R) is suggested to play important roles in cancer cell growth through cross-talk with hormone receptors and growth factor receptors. However, its clinical significance in breast cancers *in vivo* is still unclear. We examined immunohistochemically the expression of IGF1R, phosphorylated-AKT (pAKT) and phosphorylated-ERK1/2 (pERK1/2) using tissue microarray slides containing 150 cases of primary breast carcinoma. Their mutual correlation and correlation with the status of hormone receptors epidermal growth factor receptor and human epidermal growth factor receptor type 2 were also investigated. IGF1R overexpression was detected in 71 cases (47%), and was correlated with lower nuclear grade ($P = 0.03$), positive estrogen receptor (ER) and/or progesterone receptor status ($P = 0.002$). pERK1/2 expression, detected in 53 cases (35%), was correlated with positive ER ($P < 0.0001$) and lower nuclear grade ($P = 0.014$). pAKT expression, detected in 88 cases (59%), was not correlated with nuclear grade, hormone receptors status or other clinical parameters. Of the 71 IGF1R-overexpressing tumors, pERK1/2 expression was detected in 27 (56%) of 48 ER-positive cases but in only four (17%) of 23 ER-negative cases ($P = 0.022$). In contrast, pAKT expression was constantly (64% or higher) detected irrespective of hormone receptor status in IGF1R-overexpressing breast cancers. Taken together, these findings suggest that IGF1R overexpression might activate pERK1/2 and pAKT in hormone receptor-positive breast cancer, but activate only pAKT in hormone receptor-negative breast cancer. (*Cancer Sci* 2006; 97: 597–604)

Recent evidence suggests that potent cross-talk between growth factor receptor-induced signaling pathways and estrogen receptor (ER) signaling pathways is involved in the acquisition of resistance to tamoxifen therapy by breast cancer cells.[1] Estrogen-bound ER activates estrogen-regulated gene transcription through genomic action, but after long-term tamoxifen treatment, resistance can develop with the activation of tyrosine kinase receptors, such as insulin-like growth factor receptor type 1 (IGF1R), human epidermal growth factor receptor (HER) type 1 (HER1 or EGFR), and/or HER type 2 (HER2 or c-erbB2) by ER phosphorylation through non-genomic action.[1,2]

The EGFR and HER2 oncoproteins are overexpressed in 15–20% and 27–30% of primary breast cancers, respectively. Their overexpression has been shown to be correlated with high-grade and hormone receptor-negative tumors, and with poorer patient prognosis.[3–6] Whereas IGF1R overexpression is reported to be detectable in 43–50% of primary breast cancers,[7,8] its clinical and prognostic significance remains undetermined in spite of clear evidence of the biological importance of IGF1R overexpression in breast cancer cells *in vitro*.[9,10]

EGFR/HER2 and IGF1R show bidirectional signal transduction through the MAPK/ERK1/2 pathway and the PI3K/AKT pathway. The MAPK/ERK1/2 pathway plays a critical role in the regulation of cell growth, differentiation and progression. Phosphorylation of ERK1/2 is a common result of growth factor receptor activation or exposure to an oncogenic agent.[11,12] The PI3K/AKT pathway also plays a critical role in controlling the balance between apoptosis and cell survival. This balance in response to extracellular and intracellular signaling is vital for maintaining tissue homeostasis. Aberrant control of cell survival signaling can result in tumor progression and resistance to chemopreventive agents used in cancer treatment.[13,14] Recent reports have revealed that activation of the PI3K/AKT pathway under the influence of IGF1R plays an important role in maintaining the proliferation of breast cancer cells that are resistant to gefitinib, trastuzumab or chemoradiotherapy *in vitro* and *in vivo*.[15–18] Furthermore, the latest translational research has revealed that immunohistochemical overexpression of IGF1R is associated with response to lapatinib, a small molecule tyrosine kinase inhibitor of EGFR and HER2, in a clinical trial of patients with advanced or metastatic breast cancer overexpressing HER2. Therefore, IGF1R is expected to be a therapeutic predictive biomarker for the effectiveness of lapatinib.[19]

In the present study, we examined the clinicopathological implications of IGF1R expression, aiming at downstream signaling kinases, phosphorylated AKT (pAKT) and phosphorylated ERK1/2 (pERK1/2) in 150 cases of breast carcinoma by using tissue microarray (TMA) and immunohistochemistry.

Patients and Methods

Patients

The present study was carried out after approval by the internal review board and after gaining the patients' consent to use their cancer specimens for research.

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doi: 10.1111/j.1349-7006.2006.00228.x

One hundred and fifty patients who underwent resection of breast cancer at the National Defense Medical College Hospital (Tokorozama, Japan) between 1995 and 1997 were enrolled in the present study. The median patient age of the study population was 53.8 years (range 30–81 years). The median tumor diameter was 3.4 cm (range 0.7–13.0 cm). The histological type of the primary tumor was invasive ductal carcinoma in 130 cases (87%), ductal carcinoma *in situ* in 15 cases (10%), and invasive lobular carcinoma in five cases (3%). According to the nuclear grading system of the Japan Breast Cancer Society, 17 cases (11%), 84 cases (56%) and 49 cases (33%) were classified as grade 1, grade 2 and grade 3, respectively. The numbers of cases with and without axillary lymph node metastasis were 52 (35%) and 77 (51%), respectively, and lymph node status was not recorded in 21 cases (14%). ER was positive in 77 cases (51%) and negative in 73 cases (49%), whereas progesterone receptor (PgR) was positive in 87 cases (58%) and negative in 63 cases (42%). The median time of follow up was 64.9 months, ranging from 12 to 122 months. During the whole time of follow up, 39 (26%) of 150 patients relapsed distantly, whereas the other 111 (74%) patients were alive without recurrence (Table 1).

Tissue microarray construction

We reviewed all of the hematoxylin and eosin-stained sections of archived pathological specimens of primary breast cancer. The histological diagnosis, including histological type and nuclear grade, was confirmed in all cases. For the 150 available cases of breast cancer, the most representative area of each tumor was punched out to construct TMA comprising single tissue cores (diameter 2.0 mm) from the 150 original blocks. One TMA block contained a maximum of 66 tissue cores, and three TMA sets were prepared for the present study. Sections 4 μm in thickness were cut from the blocks.

Table 1. Clinicopathological features in 150 breast cancers

Clinical parameter	No. cases
Total	150 (100%)
Age (years)	
Range	30–81 (53.8 median)
<50	57 (38%)
≥50	93 (62%)
Size of tumor (cm)	
Range	0.7–13.0 (3.4 median)
<2.0	42 (28%)
≥2.0	108 (72%)
Histological type	
Invasive ductal carcinoma	130 (87%)
Ductal carcinoma *in situ*	15 (10%)
Invasive lobular carcinoma	5 (3%)
Nuclear grade	
Grade 1	17 (11%)
Grade 2	84 (56%)
Grade 3	49 (33%)
Lymph node status	
Positive	52 (35%)
Negative	77 (51%)
Unknown	21 (14%)
Estrogen receptor status	
Positive	77 (51%)
Negative	73 (49%)
Progesterone receptor status	
Positive	87 (58%)
Negative	63 (42%)
Follow-up duration (months)	11–122 (64.9 median)
Event	
No relapse	111 (74%)
Distant relapse	39 (26%)

Immunohistochemistry

In brief, the 4 μm-thick sections were deparaffinized in xylene, and dehydrated in a graded ethanol series. Antigen retrieval was carried out by incubation of the tissue sections in a microwave oven in 10 mM sodium citrate (pH 6.0) with 0.1% Tween40 at 120°C for 45 min.

In the present study, we used a rabbit polyclonal anti-IGF1R antibody (ready to use; NeoMarkers, Fremont, CA, USA), a rabbit polyclonal anti-pERK1/2 antibody (sc-7383, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a rabbit polyclonal anti-pAKT antibody (Ser473, 1:100; Cell Signaling Technology, Danvers, MA, USA). After antigen retrieval, the tissue sections were incubated in 0.3% hydrogen peroxide in methanol for 30 min, reacted with the primary antibody for 1–3 h, incubated with dextran polymer reagent conjugated with peroxidase and secondary antibody (envision; Dakocytomation, Glostrup, Denmark) for 1 h, and subsequently reacted with 3,3-diaminobenzidine tetrahydrochloride-hydrogen peroxide as the chromogen.

IGF1R staining of the cells was assessed according to both the intensity and the proportion of membrane staining (Fig. 1A–D) and was scored as follows: 0, membrane staining in less than 10% of constituent cells or no membrane staining; 1+, incomplete membrane staining that did not include the entire circumference of the membrane in 10% or more of the carcinoma cells; 2+, weak or moderate complete membrane staining along the entire circumference of the cell membrane in 10% or more of the carcinoma cells; and 3+, strong complete membrane staining along the entire circumference of the cell membrane in 10% or more of the carcinoma cells. Cases that were scored 2+ and 3+ were considered to have IGF1R overexpression.

The pERK1/2 staining of the cancer cells was evaluated according to the intensity and proportion of nuclear staining and scored on a three-point scale as follows (Fig. 1E–G): 0, nuclear staining in less than 10% of constituent cells or no nuclear staining; 1+, weak nuclear staining in 10% or more of the constituent cells; and 2+, strong nuclear staining in 10% or more of the constituent cells. Cases with a score of 2+ were considered to be pERK1/2-positive.

The pAKT staining of the cancer cells was evaluated according to the intensity and the proportion of cytoplasmic staining and scored on a three-point scale as follows (Fig. 1H–J): 0, cytoplasmic staining in less than 10% of the constituent cells or no staining; 1+, weak cytoplasmic staining in 10% or more of the constituent cells; and 2+, strong cytoplasmic staining in 10% or more of the constituent cells. Cases with a score 2+ were considered to be pAKT-positive.

Expression of EGFR, HER2, ER and PgR

The expression of EGFR, HER2, ER and PgR had already been examined immunohistochemically in the 150 tumors.[20]

doi: 10.1111/j.1349-7006.2006.00228.x

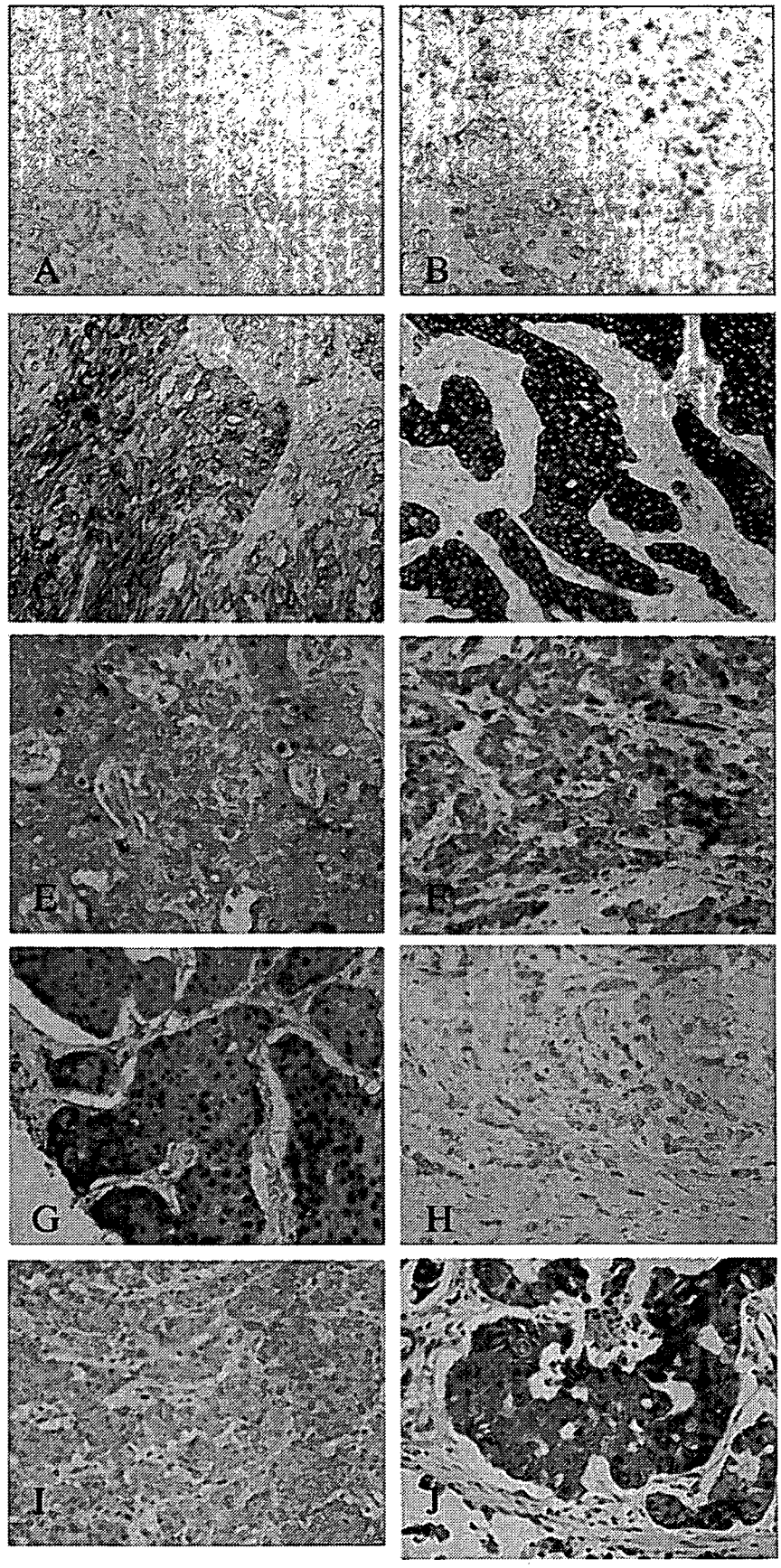

Fig. 1. Immunohistochemical staining of insulin-like growth factor receptor type 1 (IGF1R) protein in primary breast cancer scored according to area and intensity of membrane and cytoplasmic staining. (A) 0, (B) 1+, (C) 2+, (D) 3+ (×200). pERK1/2 staining was evaluated according to the intensity and proportion of nuclear staining. (E) 0, (F) 1+, (G) 2+ (×200). pAKT staining was evaluated according to the intensity and proportion of cytoplasmic staining. (H) 0, (I) 1+, (J) 2+ (×200).

Antibodies or kits used for immunohistochemistry were a PharmDx EGFR Kit for EGFR, a Herceptest for HER2, a monoclonal anti-ER antibody (clone ID5) and a monoclonal anti-PgR antibody (clone PgR636) purchased from Dakocytomation. The method used for immunohistochemistry has been described previously.[20]

The expression of EGFR and HER2 was scored as 2+ and 3+ if the entire circumference of the cell membrane was weakly or moderately stained, or strongly stained, respectively, in 10% or more of the constituent carcinoma cells. A score of 1+ was given if incomplete membrane staining was observed in 10% or more of the carcinoma cells, and a score of 0 was given if there was membrane staining in less than 10% of the constituent cells or if there was no membrane staining. Cases with a score of 2+ or 3+ were judged as showing overexpression. Hormone receptor status was classified into three groups: 0, when no cancer cell nuclei were stained; 1+, when less than 10% of cancer cell nuclei were stained; and 2+, when 10% or more of cancer cell nuclei were stained. In the present study, a score of 2+ was regarded as ER-positive or PgR-positive, and score of 0 or 1+ was regarded as ER-negative or PgR-negative.

Statistical analysis

We used the χ^2-test or Fisher's exact test to reveal the correlation of the expression of each protein with histological grade and other clinical parameters. A Cox proportional hazards model was used to test the significance of hormone receptors, IGF1R and signaling kinases as predictors of disease-free survival times. All statistical analyses were carried out using Statview 5.0 software (SAS Institute, Cary, NC, USA).

Results

Relationship of IGF1R, EGFR and HER2 with clinicopathogical parameters and hormone receptor status in breast cancer

Table 2 shows the relationship between overexpression of IGF1R, EGFR and HER2, and clinicopathological parameters and hormone receptor status in breast cancer. We have already shown the inverse correlation of EGFR and HER2 with hormone receptor status and the correlation of EGFR with higher tumor grade in our previous report.[20] In this study, we re-evaluated ER and PgR status according to the criteria described above and classified them into four groups by combining ER and PgR.

IGF1R overexpression was detected in 71 (47%) of the 150 cases. In non-cancerous mammary gland tissue, IGF1R was also expressed with a score of 1+ or 2+ localized in both luminal and myoepithelial cells. IGF1R was overexpressed in 48 (62%) of 77 ER-positive cases, 23 (32%) of 73 ER-negative cases, 54 (62%) of 87 PgR-positive cases, and 17 (27%) of 63 PgR-negative cases. When the two hormone receptors were combined, IGF1R was overexpressed in 42 (67%) of 63 ER-positive/PgR-positive cases, six (43%) of 14 ER-positive/PgR-negative cases, 12 (50%) of 24 ER-negative/PgR-positive cases, and 11 (22%) of 49 ER-negative/PgR-negative cases. ER-positive/PgR-positive cells and IGF1R-positive cells were almost identical in ER-positive/PgR-positive and IGF1R-positive tumors. There was a significant correlation of IGF1R overexpression with ER or PgR positivity, compared with ER or PgR negativity ($P = 0.0005$, $P < 0.0001$, respectively). The frequency of IGF1R overexpression with positivity for ER and PgR was significantly higher than that of IGF1R overexpression with double negativity for ER and/or PgR ($P = 0.002$).

IGF1R was overexpressed in 12 (71%) of 17 cases with grade 1, 42 (50%) of 84 cases with grade 2, and 17 (35%) of 49 cases with grade 3. IGF1R overexpression was correlated with lower nuclear grade ($P = 0.03$), but was not significantly correlated with age, tumor size, histological type or axillary lymph node status.

EGFR was overexpressed in 0 (0%) of 63 ER-positive/PgR-positive cases, 0 (0%) of 14 ER-positive/PgR-negative cases, one (4%) of 24 ER-negative/PgR-positive cases, and 11 (22%) of 49 ER-negative/PgR-negative cases. Frequency

Table 2. Correlation between insulin-like growth factor receptor type 1 (IGF1R), epidermal growth factor receptor and human epidermal growth factor receptor type 2 overexpression with clinicopathological parameters and hormone receptor status in breast cancer

Parameter	Total	Cases					
		IGF1R		EGFR		HER2	
		n	%	*n*	%	*n*	%
Total	150	71	47	12	8	23	15
Hormone receptor status							
ER$^+$	77	48	62*	0	0†	2	3‡
ER$^-$	73	23	32	12	16	21	29
PgR$^+$	87	54	62§	1	1¶	6	7**
PgR$^-$	63	17	27	11	17	17	27
Hormone receptor status combined							
ER$^+$/PgR$^+$	63	42	67††	0	0‡‡	2	3§§
ER$^+$/PgR$^-$	14	6	43	0	0	0	0
ER$^-$/PgR$^+$	24	12	50	1	4	4	17
ER$^-$/PgR$^-$	49	11	22	11	22	17	35
Histological grade							
Invasive ductal carcinoma	130	58	45	11	8	18	14
Ductal carcinoma *in situ*	15	8	53	1	7	5	33
Invasive lobular carcinoma	5	3	60	0	0	0	0
Nuclear grade							
Grade 1	17	12	71¶¶	0	0***	2	12
Grade 2	84	42	50	2	2	10	12
Grade 3	49	17	35	10	20	11	22
Age (years)							
<50	57	21	37	6	11	9	16
≥50	93	50	54	6	6	14	15
Tumor size (cm)							
<2.0	42	15	36	3	7	8	19
≥2.0	108	56	52	9	8	15	14
Lymph node status							
Positive	52	23	44	6	12	7	13
Negative	77	33	43	4	5	14	18
Unknown	21	15	71	2	10	2	10

*$P = 0.0005$, †$P < 0.0001$ and ‡$P < 0.0001$ between estrogen receptor (ER)-positive and ER-negative cases; §$P < 0.0001$, ¶$P < 0.0001$ and **$P = 0.0007$ between progesterone receptor (PgR)-positive and PgR-negative cases; ††$P = 0.002$, ‡‡$P < 0.0001$ and §§$P = 0.003$ between ER- and/or PgR-positive cases and double-negative ER and PgR cases; ¶¶$P = 0.03$, ***$P = 0.026$ between the grade 1, 2 and 3 cases.

doi: 10.1111/j.1349-7006.2006.00228.x

of EGFR overexpression in cases showing double negativity for ER and PgR was significantly higher than in cases that were positive for ER and/or PgR ($P < 0.0001$).

HER2 was overexpressed in 2 (3%) of 63 ER+/PgR + cases, 0 (0%) of 14 ER+/PgR – cases, 4 (17%) of 24 ER –/PgR + cases, and 17 (35%) of 49 ER –/PgR – cases. The frequency of HER2 overexpression in cases that were double negative for ER and PgR was also significantly higher than in cases that were positive for ER and/or PgR ($P = 0.003$).

In the group with ER-positive and/or PgR-positive breast cancers, the incidence of IGF1R overexpression was strikingly higher than that of EGFR or HER2 overexpression. In contrast, in the group of double negativity of ER and PgR, the incidence of IGF1R overexpression was almost equal to that of EGFR and was rather lower than that of HER2 overexpression (Table 2).

Relationship of pERK1/2 and pAKT with clinical parameters and hormone receptor status in breast cancer

As shown in Table 3, pERK1/2 was detected as 2+ in 53 (35%) of the 150 cases, including 41 (53%) of 77 ER-positive cases, 12 (16%) of 73 ER-negative cases, 40 (46%) of 87 PgR-positive cases, and 13 (21%) of 63 PgR-negative cases. When ER and PgR were combined, pERK1/2 was expressed in 35 (56%) of 63 ER-positive/PgR-positive cases, six (43%) of 14 ER-positive/PgR-negative cases, five (21%) of 24 ER-negative/PgR-positive cases, and seven (14%) of 49 ER-negative/PgR-negative cases. There was a significant correlation of pERK1/2 with ER or PgR positivity, compared with ER or PgR negativity ($P < 0.0001$, $P = 0.002$, respectively). pERK1/2 expression was significantly higher in cases that were positive for ER and/or PgR than in cases that were double negative for both ER and PgR ($P = 0.0001$).

pERK1/2 was positive in 42 (32%) of 130 cases with invasive ductal carcinoma, in six (40%) of 15 cases with ductal carcinoma *in situ*, and in four (80%) of five cases with invasive lobular carcinoma. There was a significant difference in correlation of pERK1/2 between invasive ductal carcinoma and invasive lobular carcinoma ($P = 0.03$).

With regard to nuclear grade, pERK1/2 was positive in 10 (59%) of 17 grade 1 cases, 32 (38%) of 84 grade 2 cases, and 11 (22%) of 49 grade 3 cases. There was a significant correlation of pERK1/2 with lower nuclear grade ($P = 0.014$). There was no correlation of pERK1/2 with age, tumor size or axillary lymph node status.

pAKT was detected as 2+ in 88 cases (59%) (Table 3). There was no significant correlation of pAKT overexpression with hormone receptor status, histological type, nuclear grade or other clinical parameters. pERK1/2-positive cancer cells and pAKT-positive cancer cells were almost identical in pERK1/2-positive and pAKT-positive tumors.

Correlation of pERK1/2 and pAKT with hormone receptor status in breast cancers overexpressing IGF1R

In Table 4, pERK1/2 positivity rates are presented for the four subsets of hormone receptors status in the 71 IGF1R-overexpressing breast cancers. pERK1/2 expression was detected in 24 (57%) of 42 ER-positive/PgR-positive cases, three (50%) of six ER-positive/PgR-negative cases, two (17%) of 12 ER-negative/PgR-positive cases, and two (18%) of 11 ER-negative/PgR-negative cases. There was a significant difference of pERK1/2 expression rates between ER-positive and ER-negative cases in breast cancers overexpressing IGF1R ($P = 0.022$).

Table 3. Correlation of pERK1/2 and pAKT with clinicopathological parameters and hormone receptor status in breast cancer

Parameter	Total	Cases			
		pERK1/2		pAKT	
		n	%	*n*	%
Total	150	53	35	88	59
Hormone receptor status					
ER^+	77	41	53*	45	58
ER^-	73	12	16	43	59
PgR^+	87	40	46†	53	61
PgR^-	63	13	21	35	56
Hormone receptor status combined					
ER^+/PgR^+	63	35	56‡	38	60
ER^+/PgR^-	14	6	43	7	50
ER^-/PgR^+	24	5	21	15	63
ER^-/PgR^-	49	7	14	28	57
Histological type					
Invasive ductal carcinoma	130	42	32§	75	58
Ductal carcinoma *in situ*6	15	6	40	10	67
Invasive lobular carcinoma	5	4	80	2	40
Nuclear grade					
Grade 1	17	10	59¶	11	65
Grade 2	84	32	38	44	52
Grade 3	49	11	22	33	67
Age (years)					
<50	57	16	28	31	54
50	93	37	40	57	61
Tumor size (cm)					
<2.0	42	12	29	17	40
2.0	108	41	38	71	66
Lymph node status					
Positive	52	18	35	28	54
Negative	77	29	38	48	62
Unknown	21	6	29	12	57

*$P < 0.0001$ between estrogen receptor (ER)-positive and ER-negative cases; †$P = 0.002$ between progesterone receptor (PgR)-positive and PgR-negative cases; ‡$P = 0.0001$ between ER- and/or PgR-positive cases and double-negative ER and PgR cases; §$P = 0.03$ between the cases with invasive ductal carcinoma and invasive lobular carcinoma; ¶$P = 0.014$ between grade 1, 2 and 3 cases.

Table 4. Correlation of hormone receptor status with pERK1/2 and with pAKT in breast cancer with overexpression of insulin-like growth factor receptor type 1 (IGF1R)

Hormone receptor status	Total	Cases			
		pERK1/2		pAKT	
		n	%	*n*	%
Total	71	33	46	48	68
ER^+/PgR^+	42	24	57*	28	67
ER^+/PgR^-	6	3	50	4	67
ER^-/PgR^+	12	2	17	9	75
ER^-/PgR^-	11	2	18	7	64

*$P = 0.022$, between the cases with estrogen receptor (ER) positivity and ER negativity irrespective of progesterone receptor (PgR) status.

In the 71 IGF1R-overexpressing tumors, pAKT was detected in 28 (67%) of the 42 ER-positive/PgR-positive cases, four (67%) of the six ER-positive/PgR-negative cases, nine (75%) of the 12 ER-negative/PgR-positive cases, and seven (64%) of the 11 ER-negative/PgR-negative cases. Thus, pAKT was detected frequently regardless of hormone receptor status in IGF1R-overexpressing breast cancers.

Significance of hormone receptors, IGF1R and signaling kinases as predictors of disease-free survival

In Table 5, proportional hazards analysis revealed that the risk of relapse of ER-negative and PgR-negative cases was approximately twice as high as for those that were ER-positive and/or PgR-positive (95% confidential interval 1.12–4.0, $P = 0.02$). Cases with IGF1R overexpression tended to have better prognosis than cases without IGF1R overexpression in hormone receptor-positive breast cancers ($P = 0.09$). However, IGF1R status was not correlated with prognosis in 49 ER-negative and PgR-negative cases. In pERK1/2 and pAKT status, the groups with pERK1/2 or pAKT expression were not correlated with prognosis regardless of ER/PgR status and IGF1R status.

Discussion

IGF1R overexpression was comprehensively detected in breast cancers showing all subsets of hormone receptor status, but was more frequent in cases that were positive for ER and/or PgR than in cases that were double negative for both ER and PgR. These results indicate that IGF1R-overexpressing breast cancers might frequently develop through cellular activation induced by hormone receptors. It has been shown that estrogens induce insulin-like growth factor type 1 (IGF-1) in ER-positive breast cancer cells, and IGF-1 stimulates their growth through IGF1R. The reason for the association between IGF1R overexpression and ER positivity can be, at least in part, explained by the presence of this loop.[21,22] However, more than 20% of ER/PgR double-negative breast cancers also overexpressed IGF1R.

With regard to downstream signaling kinases, the incidence of pAKT expression did not differ significantly among all the subsets of ER/PgR status in breast cancer. In contrast, the incidence of pERK1/2 expression was highest in ER/PgR double-positive cancers, intermediate in ER-positive/PgR-negative and ER-negative/PgR-positive cancers, and lowest in ER/PgR double-negative cancers.

Several papers have described the relationship between pAKT or pERK and clinicopatholgical characteristics of breast cancer. Kirkegaard *et al.* reported that immunohistochemical expression of pAKT (Ser473) predicted decreased overall survival, but was not significantly associated with disease-free survival.[23] Cineas *et al.* showed that a high level of pAKT measured by chemiluminesence-linked immunosorbent assay was a predictor of decreased disease-free survival.[24] However, we couldn't show a significant correlation between immunohistochemical expression of pAKT and shorter disease-free survival.

In contrast, Svensson *et al.* reported that ERK1/2 phosphorylation in breast cancer correlated with better survival and a less-aggressive phenotype.[25] We also showed that pERK1/2 was frequently expressed in breast cancers that were hormone receptor-positive with lower nuclear grade, but that pERK1/2 didn't significantly correlate with better prognosis. A part of our results was compatible with the report by Svensson *et al.*

The manifold effects of the ER pathway can be partly explained by the newly discovered intense cross-talk of ER with the growth factor receptor-regulated system. Many recent studies have emphasized the importance of this kind of cross-talk in breast cancer etiology and progression. The latest reports suggest that cross-talk between ER and growth factor receptor pathways, such as the EGFR family and IGF1R, contributes to tamoxifen resistance, which is intimately associated with the dynamic equilibrium of multiple signaling pathways.[26–29]

Table 5. Significance of hormone receptors, insulin-like growth factor receptor type 1 (IGF1R) and signaling kinases as predictors of disease-free survival

Cases	Hazard rate	95% confidence interval	*P*-value
ER^- and PgR^- vs ER^+ and/or PgR^+	2.12	1.12–4.00	0.02
ER^+ and/or PgR^+ ($n = 101$)			
$IGF1R^-$ vs $IGF1R^+$	1.9	0.85–4.83	0.09
$pERK1/2^-$ vs $pERK1/2^+$	1.12	0.45–2.78	0.81
$pAKT^-$ vs $pAKT^+$	1.25	0.51–3.07	0.63
ER^- and PgR^- cases ($n = 49$)			
$IGF1R^-$ vs $IGF1R^+$	1.22	0.41–3.69	0.71
$pERK1/2^-$ vs $pERK1/2^+$	0.57	0.17–2.0	0.38
$pAKT^-$ vs $pAKT^+$	0.76	0.28–1.84	0.49
Breast cancer cases with IGF1R overexpression ($n = 71$)			
$pERK1/2^-$ vs $pERK1/2^+$	1.2	0.35–4.09	0.78
$pAKT^-$ vs $pAKT^+$	0.64	0.17–2.38	0.5

The total number of cases was 150. Proportional hazards analysis revealed that estrogen receptor (ER)-negative and progesterone receptor (PgR)-negative cases were approximately twice as likely to relapse (95% confidential interval 1.12–4.00, $P = 0.02$) than ER-positive and/or PgR-positive cases, and that cases with IGF1R overexpression tended to have a better prognosis than cases without IGF1R overexpression ($P = 0.09$). Other groups did not show a significant correlation of hormone receptors, IGF1R and signal kinases with disease-free survival.

doi: 10.1111/j.1349-7006.2006.00228.x

The difference in distribution of pERK1/2-expressing tumors and pAKT-expressing tumors among the subsets of hormone receptor status observed in the present study appears to be informative in terms of breast cancer development. Most human mammary cancers originate in luminal mammary epithelial cells lining the mammary ducts and alveoli. These cancers are histopathologically diverse and are classified on the basis of their growth requirements as hormone-dependent or hormone-independent tumors.[30] During the process in which breast precancerous cells deviate from a hormone-dependent status, which represents a less aggressive phenotype, the MAPK signaling pathway modulated by phosphorylation of ERK1/2 might lose its dominant effect on cellular proliferation through complex remodeling of the intracellular signaling network. Instead, the PI3K/AKT signaling pathway might come to play a relatively potent role in breast cancer cells that have lost their expression of ER and/or PgR. In fact, we found that most cases of hormone receptor-negative breast cancer had a low level of pERK1/2 expression but a high level of pAKT expression, accompanied by overexpression of EGFR and/or HER2. These findings suggest that the growth of hormone receptor-negative breast cancers might be frequently regulated by activation of EGFR and/or HER2 growth factor receptors, and that phosphorylation of AKT becomes dominant in these cancers through the change in balance between pAKT and pERK1/2.

Considering the present results, it is possible that in IGF1R-overexpressing breast cancers, the levels of expression of pERK1/2 and pAKT might alter in accordance with the changes in ER and/or PgR expression during cancer progression.

Some *in vitro* experimental studies have suggested that the IGF1R signaling pathway and its function may differ between hormone-dependent and hormone-independent breast cancer cells. Bartucci *et al.* have reported that in the ER-positive cell line MCF-7, IGF1R transmits various signals, such as those for growth, survival, migration and adhesion, whereas in ER-negative MDA-MB-231 cells, the growth-related function of IGF1R is attenuated, although it is still able to control non-mitogenic processes, such as migration.[31] One of the most significant differences in IGF1R signaling between ER-negative and ER-positive cells was impaired long-term stimulation of the PI3K/AKT pathway. Although sustained AKT activity could be important for the survival of breast cancer cells, a proper equilibrium between AKT and other pathways such as ERK1/2 is also critical in determining the biological behavior of breast cancer. There is additional evidence that hyperactivation of AKT can downregulate the MAPK/ERK1/2 pathway in ER-negative breast cancer.[31,32]

A study by Surmacz has also suggested a requirement for the IGF1R pathway by the ER-negative MDA-MB-435 cell line.[30] The growth of these cell lines is not enhanced by IGF-I. Interestingly, despite the lack of a mitogenic response to IGF-I, the metastatic potential of these ER-negative breast cancer cell lines can be effectively inhibited by IGF1R antagonists.[33,34] These experimental findings *in vitro* are compatible with the present data for IGF1R-overexpressing breast cancer.

In retrospective clinical studies, a highly significant correlation between IGF1R overexpression and better patient prognosis has been reported,[35] whereas patients with ER-negative but IGF1R-positive tumors, which are reported to occur infrequently, tend to show shorter disease-free survival.[36]

In conclusion, we have found that breast cancers showing double positivity for hormone receptors have the highest level of IGF1R overexpression (67%), and that high levels of both pERK1/2 and pAKT expression are also detected frequently. However, in hormone receptor-negative breast cancers, IGF1R was overexpressed in 22% of cases, and the expression of pERK1/2 was low, although the level of pAKT expression was relatively higher than that of pERK1/2. From these findings it is evident that the role of IGF1R in hormone receptor double-negative breast cancer differs from that in hormone receptor-positive breast cancer. The balance between pERK1/2 and pAKT expression might be influenced by differential overexpression of IGF1R in the constituent cancer cells of a tumor.

This is the first report to have demonstrated a critical role for IGF1R expression *in vivo* in association with hormone receptors in breast cancer.

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doi: 10.1111/j.1349-7006.2006.00228.x

Breast Cancer
Vol. 13 No. 3 July 2006

Review Article

HER-2 (c-erbB-2) Test Update: Present Status and Problems

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HER-2 tests are routinely used for the identification of patients with metastatic breast cancer that is potentially responsive to trastuzumab (herceptin) therapy. Recently, convincing data have been published with regard to the efficacy of trastuzumab as a drug for neoadjuvant therapy or adjuvant therapy for operable primary breast cancer that overexpresses HER-2. It is also noteworthy that at the St. Gallen International Consensus Conference 2005, HER-2 protein overexpression or HER-2 gene amplification has been included as an indicator for higher risk of recurrence for both node-negative and node-positive breast cancers. To measure the HER-2 level, the worldwide consensus appears to be that immunohistochemistry (IHC) should be performed first and, if the results of IHC are uncertain, fluorescence *in situ* hybridization (FISH) should be performed later, although some investigators argue that FISH should be performed first. These tests should be performed in strict adherence to existing instructions. Quality control is of utmost importance when performing HER-2 tests, both internal and external, for routine diagnosis and in clinical protocol studies.

Breast Cancer 13:236-248, 2006.

Key words: Trastuzumab, Fluorescence *in situ* hybridization (FISH), Immunohistochemistry, Metastatic breast cancer, Prognostic factor

Molecular-targeted therapy is currently having a tremendous impact on the daily practice of clinical oncology. Above all, the development of trastuzumab (Herceptin, Hoffman-La Roche, Ltd., Basel, Switzerland; Genentech, Inc., South San Francisco, CA, USA), an anti-HER-2 (c-erbB-2) monoclonal antibody, as an effective anti-cancer agent for patients with HER-2-overexpressing breast cancer has significantly changed the treatment of metastatic breast cancer. This agent was shown to significantly prolong the progression-free survival as a single agent or in combination with standard chemotherapeutic agents[1,2].

In 2005, the results of a phase III trial that compared the pathological complete response (pCR) rate in a neoadjuvant setting between two arms of patient groups with HER-2-overexpressing operable primary breast cancer were published. The trial was stopped because of the obvious superiority of trastuzumab plus chemotherapy (65.2% pCR rate) over chemotherapy only (26% pCR rate) by the interim analysis ($p = 0.02$)[3].

Furthermore, in a late-breaking session at the 2005 annual meeting of the American Society of Clinical Oncology (ASCO), astonishing interim reports were delivered with regard to three phase III trials that compared disease-free survival rates in patients with HER-2-overexpressing operable breast cancer in an adjuvant setting[4,5]. In the combined results of NSABP B-31 and Breast Intergroup N9831 protocols, the disease-free survival rate 3 years after surgery was 75% in the arm treated exclusively with systemic chemotherapy but 87% in the arm treated with systemic chemotherapy plus trastuzumab[4]. Therefore, the addition of trastuzumab resulted in a 52% reduction of recurrence 4 years after surgery. Similarly, in the HERA trial, the addition of trastuzumab also showed a 46% risk reduction of recurrence 2 years after surgery[5]. These results clearly show that this molecular-targeted therapy will be a treatment of choice for a subset of operable breast cancers in

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Abbreviations:
DCIS, Ductal carcinoma *in situ*; ECD, Extracellular domain; EGFR, Epidermal growth factor receptor; EIA, Enzyme immunoassay; ER, Estrogen receptor; FISH, Fluorescence *in situ* hybridization; IGF-1R, Insulin-like growth factor-1 receptor; IHC, Immunohistochemistry; pCR, Pathological complete response; RT-PCR, Reverse transcription-polymerase chain reaction; SD, Standard deviation

Table 1. St. Gallen International Conference 2005 Risk Categories[7]

Risk category	Endocrine-responsive	Endocrine-non-responsive
Lower risk	Node-negative ER and/or PgR expressed, AND all of the following features: pT ≤2 cm, Grade 1, Age ≥35 years, Vessel invasion (−), AND HER-2/neu(−)	Not applicable*
Intermediate Risk	Node-negative ER and/or PgR expressed, AND at least one of the following features: pT >2 cm, Grades 2-3, Age <35 years, Vessel invasion (+), OR HER-2/neu(+) Node-positive 1-3 Vessel invasion (−), HER-2/neu(−)	Same features PLUS ER and PgR absent
High risk	Node-positive 4 or more, OR Any Node-positive with Extensive vessel invasion or HER-2/neu(+)	Same features PLUS ER and PgR absent

*Microinvasive cancer or specific types of tumor phenotypes
(e.g., medullary or apocrine and myoepithelial cancers) presenting as node-negative disease

addition to surgery, hormone therapy, chemotherapy, and radiotherapy.

In a 2001 article, we reviewed the status of HER-2 gene amplification as a prognostic and predictive factor in breast cancer patients[6]. Shortly before trastuzumab was approved as a drug of choice for HER-2-overexpressing metastatic breast cancer by the Ministry of Health, Labor, and Welfare of Japan (MHLW) in 2001, the trastuzumab pathology committee was organized in order to prepare for HER-2 testing and determining the optimal use of trastuzumab. Thereafter, several HER-2 testing kits, including immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH), were approved as external diagnostic agents to identify HER-2 protein overexpression or HER-2 gene amplification. The contents of the previous review were revised, and the present status and problems of HER-2 testing are discussed in the present article.

Prognostic and Predictive Value of HER-2 Overexpression

Prognostic Factors

In the ASCO guidelines issued in 2001, HER-2 overexpression/amplification was not included as a recommended prognostic or predictive marker for response to anthracycline-based chemotherapy[7]. However, at the 9th St. Gallen International Consensus Conference on the primary therapy of early breast cancer in 2005, HER-2 was newly included among the parameters for higher-risk node-negative and node-positive breast cancers (Table 1)[8].

In a large-scale review by Ross *et al.*[9] of 81 studies with a total of 27,161 patients, 73 (90%) studies with 25,166 (92%) total patients found that either HER-2 gene amplification or HER-2 protein overexpression predicted breast cancer outcome by either univariate or multivariate analysis depending on the study.

In node-positive breast cancer, it has been suggested that HER-2 gene amplification and/or HER-2 protein overexpression are strong prognostic indicators, independent of tumor size or the number of metastatic lymph nodes[10-12]. In a meta-analysis by Clark and McGuire, multiple studies showed the significant prognostic value of HER-2 in node-positive breast cancer[13]. Our prospective and retrospective studies showed a consistent tendency[14,15].

HER-2 gene amplification and/or protein overexpression also have been shown to be indicators of poorer patient prognosis in node-negative breast cancer in a number of papers, although this is considered to be a controversial issue by some investigators. Press *et al.* continuously demonstrated the correlation between HER-2 gene amplification and poorer prognosis of patients with node-negative breast cancer[16]. Tsutsui *et al.* also reported the prognostic implication of HER-2 overexpression in cases in Japan involving surgically resected breast cancers[17].

In node-negative breast cancers, a significant percentage (about 5 to 10%) of cases are ductal carcinoma *in situ* (DCIS). It is well known that HER-2 gene amplification is frequent (42%) in DCIS, especially high-grade DCIS, or the comedo subtype[18]. Therefore, it is reasonable to conclude that HER-2 overexpression may be an early step in the development of a distinct histological type of carcinoma of the breast; however, there was no association of HER-2 overexpression with lymph node status or recurrence[18].

Allred *et al.* divided 613 cases of node-negative breast cancer into low-risk and high-risk lesions. In low-risk lesions, which were less than 3.0 cm in diameter, estrogen-receptor (ER) positive, and lacked significant DCIS components (n = 179), HER-2 overexpression correlated significantly with poorer prognosis[19]. Based on these data, HER-2 examination as a prognostic indicator was applicable to only 29% (179 of 613) of node-negative breast cancers.

Predictive Factor of Responsiveness to Anthracycline-Based Regimens

In 1994, Muss *et al.* suggested the correlation of HER-2 overexpression with a higher response of breast cancer to anthracycline-based adjuvant therapy[20]. Paik *et al.* then reported that there was no statistical significance in a larger-scale retrospective study[21]. It has been suggested in several papers that adjuvant or neoadjuvant therapy using anthracycline-based regimens has given rise to a higher pCR rate in patients with HER-2-overexpressing breast cancer than in those with HER-2-non-overexpressing breast cancer[22,23], although negative data also exist[24].

There is an inverse correlation between HER-2 overexpression and hormone-receptor expression. HER-2 overexpression occurs frequently in breast cancer of high histological or nuclear grade (Grade 3), whereas hormone-receptor expression is frequent in breast cancers of low-to-moderate histological or nuclear grade (Grades 1 and 2)[6]. HER-2-overexpressing breast cancers generally tend to be resistant to tamoxifen therapy[25] and are not considered to be candidates for hormone therapy. However, HER-2 overexpression/gene amplification and hormone receptor expression do not appear to be always mutually exclusive. In a study of neoadjuvant endocrine therapy for hormone-receptor-positive operable breast cancer in postmenopausal patients, cases with both overexpression of HER-1 (EGFR) and/or HER-2 and expression of ER showed a response rate of 88% to letrozole, a third-generation non-steroid aromatase-inhibitor, but a response rate of only 21% to tamoxifen[26].

HER-2 Testing for Trastuzumab Therapy Indication

The most important task of the trastuzumab pathology committee was to edit and publish a guide of HER-2 testing for the clinicians and pathologists who are going to use trastuzumab or start HER-2 testing. Referring to already-published HER-2 testing guides overseas[27,28], the committee drafted a guide for HER-2 testing that included a recommendation for material preparation, a list of available reagents, instructions for evaluating the results, and an algorithm of the testing. Other activities of the committee included making approaches to the MHLW, editing the HER-2 atlas, and lecturing on HER-2 testing. The HER-2 guide was first published in 2001, and in accordance with the approval of FISH by the national health insurance thereafter, the second version was published in 2003[29]. The contents of the guide are described below.

Material Preparation

For HER-2 testing, routinely processed formalin-fixed paraffin-embedded tissue blocks should be used for the analysis. Surgically resected specimens should be fixed in 10% formalin, ideally 10% buffered formalin, overnight or for 48 h. Overfixation can produce negative results because formalin suppresses the antigenicity. Formalin fixation for 4 days or longer might produce inaccurate data by FISH, and formalin fixation for 1 week or longer might result in inaccurate IHC results (Hiroi & Tsuda, unpublished observation)

Table 2. HER-2 Testing Kits that are Approved as External Diagnostic Reagents by the MHLW, Japan

Object of Measurement	Method	Kit (Manufacturer)
Protein	IHC	Herceptest (DakoCytomation)
		Histofine HER-2 kit (Nichirei)
		Pathway HER-2 (Ventana)
		Kyowa Stain HER-2/neu (M) (Kyowa Medex/BioGenex)
	EIA	ERBB2 EIA (Nichirei)
DNA	FISH	PathVysion HER-2 DNA probe kit (Vysis)

As a FISH kit, INFORM kit (Ventana) is also available, but it is not approved in Japan as an external diagnostic reagent.

Paraffin-embedded tissue blocks should be cut into 4-μm-thick sections for IHC and 5-μm-thick sections for FISH. The sections should be mounted on silane-coated glass slides.

Because trastuzumab is approved for metastatic breast cancer, it is ideal to test the metastatic foci of breast cancer for HER-2. However, there is a general consensus to substitute the HER-2 status in the primary site for that in metastatic sites because, in a number of studies comparing the HER-2 status between the primary and the metastatic sites, the concordance rates were reported to be between 87.6% and 100%[30-33]. In a recent study of circulating tumor cells, the HER-2 status of 37.5% of patients with HER-2-negative primary breast cancer became positive when relapse occurred[34]. It has also been shown that, among distant metastatic foci, there is heterogeneity in the HER-2 status[31]. Therefore, it is not conclusive yet whether the examination of HER-2 status in the primary site only is sufficient for the eligibility to trastuzumab.

For the evaluation of HER-2 status in the primary site, the invasive component should be determined, and the non-invasive component should not be determined, because only the invasive component is believed to metastasize. The HER2 status might be different in intraductal and invasive components in a number of tumors[35].

The determination of HER-2 status by IHC should be performed by pathologists recognized as specialists in pathology by the Japanese Society of Pathology. The determination of HER-2 FISH should be performed by specialists in cell identification.

Immunohistochemistry (IHC)

Table 2 includes a list of HER-2 tests that were approved by the MHLW as external diagnostic reagents, which are in part reimbursed by health insurance. IHC tests show the location and intensity of expression of specific proteins on pathological tissue sections. The HercepTest (DakoCytomation, Carpinteria, CA, USA), the Histofine HER-2 kit (Nichirei, Tokyo), Pathway HER-2 (clone CB-11, Ventana Medical Systems, Tucson, AZ, USA) and Kyowa Stain HER-2/neu (M) (clone CB-11, Kyowa Medex, Tokyo/BioGenex Laboratories, Innc., San Ramon, CA, USA) are IHC tests available in Japan. When using these kits, the tests should be performed in strict adherence to the recommendations of the manufacturer. The duration of antigen retrieval, the dilution of the antigen, and the duration of the peroxidase reaction using diaminobenzidine and hydrogen peroxide are particularly critical. As is well known, HER-2 determination is performed not only based on localization but also on the intensity of staining. The intensity of staining could be easily altered as a result of factors related to antigen retrieval, dilution of the antigen, and diaminobenzidine-hydrogen peroxide reaction.

Table 3. Immunohistochemical HER-2 Scoring Method

Score	Staining pattern
0	No membrane staining or any membrane staining in 10% or less of tumor cells
1+	Faint/barely perceptible membrane staining is detected in more than 10% of tumor cells. The cells are only stained in part of the membrane
2+	Weak-to-moderate complete membrane staining is seen in more than 10% of tumor cells
3+	Strong complete membrane staining is seen in more than 10% of tumor cells

The criteria for IHC evaluation are the same among these IHC kits. They include scores of 0, 1+, 2+, and 3+, with score 3+ being regarded as overexpression (Table 3, Fig 1). It is very impor-

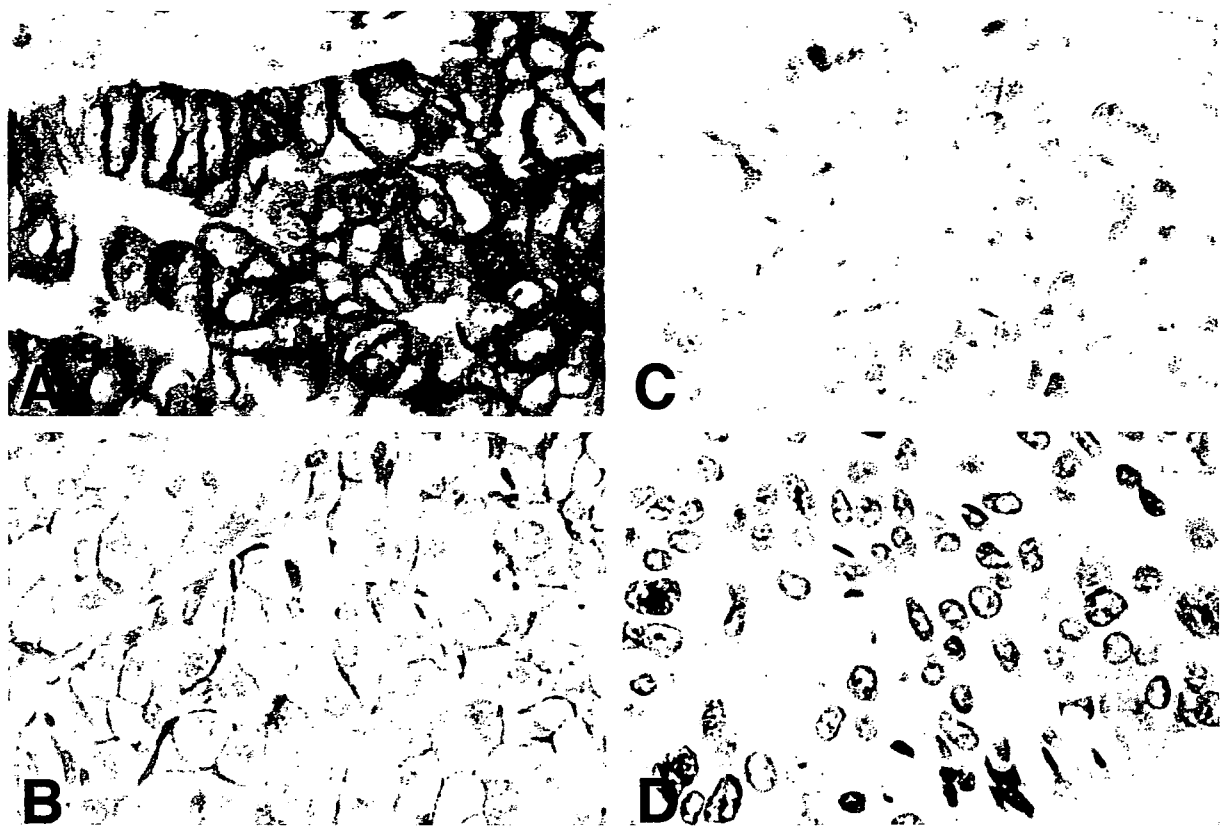

Fig 1. Evaluation of HER-2 protein overexpression by immunohistochemistry (IHC) in breast cancer. A. IHC score of 3+; B. IHC score of 2+; C. IHC score of 1+; D. IHC score of 0. (HercepTest, Original magnification ×200). The criteria for evaluation are described in Table 3.

tant to understand the criteria because tumors of HER-2 3+ intensity are candidates for trastuzumab therapy, whereas tumors with 0 or 1+ staining intensity are in principle not candidates for trastuzumab therapy.

Fluorescence in situ Hybridization (FISH)

FISH detects DNA or chromosome copies on histopathological sections by using DNA-DNA hybridization between fluorescence-labeled specific DNA probe(s) and tumor cell DNA on the tissue sections. At present, the PathVysion kit (Vysis Inc., Downers Grove, IL, USA) is approved by the MHLW as an external diagnostic agent to detect HER-2 gene amplification. The kit has been commercialized by Fujisawa Pharmaceutical Co. (Osaka, Japan; presently Asteras Pharmaceutical Co.) until September 2005, and the Japanese Agency of Abbott, Inc., will sell the kit thereafter. The PathVysion kit adopts the double-color FISH method, in which HER-2 DNA is labeled red and CEP17 (centromeric repetitive sequence on chromosome 17 as internal control) is labeled green. After hybridization, the background non-hybridized probes are repeatedly washed, nuclei are stained with 4,5-diamidino-2-phenylindole (DAPI), and specific signals are observed using a fluorescence microscope. The total signal numbers of HER-2 (red) and CEP17 (green) on 20 cancer cell nuclei are counted, and the value obtained by dividing the total number of HER-2 signals by the total number of CEP17 signals is defined as the HER-2/CEP17 ratio. A ratio of HER-2/CEP17 of 2.0 or higher should be judged as HER-2 amplification, or FISH-positive, whereas a ratio below 2.0 should be judged as FISH-negative. A schema of the calculation of the HER-2/CEP17 ratio and a representative FISH-positive breast cancer case are presented in Fig 2 and 3, respectively. The interobserver reproducibility of the FISH tests is very high[27,36].

HER-2 Testing Algorithm

An IHC score of 2+ is problematic because it may or may not indicate candidacy for the therapy. An HER-2 score of 3+ is almost always (89-94%) accompanied by HER-2 gene amplification, but the

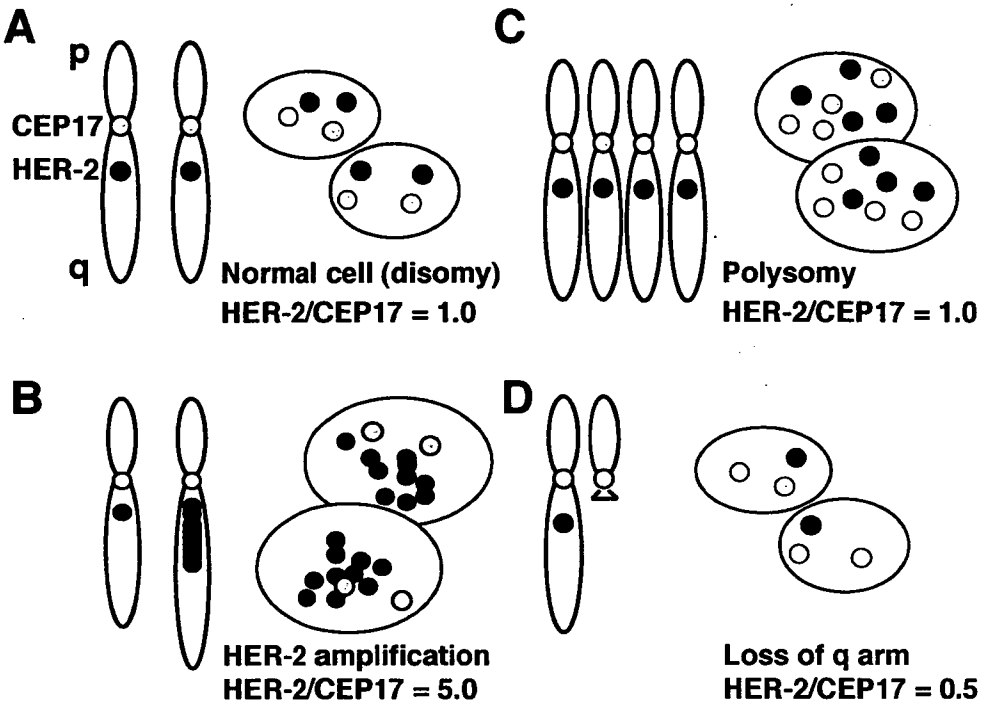

Fig 2. Patterns of chromosome 17 alterations detected by the PathVysion FISH kit. A. Normal cells or cancer cells without chromosome 17 alterations. Two signals of HER-2 (red) and two signals of CEP17 (internal control of chromosome 17, green) are visualized on each cell (in the right). It is inferred that the number of chromosome 17 is two, and there are no structural alterations (in the left). B. A case with HER-2 gene amplification. Ten signals of HER-2 and two signals of CEP17 are visualized on each cancer cell (in the right). The ratio of HER-2/CEP17 is 5.0, and the HER-2 gene is judged as amplified. In this case, the number of chromosome 17 is two (in the left). Actually, the HER-2 gene is frequently amplified on a chromosome or chromosomes other than chromosome 17. C. A case of chromosome 17 polysomy. Four signals of HER-2 and four signals of CEP17 are visualized on each cancer cell (in the right). The ratio of HER-2/CEP17 is 1.0, and the HER-2 gene is not amplified. In this case, the number of chromosome 17 is four (tetrasomy) (in the left). D. A case of loss of the long arm of chromosome 17. One signal of HER-2 and two signals of CEP17 are visualized on each cancer cell (in the right). The ratio of HER-2/CEP17 is 0.5, and a long arm of chromosome 17 on which a HER-2 gene is located is deleted. In this case, the number of chromosome 17 is two, with structural alterations in one of them (left).

percentage of HER-2 gene amplification is 3 to 7% and 3% in breast cancers with HER-2 scores of 1+ and 0, respectively[36-38]. In the HER-2 2+ group, HER-2 gene amplification was reported to be 24 to 25%[36-38]. Recent reports by Dowsett *et al.* showed a higher rate (48%) of IHC-FISH correlation in cases with IHC scores of 2+ which were examined at three reference centers, although the percentage varied markedly among the centers[39].

In a study that examined the inter-observer agreement of HER-2 IHC scores by six members of the trastuzumab pathology committee, the agreement levels for tumors with IHC scores of 3+ and 1+ or 0 were very high[40]. On the other hand, the inter-observer agreement level was poor for tumors with an IHC score of 2+[40]. For a given tumor, when a pathologist gave an IHC score of 2+, about 50% of his colleagues were likely to give an IHC score of 3+, 1+, or 0.

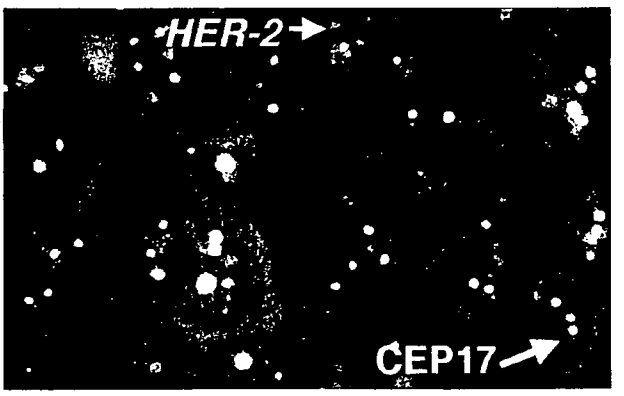

Fig 3. A case of HER-2 gene amplification. PathVysion kit. Original magnification ×1,000. (Courtesy of Dr. Y. Hiroi, National Defense Medical College).

The response to trastuzumab therapy differed

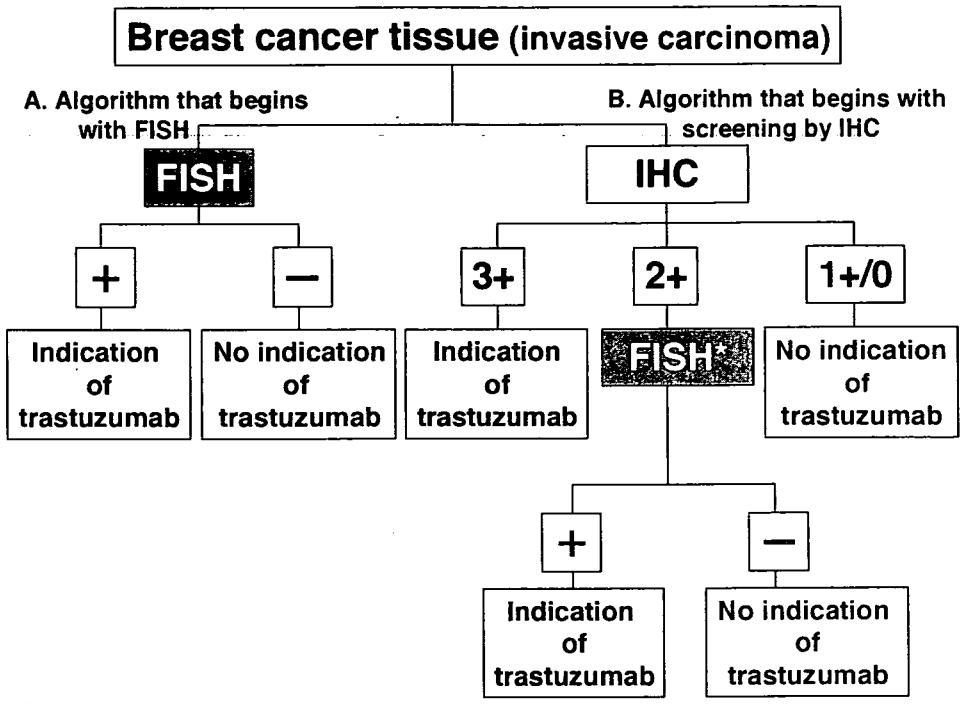

Fig 4. Algorithms for HER-2 testing in Japan[29]. Algorithm A is to perform FISH first. Algorithm B is to perform IHC first and, if the results are borderline or uncertain, to retest using FISH. Algorithm B is now more prevalent. HER-2 tests should be performed on invasive carcinomas. Patients eligible for trastuzumab therapy are those with HER-2-overexpressing and/or HER-2 gene-amplified metastatic breast cancer. *In patients with IHC 2+ in algorithm B, if the FISH test is impossible, the eligibility for trastuzumab therapy should be determined on the basis of other scientific data, e.g., serum HER-2 levels and histological or nuclear grade.

among patients with metastatic breast cancers with an HER-2 score of 3+ and those with an HER-2 score of 2+. Vogel *et al.* reported that, in HER-2-overexpressing metastatic breast cancer, the response rates to first-line trastuzumab as a single agent were 35% and 0% in IHC 3+ and IHC 2+ cases, respectively. Likewise, the response rates were 34% and 7% in FISH-positive and FISH-negative cases, respectively[1]. With regard to FISH, Press *et al.* reported that the response rates to single-agent trastuzumab therapy were 25-37%, 13-29%, and only 0-3% in women with metastatic breast cancers with HER-2/CEP17 ratios of >6.0 (high-level amplification), $\geqq 2.0$ to 6.0 (low-level amplification), and <2.0 (no amplification), respectively[41].

Taking these factors into consideration and incorporating FISH, the following HER-2 testing algorithms were recommended by the trastuzumab pathology committee. These algorithms, which were almost the same as those published overseas[27, 28], were published in Japanese in July 2003 as an HER-2 test guide (Fig 4)[29].

In the HER-2 test guide, two algorithms are presented. One is a "two-tier testing strategy[27]," in which specimens are first screened by IHC and confirmed by FISH when the results of an IHC test are uncertain or borderline. The other is a "one-tier testing strategy," in which specimens are tested by FISH. The first strategy is currently the most acceptable and realistic. When a patient has a metastatic tumor with an IHC score of 3+ or which is FISH-positive, she is eligible for trastuzumab therapy. Patients with tumors with an IHC score of 1+ or 0 are not, in principle, eligible for trastuzumab therapy. For IHC scores of 2+, retesting by FISH is recommended, and in such cases the patient should be eligible for therapy only when FISH-positive results are obtained.

Serum Testing for the HER-2 Extracellular Domain

The third type of HER-2 test is measurement of the serum HER-2 level. Enzyme immunoassay (EIA) is a quantitative method that detects shed antigens of the HER-2 extracellular domain (ECD)

in serum[42,43]. A part of the extracellular domain of the HER-2 protein can be truncated and shed into serum when the HER-2 protein is overexpressed throughout the membrane of cancer cells. Outside Japan, reports of clinical studies using sandwich EIA kits from Bayer Corporation (Tarrytown, NY, USA) and Oncogene Science, Inc. (Cambridge, MA, USA) have been published[44-46]. Recent studies have reported that EIA kits are useful for the prediction of a response to systemic chemotherapy[45] and the monitoring of disease activity after trastuzumab therapy in patients with HER-2-overexpressing metastatic breast cancer[44-47]. Esteva *et al.* reported that patients with elevated serum HER-2 ECD at baseline had a significantly higher response rate (76%) than patients with low levels at baseline (33%) in a phase II study of weekly docetaxel and trastuzumab[44]. Burstein *et al.* showed that a lack of decline in HER-2 ECD was a predictor for tumor progression after cycle 1 (8 weeks) of trastuzumab and vinorelbine as first-line therapy[45]. In a study by Fornier *et al.*, patients whose ECD normalized after 12 weeks of weekly trastuzumab and paclitaxel had a higher response (68%) than patients with persistently high ECD levels (15%, $p = 0.005$)[47].

The ERBB2 EIA kit (Nichirei, Tokyo) is approved as a serological tumor marker in Japan[43]. This kit is used as a follow-up for post-surgical patients with HER-2-overexpressing primary cancers or for monitoring disease activity in patients with HER-2-overexpressing metastatic or recurrent cancers.

EIA assays can also measure HER-2 in tumor tissue lysate; however, these assays are not clinically used at present. In HER-2 measurements from tissue lysate by EIA, false negative results can occur due to a sampling error or hard contamination of lymphocytes and/or stromal cells or proteins.

In principle, serum tests for HER-2 ECD are not used for the identification of eligibility for trastuzumab therapy. However, if it is impossible to obtain pathological tissue blocks, EIA might reveal HER-2 overexpression.

Other quantitative methods, including RT-PCR and chromosome *in situ* hybridization (or bright-field *in situ* hybridization), are limited to laboratory use at this stage.

Discussion

Level of HER-2 gene Amplification and Response to Trastuzumab

An IHC score of 2+ is considered to be borderline or uncertain. Whether such a category exists for FISH should also be examined. In fact, some authors show low-level amplification by FISH, although the definition of low-level amplification is undetermined and various values (2.0 to 2.5, 2.0 to less than 3.0, and 2.0 to 6.0) of the HER-2/CEP17 ratio have been reported. Owens *et al.* reported that the ratios of HER-2 gene amplification in cases IHC scores 0, 1+, 2+, and 3+ were 4.1, 7.4, 23.3, and 91.7%, respectively, in a large cohort of breast cancer specimens (n = 6,556) submitted to the IMPATH laboratories for HER-2 evaluation by IHC and FISH[38]. In 326 tumors with low-level amplification (HER-2/CEP17 ratio 2.0 to 2.5), only 23 (7.6%) had IHC scores of 3+, while the other 303 had IHC scores of 0, 1+, or 2+ (0 in 30 tumors, 1+ in 64, and 2+ in 209). In contrast, in 1,221 tumors with high-level amplification (HER-2/CEP17 ratio >2.5), 625 (51.2%) had IHC scores of 3+, 527 (43.1%) had IHC scores of 2+, and the remaining 69 (5.7%) had scores of 0 or 1+. Based on these results, the researchers proposed that the criteria for gene amplification should be a HER-2/CEP17 ratio greater than 2.5[38]. In our study, five out of six cases with low-level HER-2 amplification (HER-2/CEP17 ratio between 2.0 and 3.0) had HER-2 IHC scores of 0 or 1+, whereas 33 of 34 cases with higher-level HER-2 amplification (3.0 fold or higher) had an IHC score of 3+[36]. As shown above, Press *et al.* demonstrated that breast cancers with a HER-2/CEP17 ratio of 2.0 to 6.0 were frequently responsive to trastuzumab[39]. At present, it is unknown whether borderline amplification (HER-2/CEP17 ratio 2.0 to 2.5) is correlated with sensitivity to trastuzumab.

Superiority and Inferiority of FISH to IHC

The argument that FISH is superior to IHC seems to have an adequate rationale to support it. It is accepted as an alternative algorithm to perform FISH first and regard FISH-positive cases as eligible to trastuzumab therapy. An analysis of cost-effectiveness has indicated that it is more cost-effective to use FISH alone or as confirmation of all positive (scores 2+ and 3+) results rather than using FISH to confirm only weakly positive

results (2+) or using IHC alone[48]. In that paper, the frequency of HER-2 gene amplification in IHC 3+ cases was estimated to be relatively low, 67.1% (95% confidence interval: 0.547-0.795), in comparison with empirical cases, in which it was 90% or higher. This estimation might have given rise to the discrepancy in the optimal evaluation in that study and the real-world recommendations.

Yaziji *et al.* studied the concordance of FISH results with IHC results in 2,963 cases and showed a concordance rate of 98.8% between FISH-positivity and an IHC score of 3+[49]. In comparison with IHC, FISH tests showed significantly higher rates of failure (0.08% versus 5.0%), required longer time for the procedure (4 h with a 12 min standard deviation (SD) versus 36 h with a 30 min SD) and for result interpretation by a pathologist (45 s with a 13 s SD versus 7 min with a 2.5 min SD), had a higher reagent cost (10 US dollars versus 140 US dollars) and a higher projected annual cost of screening (two to six million US dollars versus 44 to 75 million US dollars, based on an estimated 211,300 invasive breast cancers in 2003)[49]. From these estimations, these authors supported a two-tier testing algorithm.

Quality Assurance in HER-2 Tests

In both the NSABP B-31 and Breast Intergroup N9831 studies, approximately the first 100 patients entered into the study were centrally reviewed for quality assurance of HER-2 tests in pathological tumor specimens submitted by the accruing institutions[50, 51]. In these studies, HER-2 tests were performed at local laboratories without confirmation by central reference laboratories. When the entered cases were re-examined at the central laboratories, 18% and 26% of them showed neither HER-2 overexpression nor HER-2 gene amplification in the NSABP B-31 and Breast Intergroup N9831 studies, respectively. After these results, the researchers decided to modify the protocol to improve the accuracy of HER-2 testing (Table 4).

Paik *et al.* reported that the reliability of the HER-2 test results tended to be lower in small-volume laboratories that processed fewer than 100 HER-2 tests a month than in laboratories that processed a larger volume of tests[50, 51]. Therefore, quality assurance at small-volume laboratories appears to be a problem with regard to improving the quality of HER-2 tests in routine practice and clinical trials. Regarding trastuzumab therapy, it is mandatory to identify eligible patients with accurate HER-2 tests and strict evaluation of the results. For that purpose, continuous action for the maintenance and improvement of HER-2 test quality is necessary in terms of the technical procedure itself and evaluation of the results.

For in-house quality control, it would be effective to use control slides to avoid technical errors and to establish a system of double checks to avoid errors in judgment. In Japan, in-house quality control is readily performed, but there is no external quality assurance system.

In an interesting and important report from the UK, a method of external quality assurance is described in detail. In the article, all clinical laboratories using assays for HER-2 as predictive or prognostic tests must participate in an appropriate external quality assurance (EQA) program, such as that run by the United Kingdom National External Quality Assessment Scheme for Immunocytochemistry (UK NEQAS-ICC)[27]. On a quarterly basis, UK NEQAS-ICC circulates unstained sections prepared from a block containing four control cell lines for HER-2 (score 3+, 2+, 0 or 1+, and 0 or 1+). Participating laboratories are asked to test the UK NEQAS sections and their own in-house control for HER-2 and to return them to the organizing center for evaluation by a panel of five expert assessors. Poorly performing laboratories are identified promptly, and the situation is rectified through appropriate action taken within a 12-month period, by the laboratories either improving until they reach an acceptable standard or being removed from the UK NEQAS participation register and losing their accreditation status for this test[27]. This kind of system appears to be worth adopting in Japan.

Future Perspectives

Based on the results of studies in 2005 with HER-2 and trastuzumab, HER-2 testing will be extended to primary breast cancers for the purpose of determining treatment from two viewpoints. One is the identification of intermediate-risk (or relatively higher risk) node-negative breast cancers and high-risk node-positive breast cancers to choose an adjuvant systemic therapy based on the St. Gallen International Conference 2005 recommendation[8]. The other is to identify patients with operable breast cancer eligible for adjuvant or neoadjuvant trastuzumab therapy according to the reports of Buzdar *et al.*[3], the NSABP B-31[4], Breast Intergroup N9831[4], and HERA[5] trials.

Table 4. HER-2 Testing and the Modification of Eligibility Criteria in the NSABP B-31 and Breast Intergroup N9831 Protocols[50,51]

	NSABP B-31	Breast Intergroup N9831
A. Number of cases	104	119
B. Initial HER-2 test for study eligibility		
Test	IHC or FISH	IHC or FISH
Eligibility criteria	1. IHC 3+ (HercepTest) 2. Strong membrane staining of >33% of the tumor cells (other IHC assays) 3. FISH (+)	1. IHC 3+ (HercepTest) 2. One third or more invasive tumor cells stain-positive (3+) (other IHC assays) 3. FISH: ≧2.0 HER-2/CEP17 ratio (PathVysion): ≧5 gene copies (INFORM)
Laboratory	Any accredited laboratory	One of 65 different local U.S. laboratories
Tests used	1. HercepTest (n = 80) 2. Other IHC (n = 20)	1. HercepTest (50%) 2. Other IHC (42%) 3. FISH (PathVysion) (7%) 4. FISH (INFORM) (1%)
C. Central testing		
Test	IHC	IHC and FISH
Eligible criteria	Same as B	Same as B
Laboratory	1. Laboratory Corporation of America Inc. (Research Triangle Park, NC, USA) (IHC) 2. NSABP Pathology Laboratory (FISH)	1. Immunohistochemistry Laboratory (Mayo Clinic, Rochester, MN, USA) (IHC) 2. Cytogenetics Laboratory (Mayo Clinic) (FISH)
Tests used	HercepTest (IHC)	HercepTest (IHC) PathVysion (FISH)
D. Concordance between local laboratory testing and central laboratory testing	82% (IHC)	74% (IHC) 66% (FISH)
E. Protocol modification of eligibility criteria	IHC score 3+ only by NSABP-approved reference laboratories or gene amplification by FISH from any laboratory	Strong overexpression of HER-2 or HER-2/neu gene amplification by central testing or by a local laboratory. To the enrollment based on local laboratory testing, then central confirmatory testing will be performed before the completion of the first chemotherapy regimen.

The revised algorithms of Fig 4 will need to be prepared soon for prognostication for patients with node-negative and node-positive breast cancers, for the identification of patients with primary operable breast cancer eligible for trastuzumab therapy in a neoadjuvant or adjuvant setting, and for the identification of patients with metastatic breast cancer eligible for conventional trastuzumab therapy. Accurate HER-2 testing and quality monitoring are increasingly important. Nearly all patients with invasive breast cancer could be largely affected by the results of HER-2 tests, and the introduction of trastuzumab therapy could significantly reduce the rate of recurrence. In addition, there are financial ramifications for patients and the national medical expenditure. A concrete nationwide strategy for quality assurance of HER-2 testing is needed.

In addition to trastuzumab, other HER-2-targeted agents are being developed. As a drug to treat trastuzumab-resistant HER-2-overexpressing breast cancer, a reversible and specific tyrosine kinase inhibitor of both HER-1 (EGFR) and HER-2, lapatinib (GlaxoSmithKline), is under investigation[52]. In a study of SKBR3 cell lines, insulin-like growth factor 1 receptor (IGF-1R) was suggested