Table 2 Choice of treatment modalities 2007 (reproduced from [1] with modifications)

HER2 (c-erbB-2) gene	Highly endocrine responsive	Incompletely endocrine responsive	Endocrine non-responsive
Negative	ET (considering CT according to risk)	ET (considering CT according to risk)	CT
Positive	ET + trastuzumab + CT	ET + trastuzumab + CT	Trastuzumab + CT

ET Endocrine therapy, *CT* chemotherapy

Table 3 The pTNM pathological classification (Reproduced from [4] with modifications)

The pTNM pathological classification system in terms of the primary tomor	Subcategories of the pTNM classification system in terms of the primary tumor
pTX	Primary tumor cannot be assessed
pT0	No evidence of primary tumor
pTis	Carcinoma in situ
	pTis(DCIS): ductal carcinoma in situ (Non-invasive ductal carcinoma)
	pTis(LCIS): lobular carcinoma in situ
	pTis(Paget): Paget disease of the nipple with no tumor[a]
pT1	Tumor 2 cm or less in greatest dimension
	pT1mic: microinvasion 0.1 cm or less in greatest dimension
	pT1a: more than 0.1 cm but not more than 0.5 cm in greatest dimension
	pT1b: more than 0.5 cm but not more than 1 cm in greatest dimension
	pT1c: more than 1 cm but not more than 2 cm in greatest dimension
pT2	Tumor more than 2 cm but not more than 5 cm in greatest dimension
pT3	Tumor more than 5 cm in greatest dimension
pT4	Tumor of any size with direct extension to chest wall or skin only as described in T4a to T4d (chest wall includes ribs, intercostals muscles, and serratus anterior muscle but not pectoral muscle)
	pT4a: extension to chest wall
	pT4b: edema (including peau d'orange), or ulceration of the skin of the breast, or satellite skin nodules confined to the same breast
	pT4c: both 4a and 4b above
	pT4d: inflammatory carcinoma

DCIS Ductal carcinoma in situ, *LCIS* non-invasive lobular carcinoma or lobular carcinoma in situ

[a] Paget disease associated with a tumor is classified according to the size of the tumor

represent a low risk even if they are of higher grade and/or affect younger patients [1, 2].

For the evaluation of the pT factor, it is important to differentiate accurately the invasive component from the non-invasive component (Fig. 2). If there are multiple invasive tumors in a breast, the diameter of the largest invasive tumor should be adopted, and the diameters of multiple invasive tumors should not be added.

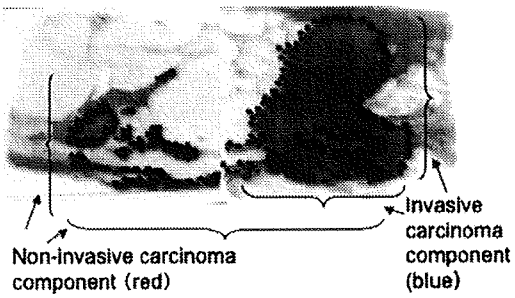

Fig. 2 Measurement of the size of the invasive component in primary breast carcinoma as determined on a stained section of breast tissue. The tumor indicated by *blue dots* is the invasive carcinoma component, and the part indicated by *red dots* is the non-invasive carcinoma component. For risk estimation, the measurement of the invasive component is necessary. H&E stain. ×1

Although the pT does not appear to have been measured very accurately, this measurement is important not only for risk estimation, but also for (1) determining the indication for primary systemic therapies (PST) based on core needle biopsy specimens, (2) assessing the therapeutic response, especially the pathological complete response (pCR), of primary tumors to primary systemic therapies based on an examination of surgically resected specimens, and (3) evaluating HER2 overexpression or gene amplification that is restricted to the invasive component.

Axillary lymph node status (pN factor)

The status of the axillary lymph nodes is the most powerful prognostic indicator in operable primary breast cancer. The outcome of patients becomes worse as the number of lymph nodes with metastasis increases [6]. The status of axillary lymph nodes has recently been classified by histopathological examination into pN0, pN1, pN2, and pN3 in the TNM classification [4]. Currently, parasternal lymph node dissection is usually not performed during breast surgery in Japan. In terms of axillary lymph node status only, pN0, pN1, pN2, and pN3 are defined as no metastasis,

Table 4 The pTNM pathological classification (Reproduced from [4] with modifications)

The pTNM pathological classification system in terms of the regional lymph nodes	Subcategories of the pTNM classification system in terms of the regional lymph nodes
pNX	Regional lymph nodes cannot be assessed (not removed for study or previously removed)
pN0	No regional lymph node metastasis[a]
pNmi	Micrometastasis (larger than 0.2 mm, but none larger than 2 mm in greatest dimension)
pN1	Metastasis in one to three ipsilateral axillary lymph node(s), and/or in ipsilateral internal mammary nodes with microscopic metastasis detected by sentinel lymph node dissection but not clinically apparent.
	pN1a: metastasis in one to three axillary lymph node(s), including at least one larger than 2 mm in greatest dimension
	pN1b: internal mammary lymph nodes with microscopic metastasis detected by sentinel lymph node dissection but not clinically apparent
	pN1c: metastasis in one to three axillary lymph nodes and internal mammary lymph nodes with microscopic metastasis detected by sentinel lymph node dissection but not clinically apparent
pN2	Metastasis in four to nine ipsilateral axillary lymph nodes, or in clinically apparent ipsilateral internal mammary lymph node(s) in the absence of axillary lymph node metastasis
pN3	Metastasis in ten or more ipsilateral axillary lymph nodes; or in ipsilateral infraclavicular lymph nodes; or in clinically apparent ipsilateral internal mammary lymph node(s) in the presence of one or more positive axillary lymph nodes; or in more than three axillary lymph nodes and in internal mammary lymph nodes with clinically negative, microscopic metastasis in internal mammary lymph nodes; or in ipsilateral supraclavicular lymph nodes

pN Regional lymph nodes

[a] Cases with only isolated tumor cells (ITCs) in regional lymph nodes are classified as pN0. ITCs are single tumor cells or small clusters of cells, not more than 0.2 mm in the greatest dimension, which are usually detected by immunohistochemistry or molecular methods but which may be verified by hematoxylin and eosin (HE) staining. ITCs do not typically show evidence of metastatic activity, e.g., proliferation or stromal reaction

metastasis to one to three lymph nodes, metastasis to four to nine lymph nodes, and metastasis to ten or more lymph nodes or to subclavicular lymph nodes, respectively (Table 4) [4].

Sentinel lymph node navigation surgery (SNNS) has become widely used for the surgical treatment of patients with early breast cancer [7]. In Japan, sentinel lymph nodes (SLNs) are identified by radioisotope-labeled colloid and/or dye, and they can be examined by intraoperative histopathology for the presence of metastasis. Axillary lymph node dissection is also carried out if metastasis is detected in SLNs, but it is not used if metastasis is absent.

Based on the diameter of the largest metastatic focus, pN0 and pN1 are sub-classified into pN0, pN0(i+), pN1 mi, and pN1a. pN0(i+) is defined as the presence of isolated tumor cells (ITC), which are tumor cell clusters with a diameter of $\leq$0.2 mm (Fig. 3). If the diameter of a metastatic tumor focus is >0.2–2.0 mm, the case is defined as pN1 mi, i.e., micrometastasis. pN1a is defined as metastasis to one to three lymph nodes with at least one node measuring >2.0 mm in diameter. Detailed cutting of SLNs (at 2-mm intervals) and accurate measurements of metastatic foci are needed for reliable SNNS [4, 7].

In patients with pN1a, the presence of HER2 overexpression/amplification is a feature of the high-risk group, whereas cases of pN1a without HER2 overexpression/amplification are classified as being of intermediate risk. All pN2 and pN3 cases are classified into the high-risk group in the 2007 St Gallen consensus [1].

Hormone receptor status

Endocrine therapies involving the use of effective drugs, such as anti-estrogens, aromatase inhibitors, and luteinizing hormone-releasing hormone (LHRH) analogues, have recently been developed, and the role of endocrine therapy in primary breast cancer is becoming increasingly important [8, 9]. Current approaches now always include the testing of ER and PgR by immunohistochemistry (IHC). If the tumor is ER- and/or PR-positive, the patient is eligible for preoperative or postoperative hormonal therapy.

There are several criteria for evaluating the results of ER and PR tests [1, 2, 10, 11]. In a classification recommended by the Japan Breast Cancer Society, the results are assessed according to the proportion of cells showing positive nuclear staining irrespective of the intensity of stained nuclei [11]: score 0 when there are no positive cells, score 1+ when the proportion of positive cells is <1%, score 2+

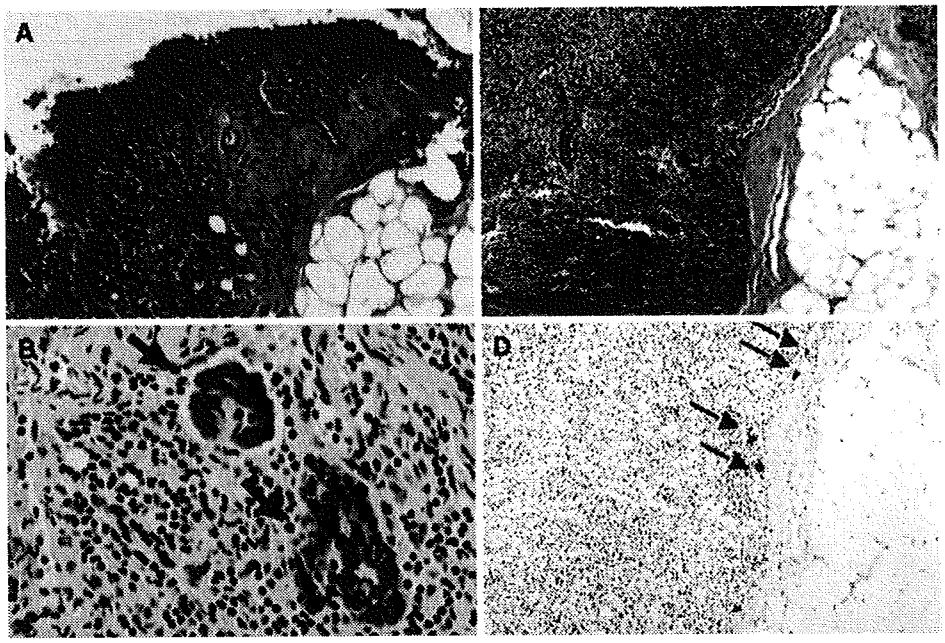

Fig. 3 Detection of micrometastasis of breast cancer cells in sentinel lymph nodes (SLNs). **a**, **b** Micrometastasis. A tumor cell nest is detectable by HE (**a**), and the tumor cells are confirmed to be positive for cytokeratin by immunohistochemistry (IHC) (**b**). *Arrows* indicate tumor cells. **c**, **d** Isolated tumor cells (ITCs). Carcinoma cells are not visible on the HE section (**c**), but single cytokeratin-positive cells (*arrows*) are detectable by IHC. **a**, **c** H&E stain. **b**, **d** Immunoperoxidase stain. **a**, **c**, **d** ×40, **b** ×200

when positive cells account for 1% to <10% of the cells tested, and score 3+ when positive cells account for 10% or more of the cells tested. A score of 3+ corresponds to a positive result, and 1+ and 2+ correspond to equivocal or borderline results. A score of 0 is negative.

The Allred score is another major scoring system that takes both the proportion and intensity of positively stained cells into account (Fig. 4) [11]. The Allred score is the sum (0, 2–8) of the proportional score (0–5) and the intensity score (0–3). In Japan, Allred scores for ER and PR are frequently requested by clinicians because of evidence that the Allred score for ER is correlated with (1) disease-free survival of patients receiving adjuvant endocrine therapies [12] and (2) the response rate of the primary tumor to neoadjuvant endocrine therapy using letrozole (an aromatase inhibitor) or tamoxifen [13].

At the St Gallen meetings, endocrine therapy was recommended for ER- or PR-positive breast cancer (Table 2) [1, 2]. In the report of the 2007 St Gallen meeting, hormone status was classified into highly endocrine responsive, incompletely endocrine responsive, and endocrine nonresponsive; incompletely responsive (previously referred to as endocrine response uncertain) is when there is some expression of steroid hormone receptors but at lower levels, or when either ER or PgR was lacking. For patients with highly endocrine-responsive breast cancer and those with incompletely endocrine-responsive breast cancer, adjuvant endocrine therapy is recommended, with chemotherapy being considered according to the risk presented (see Table 1 [1]).

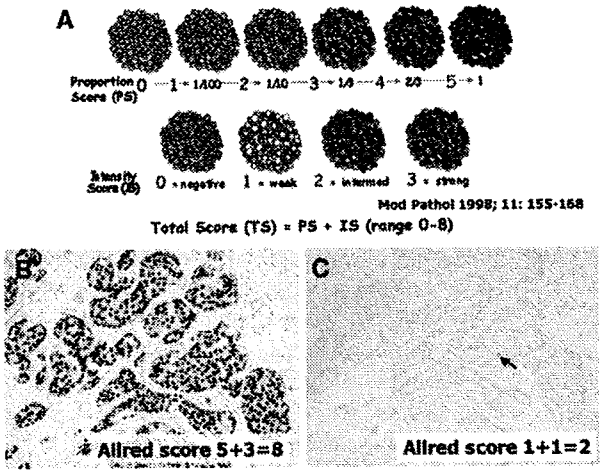

Fig. 4 Allred score system for the evaluation of the estrogen receptor (ER). **a** Schematic presentation of the system. Reproduced from Allred et al. [11]. **b** A case of strongly positive ER. Scores of intensity and proportion are 3 (strong) and 5 (67–100%), respectively, and the Allred score is 8. In the Japanese classification, the score is 3+ (see text). **c** A case of negative ER. *Arrow* Tumor cell. Scores of intensity and proportion are 1 (weak) and 1 (<1%), respectively, and the Allred score is 2. In the Japanese classification, the score is 1+, but in the St Gallen consensus, this case may be incompletely endocrine responsive

Grade

The histological grade of the malignancy of breast cancer is usually evaluated as the sum of tubule formation, nuclear pleomorphism, and mitotic count (histological grade), or as the sum of the latter two (nuclear grade), based on an examination of hematoxylin-eosin (HE)-stained histopathological sections. In 1957, Bloom and Richardson reported the important role of histological grade in the prognostication of primary breast cancer, and a modification of their criteria by Elston and Ellis is now widely used [14]. In Japan, a method of nuclear grading is presented in the "general rules" (Table 5, Fig. 5) [15].

Histological or nuclear grading is applied mainly to invasive ductal carcinoma for the purpose of estimating the risk of recurrence and determing the choice of adjuvant therapies [1, 2]. Histological grade and nuclear grade have almost the same prognostic relevance [16]. Histological grade is a powerful prognostic indicator independent of tumor size or lymph nodal status, but it has a strong correlation with HER2 amplification, nuclear p53 immunoreaction (i.e. inactivation of the tumor-suppressor function of p53 protein), hormone receptor negativity, and accumulation of chromosome alterations.

HER2 amplification and overexpression

The *HER2* gene was first cloned as a proto-oncogene homologous to the HER1 [c-erbB-1, or epidermal growth factor receptor (EGFR)] proto-oncogene that encodes tyrosine kinase growth factor receptor localized through cell membrane [17]. The *HER2* gene is located on chromosome arm 17q21.1, and genomic amplification of 17q12-q21.2 containing the *HER2* locus occurs in 10–30% of human breast cancers. *HER2* gene amplification causes overexpression of the HER2 protein and plays a role in the transduction of growth signals to the nucleus [18].

The clinicopathological implications of *HER2* gene amplification and overexpression of its protein are: (1) frequent occurrence in grade 3 carcinomas, comedo-type DCIS, Paget's disease, and inflammatory breast cancer, (2) correlation with poor prognosis independently of tumor size or nodal status, (3) indication for trastuzumab

Table 5 Nuclear grading system (Reproduced from [15] with modifications)

Nuclear grading system

Nuclear atypia

Score 1. Nuclei are uniform size and shape. The nuclei are not hyperchromatic or may be hyperchromatic with evenly dispersed chromatin or with finely granular chromatin without clumping

Score 2. Between scores 1 and 3

Score 3 Pleomorphic nuclei of varying sized showing hyperchromatism with coarse and irregular distribution often associated with large nucleoli

Mitotic counts

After choosing the fields that appear to contain largest number of mitotic figures:

Score 1: <5 per 10 high-power fields (400×)

Score 2: 5–10 per 10 high-power fields

Score 3: ≥ 11 per 10 high-power fields

Sum or scores in nuclear atypia and mitotic counts[a]

2, 3: Nuclear grade 1

4: Nuclear grade 2

5, 6: Nuclear grade 3

Visual number	Mitotic counts per 10 high-power fields (400×)			Eyepiece
	Score 1	Score 2	Score 3	
20	0–4	5–10	≥11	WHK 10×
21	0–5	6–11	≥12	CFW 10×, CFWN 10×
22	0–5	6–12	≥13	CFI 10×, WH 10×
25	0–7	8–15	≥16	CFIUW 10×
26.5	0–8	9–17	≥18	SWH 10×, SWHK 10×
27	0–9	10–18	≥19	CFUWN 10×

[a] Adjustment of criteria for mitotic counts according to the properties of eyepieces

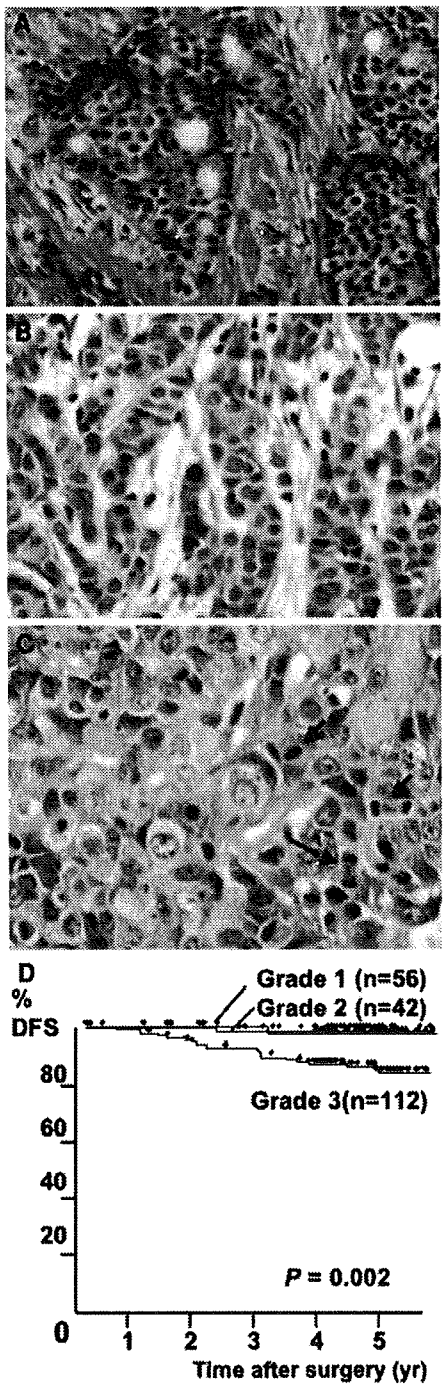

Fig. 5 Nuclear grade of pN0 (regional lymph nodes) invasive ductal carcinoma. Histopathological view of nuclear grade 1 (**a**), grade 2 (**b**), and grade 3 (**c**). **a** Nuclei are uniform, and mitotic figures are not seen. **c** Nuclei are pleomorphic, and there are a lot of mitotic figures (*arrows*). **b** is intermediate between **a** and **c**. H&E stain. ×200. **d** Disease-free survival curves for patients with pN0 breast cancer stratified by nuclear grades. Curves differ significantly ($P = 0.002$). *%DFS* Disease-free survival (%)

(Herceptin) therapy, and (4) high response rate to anthracycline-based chemotherapies [17].

Criteria for *HER2* overexpression and amplification have been established. *HER2* expression status, tested by IHC, is classified as score 0, 1+, 2+, or 3+ (Fig. 1 in [17]). An IHC score of 3+ is assessed to indicate overexpression, or *HER2*-positive; a score of 0 or 1+, as *HER2*-negative; a score of 2+, as equivocal, with a recommendation for retesting by fluorescence in situ hybridization (FISH).

There are two types of FISH tests, single-color FISH and dual-color FISH, but only the latter is approved for diagnostic testing in Japan. Dual-color FISH (PathVysion, Vysis/Abbott) visualizes concurrently red signals of *HER2* on 17q21.1 and green signals of *CEP17* on the centromere of chromosome 17. The sum of signals on 20 nuclei of cancer cells is counted for both *HER2* and *CEP17*, and the *HER2/CEP17* ratio is calculated by dividing the total signal number of *HER2* by that of *CEP17*. If the *HER2/CEP17* ratio is 2.0 or higher, the tumor is assessed to be FISH positive, whereas a ratio of less than 2.0 is taken to indicate that the tumor is FISH negative (Fig. 2 in [17]). *HER2*-positive cases are considered to be eligible for trastuzumab therapy [19].

In 2006, revised criteria for *HER2* gene amplification were recommended by the NCCN HER2 Testing in Breast Cancer Task Force as follows: a tumor with an IHC score of 0 or 1+, or with a *HER2/CEP17* ratio of less than 1.8 by dual-color FISH, is *HER2*-negative; a tumor with an IHC score of 3+, or with a *HER2/CEP17* ratio of more than 2.2 by dual-color FISH, is *HER2*-positive; a tumor with an IHC score of 2+ should be further tested using FISH, with its *HER2* status determined on the basis of the FISH result. Tumor samples with a *HER2/CEP17* ratio of 1.8 to 2.2 are considered to be borderline [20]. In Japan, a cut-off value of 2.0 for the *HER2/CEP17* ratio is still used.

Amplification of the *HER2* gene and overexpression of its protein is correlated with a poorer prognosis of patients with pN0 invasive breast cancer as well as patients with node-positive breast cancer. In a review, Ross et al. stated that *HER2* amplification or overexpression was correlated with poorer prognosis by univariate and/or multivariate analyses in 73 of 81 studies (25,166 of 27,161 patients) published between 1987 and 2003 [21]. Although *HER2* is frequently positive in DCIS of higher grade and with an accumulation of molecular alterations, i.e., comedo-type DCIS and Paget's disease, *HER2* is not a prognostic factor in DCIS.

A correlation between *HER2* overexpression and response to adjuvant or neoadjuvant anthracycline-based chemotherapy has been reported in many studies [22]. In the JBCRG-01 protocol, *HER2* overexpression was

confirmed to be a significant predictive factor of tumor response to anthracycline-based PST [23]. Hayes et al. [24] reported that a node-positive breast cancer showing amplification or overexpression of *HER2* can benefit from the addition of paclitaxel therapy after adjuvant treatment with doxorubicin plus cyclophosphamide, regardless of the ER status. In contrast, they considered that patients with *HER2*-negative, ER-positive, node-positive breast cancer would gain little benefit from the administration of paclitaxel after adjuvant chemotherapy with doxorubicin plus cyclophosphamide.

Lymphovascular involvement

Although lymphatic and vascular invasion (abbreviated as ly and v) is generally considered to be a parameter of aggressiveness of cancers in various organs, the significance of ly or v as a prognostic factor has been the subject of controversy because (1) ly(+) is frequently difficult to differentiate histologically from tumor nests in pseudolymphatic spaces emerging as artifacts, (2) ly(+) or v(+) can be confused with an intraductal component, and (3) pathologists tend to evaluate a cancer as ly(+) unconditionally if lymph node metastasis is present.

Lymphatic and vascular invasion have been excluded from the items routinely described in the "general rules". Nonetheless, obvious lymphatic permeation does exist and is sometimes diffuse in highly aggressive breast cancers, such as inflammatory carcinoma. Yoshimoto et al. [25] defined lymphovascular invasion as being strongly positive if four or more ly or v foci were present, positive if three or fewer ly or v foci were present, and negative if no ly or v focus was evident in all case slides. In their series of cases, the percentages of those that were ly or v strongly positive, positive, and negative were 13, 13, and 74%, respectively, and the corresponding recurrence rates were 60, 40, and 26%, respectively [25].

In the 2007 St Gallen meeting consensus, extensive peritumoral vascular invasion was considered to be one of the parameters for assessing whether pN0 breast cancer is of low risk or intermediate risk. In pN1a breast cancers, however, extensive lymphovascular invasion has been removed from the risk factors [1]. Extensive peritumoral vascular invasion is defined as the presence of neoplastic emboli in two or more blocks, which could be interpreted as both extensive ly(+) and v(+). Intratumoral lymph vessels in invasive breast cancer are suggested to be absent [26], and it is recommended that peritumoral lymphovascular invasion should be taken into account. Recently, several reports have mentioned the utility of D2-40 or vascular endothelial growth factor (VEGF)-C in combination with IHC to visualize lymphatic ducts, and the diagnostic application of these molecules is expected [27, 28].

Future perspectives

According to the recommendation of the St Gallen meetings, more than 70% of pN0 breast cancers are included in the intermediate-risk group and considered eligible for adjuvant chemotherapy, despite the fact that the 10-year disease-free survival rate of patients with pN0 breast cancer in Japan is 10–15% [4]. It is expected that recurrence risk in pN0 breast cancer will be evaluable more accurately based on the properties of the resected tumors. On the basis of estimations from the categories established at the St Gallen consensus meeting, the 10-year recurrence rate of pN0 intermediate-risk cancer would be 13–20%, whereas that of pN0 low-risk cancer would be 5% or less. It is ideal that the pN0 group is classified as representing 33% of the high-risk group, with a recurrence rate of 30–45%, and 67% of the low-risk group with a recurrence rate of 5% or less (Fig 6). To establish a more ideal classification of early breast cancer, various tests are being undertaken that are based on molecular features.

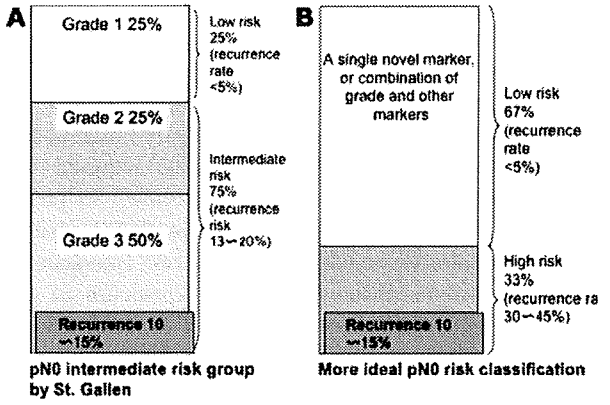

Fig. 6 Comparison between the pN0 intermediate-risk group (**a**) and the ideal pN0 high-risk group (**b**). In **a**, the intermediate-risk group comprises 75% of all patients with pN0 breast cancer and is estimated to show 13–20% of recurrence. Actually, most of pN0 breast cancers have an invasive component that is 2.0 cm or less in size. Lymphovascular invasion and *HER2* overexpression are shown by some researchers to be significant prognostic indicators with a relatively weak impact in pN0. Therefore, the overwhelming risk factor of pN0 breast cancer appears to be histological/nuclear grade. In **b**, the putative higher risk group comprises 33% of all patients with pN0 breast cancer and is estimated to show 30–45% of recurrence. For the concentration of cases, a single novel molecular marker or a combination of grade and other molecular markers would be effective. These molecular markers will be assayed by DNA microarray, Oncotype DX, array CGH, or other tools in the near future

DNA microarray

Using high-throughput microarray analysis, a 70-gene signature has been identified that can accurately select early-stage breast cancer patients who are highly likely to develop distant metastases and who, therefore, may obtain maximal benefit from adjuvant chemotherapy [29]. Validation and feasibility studies of the 70-gene profile (Mammaprint; Agendia) to patients of 60 years or younger with pN0 Stage I, II breast cancer are ongoing in the large adjuvant MINDACT (microarray in node-negative disease may avoid chemotherapy) clinical trial [29].

Oncotype DX

Although patients with pN0 and ER-positive breast cancer have an excellent prognosis, about 15% show relapse after 5 years of endocrine therapy. Clinical trials have provided evidence that these patients gain a significant benefit from chemotherapy; however, it would be significant overtreatment if it were applied to all of them. Strategies for evaluating tumors in a clinical setting have been developed using smaller sets of genes. One such strategy is the 21-gene assay (Oncotype DX; Genomic Health, Redwood City, CA), which is currently in commercial use in the USA. In Oncotype DX, a 21-gene recurrence score (RS) has been developed based on the monitoring of mRNA expression levels of 16 cancer-related genes in relation to five reference genes. One advantage of this test is the use of paraffin-embedded blocks in contrast to previous methods that required fresh frozen tissue. Oncotype DX has been shown to predict 10-year distant recurrence in patients with ER-positive, axillary lymph node-negative breast cancer. This genomic assay has also been shown to predict response to chemotherapy and endocrine therapy. A prospective study—the Trial Assigning Individualized Options for Treatment (Rx) (TAILORx)—to examine whether chemotherapy is required for the intermediate-risk group defined by the RS is accruing in North America [30, 31].

Array CGH (cancer array 800)

Comparative genomic hybridization (CGH) has already made a significant impact on cancer cytogenetics. In array-based CGH, DNAs spotted in a CGH-array contain sequence information directly linked to the genome database, and particular biological aspects of genes that lie within regions involved in copy-number aberrations can be noted. The application of array-based CGH technology for the diagnosis of breast cancer is awaited [32].

Basal-like type

With the use of gene microarrays, different subtypes of breast cancer have been characterized. These subtypes include the basal, ERBB2+, and luminal A, B and C subtypes [33, 34]. Basal-like-type breast cancer was determined by DNA microarray to be a group of tumors that were ER-negative, *HER2*-negative, and positive for myoepithelial/basal markers, such as vimentin, alpha-smooth muscle actin, EGFR, cytokeratin (CK) 5/6, CK14, and/or CK17 [33, 34]. It has also been reported that breast cancers arising in patients with familial breast/ovarian cancer carry germ-line *BRCA1* mutations [35].

There are at least four histologically distinct breast cancer groups with undifferentiated features, and these frequently show bidirectional differentiation toward luminal epithelial and myoepithelial/basal lineages [36, 37]. These groups comprise (1) invasive ductal carcinoma with a large central acellular zone (central acellular carcinoma), (2) atypical medullary carcinoma, (3) matrix-producing carcinoma, and (4) carcinoma with spindle-cell metaplasia (Fig. 7). In these four cancer types, KIT (CD117) expression and EGFR overexpression were detected in 34% and 88% of cancers with frequent expression of myoepithelial/basal markers but a low frequency of *HER2* overexpression or ER/PgR expression (Fig. 8) [37]. For the identification of a basal-like phenotype, confirmation of positivity for basal cytokeratins, i.e., either CK5/6 or CK14, by IHC is recommended [38].

Empirically, cases of node-negative breast cancer showing early recurrence appear to frequently contain the basal-like type [39]. However, Fulford et al. have reported that in node-negative patients, prognosis was similar between basal-like and other Grade 3 invasive ductal carcinomas, whereas basal-like grade 3 invasive ductal carcinoma showed a poorer prognosis than other types in patients who were positive for node metastasis [40].

One important current issue is the so-called triple-negative (i.e., ER/PgR-negative, *HER2*-negative) breast cancer, for which endocrine therapy or trastuzumab is not applicable. Approximately 5–15% of breast cancers are in this category. If systemic chemotherapies are not effective for "triple-negative" breast cancer, there are few treatment choices for this group and, in fact, chemotherapy is frequently not effective. A substantial percentage of "triple-negative" breast cancers appear to be of the basal-like type. The characterization of basal-like breast cancer on the basis of histological characteristics and molecular alterations would be useful for prognostication and treatment selection and also for the identification of targets for molecular therapy. It might be worth investigating whether therapies against activated KIT and/or EGFR are effective for cancers of the above-mentioned four histological types.

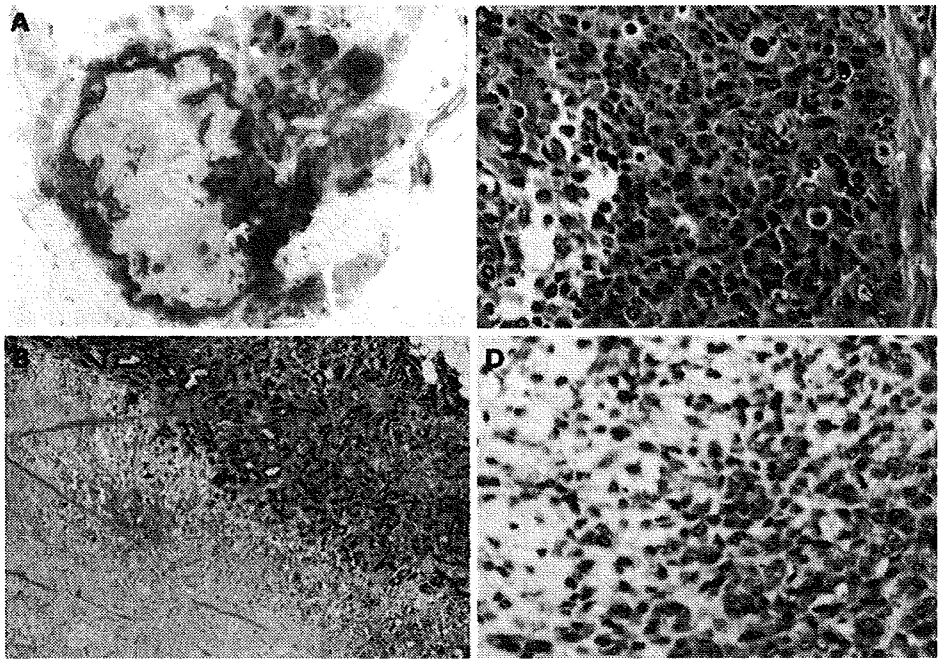

Fig. 7 Distinct breast cancer types of undifferentiated features, that appear to be representative of the basal-like phenotype. **a**, **b** Invasive ductal carcinoma with a large central acellular zone (central acellular carcinoma). **c** atypical medullary carcinoma, **d** matrix-producing carcinoma. Carcinoma with spindle-cell metaplasia is also included in this group. H&E stain. **a** ×1, **b** ×40, **c**, **d** ×200

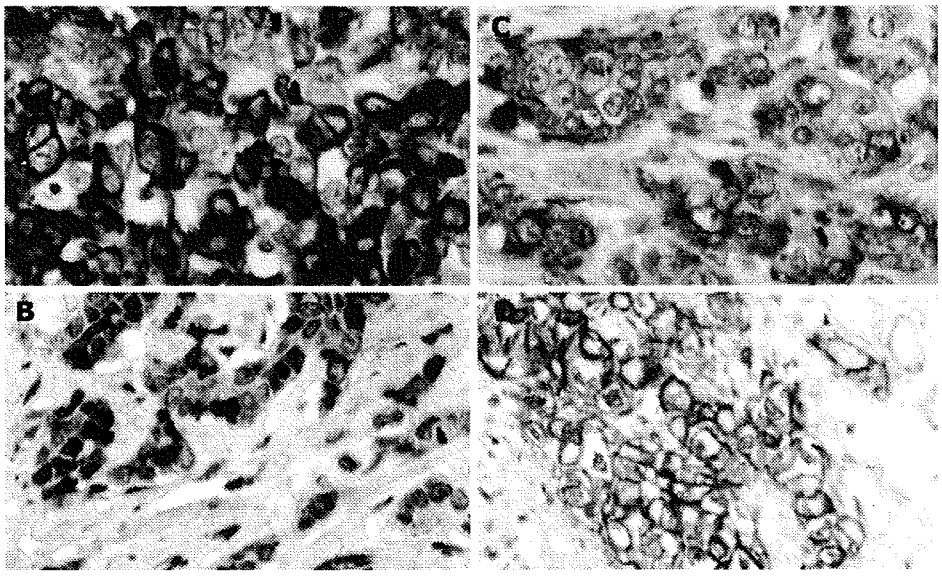

Fig. 8 Expression of myoepithelial/basal markers in undifferentiated-type breast carcinomas. **a** Vimentin, **b** KIT, **c** α-smooth muscle actin (SMA), **d** epidermal growth factor receptor (EGFR). The positive rates of expression of vimentin, α-SMA, KIT (CD117), and EGFR were 88, 41, 34, 88%, respectively, in undifferentiated types, whereas these were 5, 0.7, 5, and 5%, respectively, in other types [37]. Immunoperoxidase stain. ×200

Today, systemic chemotherapy, endocrine therapy, and trastuzumab are very effective, not only for metastatic breast cancer but also operable early breast cancer. Certain chemotherapeutic regimens with or without trastuzumab can achieve a pCR in the primary tumor in a proportion of cases. Nevertheless, these therapies are not perfect; they are not always effective, they may result in adverse events or late complications, and they may induce tumor resistance to the therapy in due course.

To achieve a greater decrease in the incidence of recurrence and death in patients with early breast cancer and those suffering adverse effects from the therapies, it is important to discriminate the higher risk group from the lower risk group more accurately in intermediate-risk

node-negative breast cancer. Furthermore, regardless of nodal status, the establishment of a diagnosis and treatment strategy for "triple-negative" breast cancers, especially the basal-like type, is desirable. To this end, molecular markers and tools, such as Oncotype Dx, Mammaprint, array CGH, and/or IHC markers for the basal-like type, would be effective. In addition, from a pathologist's viewpoint, proposals for the accurate evaluation of pT, pN, grade, ly, v, ER, PgR, and *HER2* have only recently been put forward. Therefore, there are few adequate follow-up data based on accurate descriptions of these pathological parameters. In the future, prospective clinical data as well as the revision of archival cases based on accurate histopathological evaluation might prove to have considerably higher value than expected hitherto, as exemplified by the proverb "it is always dark at the foot of the lighthouse".

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What Causes Discrepancies in HER2 Testing for Breast Cancer?

A Japanese Ring Study in Conjunction With the Global Standard

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Key Words: Breast cancer; HER2 testing; Assessment; Ring study; Multicenter study; Immunohistochemistry; Fluorescence in situ hybridization

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CME

Upon completion of this activity you will be able to:
- apply the ASCO/CAP guidelines for HER2 testing of breast carcinomas.
- define the immunohistochemical reactivity rate for HER2 in breast carcinoma that should trigger additional testing by FISH.
- analyze causes of discrepancy in HER2 testing of breast carcinomas by IHC.
- outline a protocol for single institutional guidelines for combined use of IHC and FISH for HER2 testing in breast carcinomas, incorporating quality assurance considerations.

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Questions appear on p 1006. Exam is located at www.ascp.org/ajcpcme.

Abstract

We assessed interinstitutional and interobserver consistency of human epidermal growth factor receptor type-2 (HER2) testing using immunohistochemical analysis and fluorescence in situ hybridization (FISH) in a set of 20 breast cancer samples among 10 institutions in Japan and a Herceptin adjuvant study participating laboratory in Germany and identified factors that may lead to discordant results.

We found a good agreement in immunohistochemical HER2 scoring between the coordinating institution and 10 participating laboratories ($\kappa = 0.718$) and excellent agreement for FISH ($\kappa = 0.900$). The results of a comparison between 10 Japanese laboratories and the German laboratory was good for immunohistochemical studies ($\kappa = 0.713$) and excellent for FISH ($\kappa = 0.887$). FISH retesting of equivocal samples (2+ immunohistochemically) improved agreement. Discrepancies between results were attributed to the evaluation process in 33.0% of the samples, staining procedures in 25.0%, and a combination of the two in 41.7%. Evaluation of samples according to the American Society of Clinical Oncology/College of American Pathologists guideline increased the number of 2+ immunohistochemical scores. By performing FISH retesting for these samples, consistency among multiple institutions could be archived. The quality of the staining procedures performed and the consistency of evaluations require regular assessment.

The human epidermal growth factor receptor type-2 (*HER2*) gene encodes a protein (185 kDa) that is a cell surface receptor with tyrosine kinase activity.[1] Amplification of the *HER2* gene and/or overexpression of its protein product has been shown in 25% to 30% of breast cancers.[2,3] Moreover, HER2 status is an important factor in predicting prognosis[2,4] and selection of systemic therapies for treatment.[5-9] Overall, *HER2* gene amplification is associated with a poor clinical outcome,[2,4] and, accordingly, HER2 status has been added to the risk category of the St Gallen consensus recommendation.[10] Overexpression of HER2 protein is also associated with resistance to endocrine therapy that may be specific to selective estrogen receptor modulator therapies, such as tamoxifen, but not to aromatase inhibitors.[5,6,11] In contrast, HER2 overexpression correlates with a response to treatment with anthracyclines and taxanes.[7-9]

Accumulating evidence indicates that trastuzumab (Herceptin) is effective not only for the treatment of metastatic breast cancer but also for early breast cancer with HER2 overexpression. International clinical trials[12-16] have revealed that trastuzumab treatment for primary breast cancer in the adjuvant setting reduced the risk of recurrence and mortality. Based on the results of these trials, trastuzumab has been included in the National Comprehensive Cancer Network guidelines and the St Gallen consensus recommendations. In 2006, the European Medicine Agency and the US Food and Drug Administration approved trastuzumab for primary breast cancer in the adjuvant setting. With these approvals, an increased number of patients may be able to receive treatment with trastuzumab based on HER2 testing results.

Misdiagnosis of HER2 overexpression can result in the loss of opportunity for patients to receive the benefits of trastuzumab treatment or in patients being overtreated. Therefore, accuracy in HER2 testing is of significant clinical benefit.

It has been reported that the efficacy of trastuzumab depends on the extent of HER2 overexpression: A tumor with a 2+ immunohistochemical score has a response rate of 0%, whereas a tumor with a 3+ immunohistochemical score has a response rate of 35%.[17] Although the significance of accurate HER2 testing has been emphasized, HER2 testing is not subject to external quality assurance in all countries, despite the fact that evaluation of HER2 serves as a major conclusive factor in the decision to treat with trastuzumab.

HER2 gene amplification was first examined by using Southern blotting in the early phase of a clinical study by Slamon et al.[2] An alternative method for HER2 detection is fluorescence in situ hybridization (FISH). In the early phase of a validation study, HER2 protein overexpression was examined immunohistochemically using anti-HER2 monoclonal antibodies 4D5 and CB11 (denoted the Clinical Trial Assay).[16] HER2 detection using a polyclonal antibody can be more sensitive but is less specific than using a monoclonal antibody. Although immunohistochemical analysis is now relatively inexpensive and universally available in research laboratories, it does not produce results as reliably consistent as those observed with FISH. In 3 clinical studies, the population of patients with tumors categorized as 2+ immunohistochemically varied from 12.7% to 39.5%, and the rate of *HER2* gene amplification in tumors scored 2+ immunohistochemically varied from 17.9% to 48.1%.[16,18,19] Although these data may represent a significant diversity in breast cancer tissue samples with regard to HER2, it is important to consider the sensitivity and specificity of immunohistochemical and FISH analyses.

No assessment system for the standardization of immunohistochemical or FISH analysis of HER2 has been established in Japan thus far. However, in other countries, a standardization process is in place, ie, the Nordic Immunohistochemical Quality Control (http://www.nordiqc.org/news.htm), United Kingdom National External Quality Assessment Service (http://www.ukneqas.org.uk/), the College of American Pathologists (CAP; http://www.cap.org/apps/cap.portal), and the Royal College of Pathologists of Australia Quality Assurance Program (http://www.rcpaqapa.netcore.com.au/index.html).

To investigate the consistency of HER2 testing in Europe, Dowsett et al[20] conducted an international ring study with 5 pathologists, each from a different country, applying immunohistochemical analysis and FISH to 20 slide sets. We conducted a Japanese ring study with 10 participating laboratories, responsible for diagnosing approximately 80% of breast cancer samples in Japan, and 1 laboratory in Germany that participated in the Herceptin adjuvant (HERA) trial.

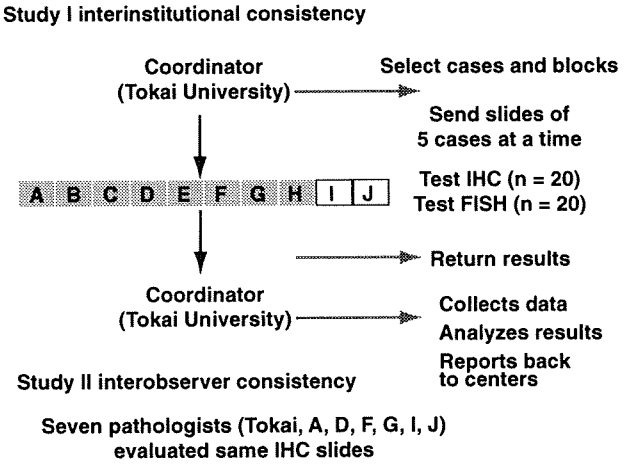
Materials and Methods

Participants

To compare and assess immunohistochemical and FISH analyses of HER2 expression by different laboratories, 7 institutions in Japan (Tokai University School of Medicine, Isehara; The Cancer Institute Japanese Foundation for Cancer Research, Koto; Niigata Cancer Center Hospital, Niigata; the Saitama Cancer Center, Kita-adachi; Tohoku University School of Medicine, Sendai; Kitakyushu Municipal Medical Center, Kitakyushu; and the National Defense Medical College, Tokorozawa), 3 commercial laboratories (SRL, Tachikawa; BML, Kawagoe; and Mitsubishi Chemical Medience, Itabashi), and 1 laboratory that was a site for a HERA trial (Institut für Pathologie, Klinikum Kassel, Targos Molecular Pathology, Kassel, Germany) participated in this ring study ▮**Figure 1**▮. The study was coordinated by the Tokai University School of Medicine and approved by the institutional review board of Tokai University.

Sample Selection and Distribution

We selected 20 cases of invasive breast cancer from the surgical pathology files of Tokai University Hospital. The breast cancer tissue samples had previously been tested and were selected to represent a relatively higher proportion of equivocal cases for the purpose of assessment. All of the specimens had been fixed with formalin (12-48 hours) and embedded in paraffin blocks. Tissue sections,



▮**Figure 1**▮ Study designs for studies I and II. Study I examined interinstitutional consistency, and study II examined interobserver consistency of HER2 testing. Institutions A-H participated in immunohistochemical analysis (IHC) and fluorescence in situ hybridization (FISH) analysis, and institutions I and J evaluated IHC results.

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4 to 6 μm thick, were mounted on silane-coated slides. A set of 5 cases, with 2 unstained slides for each case, was sent every 2 weeks to participants for immunohistochemical and FISH analyses. The slides for FISH analysis were sent after receiving the results of the immunohistochemical analysis to avoid bias from the FISH results. In this way, an identical series of 20 cases was evaluated independently for immunohistochemical and FISH detection of HER2 expression. Of the 10 participating, 8 institutions performed HER2 testing by immunohistochemical analysis (HercepTest, DakoCytomation, Carpinteria, CA) and FISH (PathVysion, Vysis, Downers Grove, IL) analyses; 2 participants performed immunohistochemical analysis only.

Study Design and Data Analysis

This study was designed to examine interinstitutional consistency (study I) and interobserver consistency (study II) in the analysis of tissue samples. Sample selection and distribution of sections for study I was described in the preceding section, with the evaluated results analyzed by the study coordinator. For study II, the goal was to examine interobserver consistency. Seven pathologists each evaluated one set of 20 cases that were stained at the Tokai University School of Medicine, the same set of 20 cases evaluated in study I. To evaluate the significance of the assessment system proposed by the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines for interobserver consistency in immunohistochemical analysis, evaluation systems described in the manufacturer's protocol (study IIA) and those of the ASCO/CAP guideline were compared (study IIB).

The results were estimated by concordance rate and κ value. A κ value of more than 0.75 represented excellent agreement, values from 0.4 to 0.75 represented fair to good agreement, and values less than 0.4 represented poor agreement beyond chance. Concordance rates between the coordinating laboratory and participating Japanese laboratories and between the HERA laboratory and the Japanese laboratories were evaluated.

Immunohistochemical Analysis and FISH

Immunohistochemical detection kits for HER2 containing the same lot of polyclonal antibody (HercepTest) were distributed to all participating laboratories. Sections were stained according to the manufacturer's protocols. Staining results were evaluated using the criteria 0, 1+, 2+, and 3+ according to the HercepTest kit instructions, which were the standardized criteria at the time, for studies I and IIA **Image 1**. In study IIB, the ASCO/CAP guidelines were used as the staining criteria.[21]

For FISH detection of HER2, HER2/CEP17 probe kits (Vysis) were distributed and used according to the manufacturer's protocol. Signal numbers for the *HER2* gene (labeled with SpectrumOrange, Vysis) and the *CEP17* gene (labeled with SpectrumGreen, Vysis) were counted in more than 20 tumor cells from each site, and the ratio of the HER2/CEP17 signal numbers was calculated. The results were interpreted as positive when the signal ratio of HER2/CEP17 was equal or greater than 2.0 and negative when it was less than 2.0 according to the manufacturer's protocol.

Results

Interinstitutional Consistency

The results of study I are shown in **Table 1** and **Table 2**. Of 20 samples analyzed immunohistochemically, 14 (70%) tumors were grouped in the same category when the results were categorized into 2 groups as 0, 1+/2+ and 3+; and 8 (40%) of 20 scores were consistent when the results were categorized as negative (0, 1+), equivocal (2+), and positive (3+) (Table 1). The recorded immunohistochemical results were in good agreement between the participating Japanese laboratories and the coordinating laboratory (κ = 0.718), and agreement was also good between participants and the HERA laboratory (institution E in Table 1) (κ = 0.713). For FISH analyses, results for 17 (85%) of 20 samples were consistent for all participants (Table 2). Discrepancies in results were mainly observed for samples with a HER2/CEP17 signal ratio close to 2.0. The FISH results were in excellent agreement between participants and the coordinator (κ = 0.900) and between participants and the HERA laboratory (institution E in Table 2) (κ = 0.887).

FISH Retesting for Cases Immunohistochemically Scored as 2+ Increased Agreement

To verify the algorithm for "Indication of Trastuzumab and HER2 Testing,"[22] simulation analyses were performed. Initially, analysis of study I was conducted to determine whether retesting of FISH for cases scored immunohistochemically as 2+ would improve agreement. There were 11 cases that were determined to be 2+ by at least 1 institution. When these cases were retested by FISH, agreement in scoring improved in 8 of the 11 cases. In the remaining 3 cases, scores were lowered or unchanged **Table 3**. A second analysis was used to validate whether the 2 trees of algorithms are acceptable from the perspective of clinical benefit. According to the distribution of results organized into the FISH tree **Figure 2**, 46.7% of cases had indications for trastuzumab treatment. Based on the distribution of results into the immunohistochemical analysis tree (Figure 2), 45.6% had indications for trastuzumab treatment. Retesting samples by FISH closed the gap further, demonstrating that

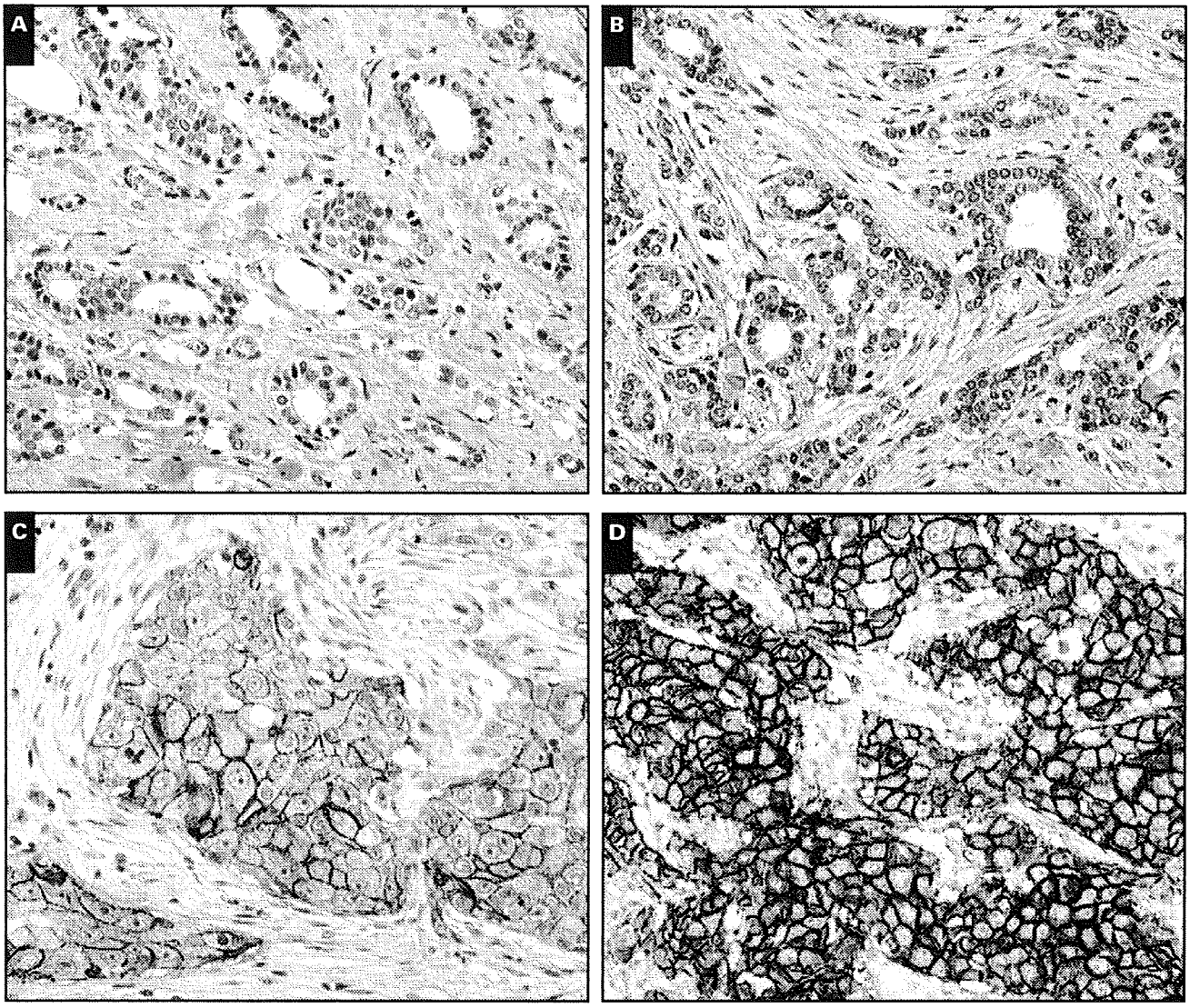

Image 1 Scoring criteria for immunohistochemical examination. **A**, Carcinoma cells lack positive reactivity for HER2 protein (score 0; ×100). **B**, A weakly positive reaction is found, but they are not completely bound to the cell membrane (score 1+; ×100). **C**, Weak to moderate, complete membrane staining is detected in >10% of tumor cells (score 2+; ×100). **D**, Strong, complete membrane staining is observed in >10% of tumor cells (score 3+ in studies I and IIA) and in >30% of tumor cells (score 3+ in study IIB) (×100).

retesting by using FISH can minimize the prescription of treatment that will have no effect or too much of an effect, depending on the type of cancer present.

Interobserver Discrepancies and Their Causes

To analyze the cause of discrepancies that occur in evaluating the pathology of tissue samples, interobserver consistency was examined in study II. By using the evaluations provided by each of the 7 pathologists on the Japanese Pathology Board for Optimal Use of Trastuzumab for 1 set of the 20 cases, in which the variable factor of staining procedures had been excluded, we examined interobserver discrepancy. Nine of the evaluations were inconsistent, with discrepancies between immunohistochemical scores of 2+ and 3+ in 2 samples (cases 4 and 6) **Image 2** and between immunohistochemical scores of 2+ and 1+ in 7 samples (cases 3, 5, 7, 9, 10, 18, and 15; study IIA) **Table 4**. The analysis of discrepancies from study I suggested that a complexity of factors, including interobserver diversity and staining procedures (Table 4, study I), accounted for the differences. However, of the 12 samples in which the results were not consistent in study I, 3 (25%) showed complete agreement in study IIA, and interinstitutional concordance was lower in study I. We hypothesize that

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Table 1
Analysis of Interinstitutional Concordance by Immunohistochemical Scoring Results*

Case No.	Coordinator	Institution										Concordance (%)
		A	B	C	D	E	F	G	H	I	J	
11	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	100
12	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	100
13	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	100
19	3+	3+	3+	3+	3+	3+	2+	3+	3+	3+	3+	91
20	3+	3+	3+	3+	3+	3+	2+	3+	3+	3+	3+	91
4	3+	2+	3+	2+	3+	2+	2+	3+	2+	2+	2+	64
8	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	2+	91
6	2+	3+	3+	3+	2+	3+	2+	2+	2+	2+	2+	64
5	2+	2+	2+	3+	3+	2+	2+	2+	2+	2+	2+	82
7	2+	2+	1+	2+	2+	2+	2+	1+	2+	2+	1+	73
9	2+	2+	2+	2+	2+	2+	2+	1+	2+	2+	1+	82
10	2+	1+	1+	2+	0	2+	1+	1+	2+	1+	1+	64
18	1+	2+	1+	2+	2+	2+	1+	1+	1+	2+	1+	55
15	1+	1+	1+	2+	1+	1+	1+	1+	2+	2+	1+	73
3	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	0	100
16	1+	1+	1+	2+	0	1+	0	1+	1+	1+	0	91
2	1+	1+	1+	0	1+	1+	0	1+	1+	0	0	100
14	1+	1+	1+	1+	0	1+	0	0	0	0	0	100
1	0	0	1+	0	0	0	1+	1+	0	0	0	100
17	0	0	0	0	0	1+	0	0	0	0	0	100

* 0 and 1+ were considered negative; 2+, equivocal; and 3+, positive.

Table 2
Analysis of Interinstitutional Concordance by Fluorescence In Situ Hybridization Results*

Case No.	Coordinator	Institution								Concordance (%)
		A	B	C	D	E	F	G	H	
11	7.1	7.4	3.8	18.7	8.3	4.9	10.0	6.2	7.3	100
12	4.6	9.4	4.5	13.9	4.4	4.6	7.8	4.2	6.8	100
13	7.3	9.8	9.6	14.1	8.4	7.4	10.3	4.2	5.2	100
19	6.7	6.3	8.1	11.5	3.9	5.5	8.0	13.3	6.2	100
20	6.6	7.7	5.9	7.4	5.4	5.6	6.7	4.5	4.5	100
4	6.1	8.9	4.7	10.7	5.6	5.2	7.9	3.9	5.3	100
8	3.2	4.9	2.7	12.5	2.4	5.0	4.4	5.4	2.7	100
6	6.5	4.4	4.4	20.9	5.4	3.9	4.6	3.9	2.7	100
5	2.5	4.1	3.5	24.7	2.5	2.6	1.6	1.6	1.9	67
7	2.5	2.2	2.1	1.8	1.9	1.3	2.6	3.0	1.8	56
9	1.3	1.2	1.3	1.5	1.5	1.0	1.5	1.2	1.5	100
10	1.6	1.2	1.4	2.0	1.2	1.0	1.0	1.4	1.3	89
18	1.2	1.2	1.2	1.0	1.2	1.2	1.7	1.0	1.1	100
15	1.1	1.3	1.3	1.0	1.0	0.9	1.4	1.4	1.0	100
3	1.3	0.9	1.2	1.0	1.3	1.0	1.4	1.5	1.2	100
16	1.4	1.2	1.0	1.1	1.2	1.1	1.2	1.5	1.1	100
2	1.8	1.3	1.0	1.0	1.5	1.1	1.1	1.0	1.2	100
14	1.9	1.4	1.1	1.1	1.0	1.2	1.3	1.1	1.1	100
1	1.2	1.4	1.2	1.0	1.4	1.1	1.9	1.2	0.9	100
17	1.1	1.2	1.1	1.0	1.0	1.1	1.1	1.1	0.9	100

* Data are given as the HER2/CEP17 ratio. A ratio <2.0 was considered negative, and a ratio ≥2.0, positive.

staining procedures were the cause. In 4 of 12 cases, the interobserver discrepancy present in study IIA was identical to the concordance found in study I. For these cases, interobserver discrepancy was considered to be the cause. In the other 5 of 12 cases, both interobserver and interinstitutional discrepancies suggest that staining procedures and interobserver discrepancies are possible causes.

Significance of ASCO/CAP Guideline

To study the significance of the ASCO/CAP interpretive criteria, we conducted study IIB. The variable factor is the only evaluation system in study II; therefore, we can analyze the significance of the proposed criteria in comparison with previous criteria and the ASCO/CAP guidelines while excluding the influences of staining procedures. Revised

Table 3
Analysis of FISH Retesting for Samples With Immunohistochemical Scores of 2+*

			Institution											
	Coordinator		A		B		C		D		E		F	
Case No.	IHC	FISH	IHC	FISH	IHC	FISH	IHC	FISH	IHC	FISH	IHC	FISH	IHC	FISH
11	3+	7.1	3+	7.4	3+	3.8	3+	18.7	3+	8.3	3+	4.9	3+	10.0
12	3+	4.6	3+	9.4	3+	4.5	3+	13.9	3+	4.4	3+	4.6	3+	7.8
13	3+	7.3	3+	9.8	3+	9.6	3+	14.1	3+	8.4	3+	7.4	3+	10.3
19	3+	6.7	3+	6.3	3+	8.1	3+	11.5	3+	3.9	3+	5.5	2+	8.0
20	3+	6.6	3+	7.7	3+	5.9	3+	7.4	3+	5.4	3+	5.6	2+	6.7
4	3+	6.1	2+	8.9	3+	4.7	2+	10.7	3+	5.6	2+	5.2	2+	7.9
8	3+	3.2	3+	4.9	3+	2.7	3+	12.5	3+	2.4	3+	5.0	3+	4.4
6	2+	6.5	3+	4.4	3+	4.4	3+	20.9	2+	5.4	3+	3.9	2+	4.6
5	2+	2.5	2+	4.1	2+	3.5	3+	24.7	3+	2.5	2+	2.6	2+	1.6
7	2+	2.5	2+	2.2	1+	2.1	2+	1.8	2+	1.9	2+	1.3	2+	2.6
9	2+	1.3	2+	1.2	2+	1.3	2+	1.5	2+	1.5	2+	1.0	2+	1.5
10	2+	1.6	1+	1.2	1+	1.4	2+	2.0	0	1.2	2+	1.0	1+	1.0
18	1+	1.2	2+	1.2	1+	1.2	2+	1.0	2+	1.2	2+	1.2	1+	1.7
15	1+	1.1	1+	1.3	1+	1.3	2+	1.0	1+	1.0	1+	0.9	1+	1.4
3	1+	1.3	1+	0.9	1+	1.2	1+	1.0	1+	1.3	1+	1.0	1+	1.4
16	1+	1.4	1+	1.2	1+	1.0	2+	1.1	0	1.2	1+	1.1	0	1.2
2	1+	1.8	1+	1.3	1+	1.0	0	1.0	1+	1.5	1+	1.1	0	1.1
14	1+	1.9	1+	1.4	1+	1.1	1+	1.1	0	1.0	1+	1.2	0	1.3
1	0	1.2	0	1.4	1+	1.2	0	1.0	0	1.4	0	1.1	1+	1.9
17	0	1.1	0	1.2	0	1.1	0	1.0	0	1.0	1+	1.1	0	1.1

FISH, fluorescence in situ hybridization; IHC, immunohistochemical analysis.
* Data are given for IHC as immunohistochemical scores (0 and 1+, negative; 2+, equivocal; 3+, positive) and for FISH as the HER2/CEP17 ratio (<2.0, negative; ≥2.0, positive).

assessments by at least 1 pathologist were detected in 3 cases **Table 5**. As a result, concordance between sample designations increased, decreased, and remained unchanged for the 3 cases, respectively. It is noteworthy that most of the changes were from an immunohistochemical score of 3+ to 2+.

The FISH results were reviewed according to the ASCO/CAP criteria, and interpretations of equivocal (1.8-2.2) were frequently identified in discrepant cases according to manufacturer's criteria (cases 5 and 7).

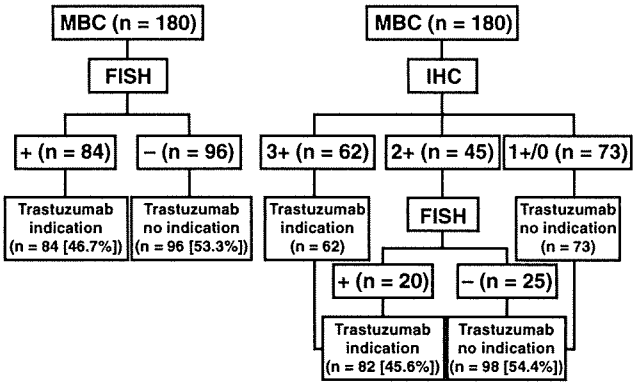

Figure 2 Simulation analysis for retesting fluorescence in situ hybridization (FISH) samples for immunohistochemical analysis (IHC) scores of 2+ according to the HER2 testing algorithm. MBC, metastatic breast cancer.

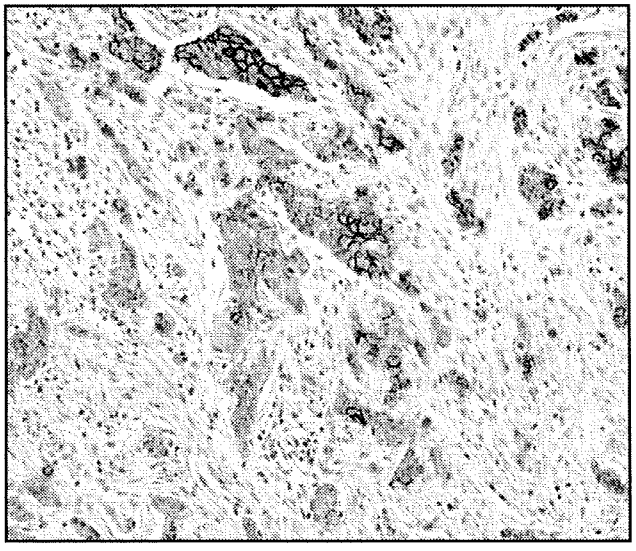

Image 2 (Case 4) Immunohistochemical features. Tumor cells with varied intensity for HER2 protein are heterogeneously distributed (×100).

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Institution					
G		H		Concordance (%)	
IHC	FISH	IHC	FISH	IHC	ICH2+/FISH
3+	6.2	3+	7.3	100	
3+	4.2	3+	6.8	100	
3+	4.2	3+	5.2	100	
3+	13.3	3+	6.2	89	100
3+	4.5	3+	4.5	89	100
3+	3.9	2+	5.3	56	100
3+	5.4	3+	2.7	100	
2+	3.9	2+	2.7	56	100
2+	1.6	2+	1.9	78	67
1+	3.0	2+	1.8	78	67
1+	1.2	2+	1.5	89	100
1+	1.4	2+	1.3	56	89
1+	1.0	1+	1.1	56	100
1+	1.4	2+	1.0	78	
1+	1.5	1+	1.2	100	89
1+	1.5	1+	1.1	89	100
1+	1.0	1+	1.2	100	
0	1.1	0	1.1	100	
1+	1.2	0	0.9	100	
0	1.1	0	0.9	100	

Discussion

Accuracy in HER2 testing is very important for the treatment of patients. Large clinical trials such as the North Central Cancer Treatment Group, National Surgical Adjuvant Breast and Bowel Project, and HERA require a standardization of HER2 testing, thus emphasizing global quality control. The present Japanese ring study demonstrates good agreement for immunohistochemical detection of HER2 and excellent agreement for HER2 detection using FISH despite a higher proportion of equivocal ratings (2+). Agreement levels between participants and the coordinator (κ values for immunohistochemical analysis and FISH of 0.718 and 0.900, respectively) and the HERA laboratory (κ values for immunohistochemical analysis and FISH of 0.713 and 0.887, respectively) were almost identical.

In the present study, an attempt was made to exclude variable factors in the technical procedures by use of a detection kit containing the same lot of antibody for immunohistochemical analysis and FISH probes and also the use of the same protocols. However, the tissue processing (eg, fixation of tumor samples, absorbance, tissue embedding) before immunohistochemical analysis was conducted in different laboratories in different countries and was not controlled. Therefore, tissue preparation variables, if present, were maintained in this study. Irrespective of these variable regional factors, however, agreement was obtained during analysis of pathology between laboratories in Japan and Germany. In a previous study, Dowsett et al[20] conducted an international ring study with 5 participants from different countries—the Netherlands, Canada, France, Belgium, and Germany—using 20 sets for immunohistochemical analysis and FISH. The

Table 4
Interobserver Discrepancies in Immunohistochemical Results and Causes of Discrepant Results*

	Study I								Study II							
		Institution								Institution						
Case No.	Coordinator	A	D	F	G	I	J	Concordance (%)	Coordinator	A	D	F	G	I	J	Concordance (%)
11	3+	3+	3+	3+	3+	3+	3+	100	3+	3+	3+	3+	3+	3+	3+	100
12	3+	3+	3+	3+	3+	3+	3+	100	3+	3+	3+	3+	3+	3+	3+	100
13	3+	3+	3+	3+	3+	3+	3+	100	3+	3+	3+	3+	3+	3+	3+	100
19	3+	3+	3+	2+	3+	3+	3+	86†	3+	3+	3+	3+	3+	3+	3+	100†
20	3+	3+	3+	2+	3+	3+	3+	86†	3+	3+	3+	3+	3+	3+	3+	100†
4	3+	2+	3+	2+	3+	2+	2+	57‡	3+	2+	2+	3+	2+	2+	3+	57‡
8	3+	3+	3+	3+	3+	3+	2+	86†	3+	3+	3+	3+	3+	3+	3+	100†
6	2+	3+	2+	2+	2+	2+	2+	86‡	2+	2+	2+	2+	2+	2+	3+	86‡
5	2+	2+	3+	2+	2+	2+	2+	86‡	2+	2+	1+	2+	2+	2+	2+	86‡
7	2+	2+	2+	2+	1+	2+	1+	71§	2+	1+	2+	2+	2+	2+	2+	86§
9	2+	2+	2+	2+	1+	2+	1+	71§	2+	2+	2+	2+	1+	2+	2+	86§
10	2+	1+	0	1+	1+	1+	1+	86§	2+	2+	1+	2+	1+	2+	2+	71§
18	1+	2+	2+	1+	1+	2+	1+	57§	1+	1+	1+	1+	1+	1+	2+	86§
15	1+	1+	1+	1+	1+	2+	1+	86‡	1+	2+	1+	1+	1+	1+	1+	86‡
3	1+	1+	1+	1+	1+	1+	0	100§	1+	1+	1+	1+	1+	1+	2+	86§
16	1+	1+	0	0	1+	1+	0	100	1+	1+	1+	1+	0	0	1+	100
2	1+	1+	1+	0	1+	0	0	100	1+	0+	0	0	0	0	0	100
14	1+	1+	0	0	0	0	0	100	1+	1+	1+	1+	0	0	1+	100
1	0	0	0	1+	1+	0	0	100	0	0	0	0	0	0	0	100
17	0	0	0	0	0	0	0	100	0	0	0	0	0	0	0	100

* Data are given as immunohistochemical scores (0 and 1+, negative; 2+, equivocal; 3+, positive).
† Complete agreement in interobserver consistency in study II, with lower concordance in study I.
‡ Identical concordance between studies I and II.
§ Different concordance between studies I and II.

Table 5
Significance of Assessment According to the ASCO/CAP Guidelines*

Case No.	Coordinator	Institution						Concordance (%)
		A	D	F	G	I	J	
Study IIA								
4	3+	2+	2+	3+	2+	2+	3+§	57†
8	3+	3+	3+§	3+	3+	3+	3+§	100‡
6	2+	2+	2+	2+	2+	2+	3+§	86
Study IIB								
4	3+	2+	2+	3+	2+	2+	2+§	71†
8	3+	3+	2+§	3+	3+	3+	2+§	71‡
6	2+	2+	2+	3+	2+	2+	2+§	86

ASCO/CAP, American Society of Clinical Oncology/College of American Pathologists.
* For study IIA, immunohistochemical staining results were evaluated by using the criteria 0, 1+, 2+, and 3+ (0 and 1+, negative; 2+, equivocal; 3+, positive) according to the HercepTest kit instructions. In study IIB, the ASCO/CAP guidelines were used as the evaluation criteria. According to the ASCO/CAP guidelines, the concordance rate was increased† or decreased,‡ and the interpretations from 3+ to 2+ are increased.§

concordance rate for immunohistochemical analysis was 45% (9/20) in categories of negative, equivocal, and positive; and for FISH, the rate was 80% (16/20). In our Japanese ring study, despite the increased number of participants (11 including the coordinator), the concordance rate was similar to that in the study by Dowsett et al.[20]

The goal for this study was to identify causes of discrepancies in HER2 detection in breast cancer samples. By using interinstitutional and interobserver conditions for analysis of the same series of tumors, we tried to pinpoint factors that contribute to discrepant results. Interinstitutional discrepancies in immunohistochemical analysis were identified in 6 samples categorized as 2+ and 3+ and also in 5 samples categorized as 2+ and 1+. In both cases, discrepancies were related to technical and evaluation methods. In these 12 samples, interobserver study showed that 3 samples (3/12 [25%]) were 100% consistent when the pathologists evaluated sections stained by the same method. Based on these conclusions, discrepancies in results from the interinstitutional study were assumed to be related to tissue processing and staining procedures. Interobserver diversities were identified in 4 samples (4/12 [33%]), and the percentage of discord in the interinstitutional study was the same. Thus, it was assumed that interobserver diversity was the major cause of subsequent discrepancies. The remaining 5 samples (5/12 [42%]) were discordant owing to complex causes of technology and evaluation because interobserver discrepancy was present, and the interinstitutional concordance rate was lower or higher than the interobserver concordance rate. Table 4 shows that the staining procedure was most frequently identified as the cause of discrepancy between cases scored immunohistochemically as 2+ vs 3+, and that technical methods and interobserver diversity were more frequently identified for differences between cases scored immunohistochemically as 1+ vs 2+.

Discrepancies between immunohistochemical evaluations of 1+ and 2+ are clinically critical; therefore, assessment of both staining procedures and evaluation methods should be well controlled. To our knowledge, this is the first ring study designed to clarify the cause of discrepancies in HER2 analysis by immunohistochemical analysis and FISH by minimizing variable factors.

The ASCO/CAP "Guideline for HER2 Testing in Breast Cancer"[21] recently proposed the category of "equivocal" for tumors identified with an immunohistochemical designation of 2+ and a FISH ratio of 1.8 to 2.2. For these samples, reexamination by FISH is recommended. In addition, the criterion for immunohistochemical results of 3+ was redefined "as uniform intense membrane staining of >30% of invasive tumor cells." The present study IIB clearly showed that the new definition increased the proportion of cases designated immunohistochemically as 2+, which would be subsequently examined by FISH according to the ASCO/CAP guidelines. As shown in the simulation analysis, retesting by FISH of samples scored 2+ immunohistochemically increased the concordance rate. Thus, the currently proposed ASCO/CAP guideline can improve evaluation consistency among multiple institutions and provide more reliable identification of the most appropriate patients for trastuzumab treatment.

We assessed the quality and consistency of HER2 testing performed by laboratories in Japan that are responsible for evaluating approximately 80% of breast cancer tissue samples submitted for pathology studies. We found good to excellent agreement among the participants and in comparison with results from a HERA laboratory in Germany. This is the first ring study to evaluate the causes of discrepancies in analysis of breast cancer pathology with regard to HER2 expression by comparing interinstitutional and interobserver results with an effort to minimize technical variables.

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Predictive implications of nucleoside metabolizing enzymes in premenopausal women with node-positive primary breast cancer who were randomly assigned to receive tamoxifen alone or tamoxifen plus tegafur-uracil as adjuvant therapy

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Abstract. Recent studies have demonstrated that tegafur-uracil (UFT) is useful for the adjuvant treatment of various types of cancers. To determine whether nucleoside metabolizing enzymes could be used to predict the response to UFT treatment in women with primary breast cancer, we retrospectively analyzed archived tumor tissue samples obtained from the 3rd Adjuvant Chemo-Endocrine Therapy for Breast Cancer (ACETBC) study, in which adjuvant treatment with tamoxifen (TAM) plus UFT for 2 years was compared with TAM alone for 2 years. Samples of tumor tissue were obtained from 192 premenopausal women with node-positive invasive breast cancer. The tissue samples were examined immunohistochemically to study the expression of thymidylate synthase (TS), thymidine phosphorylase (TP), and dihydropyrimidine dehydrogenase (DPD), as well as the expression of HER2 and p53. In patients with TS-positive tumors, the risk of relapse was significantly lower in the tamoxifen plus UFT group than in the tamoxifen alone group. After 2 years, however, there was a trend towards a decrease in the relative predictive value (RPV) of TS with time. No relationship to outcome was detected for TP or DPD. Expression of HER2 or p53 was a significant prognostic indicator in the tamoxifen alone group. TS, but not TP or DPD, may be a useful predictor of response to UFT therapy. After 2 years, the RPV of TS decreased with time, suggesting that 2 years of treatment with oral fluorouracil derivatives may be inadequate. Further studies are required to investigate this possibility.

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Key words: tegafur-uracil, predictive factor, thymidylate synthase, thymidine phosphorylase, dihydropyrimidine dehydrogenase, breast cancer

Introduction

UFT is an oral formulation combining tegafur, a prodrug of 5-fluorouracil, with uracil, an inhibitor of dihydropyrimidine dehydrogenase (DPD), the rate-limiting enzyme governing the metabolism of 5-fluorouracil. Recently, many studies have demonstrated that adjuvant treatment with tegafur-uracil (UFT) is effective against lung cancer and other types of solid tumors (1-4). In breast cancer, the therapeutic usefulness of adjuvant chemotherapy with tegafur preparations has been studied in Japan and other countries for more than 20 years (5,6). Recently, Noguchi *et al* (7) reported the results of a pooled analysis of 6 randomized clinical trials in women with node-negative breast cancer. Their analysis demonstrated that survival was significantly longer in patients who received UFT than in those who did not. In addition, the effects of combined treatment with UFT and tamoxifen were found to be additive. These findings suggested that UFT may be useful for the management of primary breast cancer, although controlled studies with commonly used regimens for polychemotherapy, such as anthracycline plus cyclophosphamide (AC) and cyclophosphamide plus methotrexate plus fluorouracil (CMF), have yet to be reported.

Recent studies have shown that S-1, a combination of tegafur and 5-chloro-2,4-dihydropyrimidine (CDHP), a more potent inhibitor of DPD than uracil, has high antitumor activity against metastatic breast cancer (8). Other studies with 5-fluorouracil derivatives have demonstrated that combined treatment with capecitabine and docetaxel significantly prolongs survival among women with anthracycline-resistant breast cancer, as compared with docetaxel alone (9). Various