Table 3 Paired Ki67 data before and after letrozole/tamoxifen therapy according to aromatase protein expression status in breast cancer cells/stromal cells

Ki67 [Geometric mean (95% CI)][a]	Aromatase protein expression			
	Cancer cells		Stromal cells	
	Negative	Positive	Negative	Positive
Letrozole only				
Pre	5.54 (2.54–12.08)	3.56 (2.47–5.14)	3.64 (1.70–7.82)	3.92 (2.69–5.72)
Post	0.70 (0.33–1.49)	0.49 (0.31–0.75)	0.88 (0.38–2.04)	0.44 (0.29–0.68)
P-value[b]	0.0037	0.0001	0.0083	0.0001
Tamoxifen only				
Pre	5.97 (3.17–11.24)	5.63 (4.18–7.58)	7.75 (4.46–13.47)	4.67 (3.39–6.44)
Post	1.72 (0.75–3.97)	1.36 (0.88–2.09)	1.61 (0.76–3.39)	1.23 (0.77–1.95)
P-value[b]	0.0117	0.0001	0.0007	0.0001

[a] 95% CI: confidence Interval

[b] Wilcoxon signed rank test was used to compare paired Ki67 data within each group defined by aromatase expression status

major reason for this is that the low abundance of aromatase in the breast requires sophisticated, time-consuming and labor intensive methodology and relatively large amounts of fresh tissue. This has precluded routine use in large clinical trials. However the availability of an antibody which can specifically detect aromatase in fixed archival breast cancers has changed this. We can now report results on the presence (and semi-quantitative levels) of aromatase in tumor material obtained from a randomized trial of neoadjuvant endocrine therapy (P024).

Before discussing the findings it is worth considering methodological issues and potential limitations of the study. Firstly, IHC estimation of protein provides no information on activity and protein may be present that is deactivated or inhibited [20]. This certainly will be the case in patients treated with aromatase inhibitors. For this reason we have excluded outcome correlations with aromatase status in "on treatment" samples because we have not validated relationships between aromatase activity and expression in the presence of an endocrine agent. Secondly, because aromatase is present in different compartments of the breast (and at different levels) complete assessment requires quantification of multiple tissue types and an estimate of the relative amounts of each compartment. In this study, to simplify these confounders, we have not used assessments in adipose and benign tissue of tissue sections which were generally low in staining score and proportion. Aromatase scores were highest in the malignant and stromal compartments of breast cancers. However, these were highly related in breast cancers suggesting a field effect of trophic factors regulating aromatase. We have therefore restricted our correlations to the status of the cancer cells which were reliably present in all the samples eligible for analysis and therefore more consistent to score.

In terms of demographics we have combined the two arms of the P024 trial for long term outcome analysis, not discriminating between patients subsequently treated with tamoxifen or letrozole since all patients received tamoxifen as adjuvant therapy. This has formed a database which represents the largest published series of breast cancers assessed by aromatase IHC. The results show that tumor aromatase was positively and significantly related to smaller tumor size and ER level/status. These findings would be consistent with data published by members of the group on aromatase activity [4] but not with others using IHC with a different antibody [7]. A significant inverse correlation was observed with the proliferation marker, Ki67. To the best of our knowledge there have been no other published studies relating tumor aromatase to proliferation.

In terms of endocrine responsiveness, no significant association was detected between tumor aromatase and clinical response to either letrozole or tamoxifen. While positive correlations have been reported between the presence of in vitro and in vivo aromatase activity and response to aromatase inhibitors, these relationships were not strong and were observed in advanced disease, not in the neoadjuvant setting [18, 19] Other studies on response to tamoxifen have been negative. Thus, the response to endocrine therapy does not appear to be strongly modulated by whether the source of estrogen is autocrine or endocrine.

Despite a failure to observe significant relationships between aromatase expression and clinical or biomarker response to treatment in the neoadjuvant phase of the study, significant associations were found between the presence of tumor aromatase expression and long-term outcome following neoadjuvant treatment. Thus, tumors with positive aromatase scores had significantly greater

Table 4 Univariate and multivariate analysis of pathological tumor size, node status, post-treatment Ki67, post-treatment ER and pre treatment aromatase status[a]

A

Factor definitions	No. of patients in each group	No. of events/ No. of patients	Relapse-free survival			
			Univariable analysis		Multivariate analysis	
			HR (95% CI)	P	HR (95% CI)	P
Tumor size[a] (T1/2 vs. T3/4)	138/33	47/171	2.7 (1.4–5.0)	0.002	2.82 (1.36–5.85)	0.006
Node status (Yes vs. No)	90/69	44/159	3.9 (1.8–8.4)	0.0005	3.44 (1.58–7.48)	0.002
Ki67 level, per 2.7 fold increase[b]		48/174	1.4 (1.2–1.6)	0.0002	1.1 (1.02–1.09)	0.003
ER Allred[c] (0.2 vs. 3–8)	16/157	48/173	2.4 (1.0–5.3)	0.04	2.74 (1.1–6.67)	0.03
Aromatase status[d] (not present versus present)	37/132	48/169	1.88 (1.01–3.47)	0.04	2.34 (1.2–4.58)	0.01

B

Factor definitions	No. of patients in each group	No. of events/ No. of patients	Breast cancer-specific survival			
			Univariable analysis		Multivariate analysis	
			HR (95% CI)	P	HR (95% CI)	P
Tumor size (T1/2 vs. T3/4)	138/33	24/171	3.5 (1.5–8.3)	0.004	3.42 (1.21–9.66)	0.02
Node status (Yes vs. No)	90/69	22/159	4.6 (1.4–15.8)	0.01	4.05 (1.14–14.38)	0.03
Ki67 level, per 2.7 fold increase		25/174	1.4 (1.1–1.7)	0.009	1.05 (1.0–1.11)	0.06
ER Allred (0.2 vs. 3–8)	16/157	25/173	4.3 (1.6–11.7)	0.005	7.98 (2.58–24.7)	0.0003
Aromatase status (not present versus present)	37/132	24/169	2.82 (1.2–6.63)	0.02	3.76 (1.42–9.98)	0.008

[a] The four elements of the preoperative endocrine relapse index (PEPI) score (pathological T and N stage, surgical specimen ER and Ki67 status has been previously described [14]

[b] Surgical specimen Ki67 was analyzed as the natural log interval, or per 2.7 fold increase according to the original scale of percentage values [14]

[c] The ER analysis refers to the post-treatment values, before treatment all the tumors in this data set were ER positive. In the PEPI model, an Allred cut off of 0 or 2 is used to define ER negative

[d] The aromatase expression status was defined as present or positive if any positive staining presented in invasive breast cancer cells. Table 4A and B shows the RFS data and BCSS data, respectively

RFS and BCSS. It is not possible to ascertain whether this is directly caused by increased sensitivity to endocrine therapy in the adjuvant setting. However, the lack of association of response in the neoadjuvant situation would not be compatible with this. Furthermore the positive correlations with small clinical size and ER status levels (favorable prognostic biomarkers) and the inverse correlation with Ki67 (a poor prognosis biomarker) suggest that aromatase positive tumors may be inherently less aggressive. This is supported by data from multivariable analyses in which tumor aromatase scores predicted for long-term outcome independently of other factors that have been shown to be predictive for outcome in the post neoadjuvant endocrine therapy setting. This finding also implies that the most accurate models for the prediction of outcomes for patients with ER+ disease may combine baseline prognostic biomarker analysis, in combination with the "on-treatment" predictive biomarker analysis derived from an analysis of the tumor after several months of endocrine treatment [14].

It is therefore suggested that routine IHC measurements of aromatase in breast cancer will not generally aid

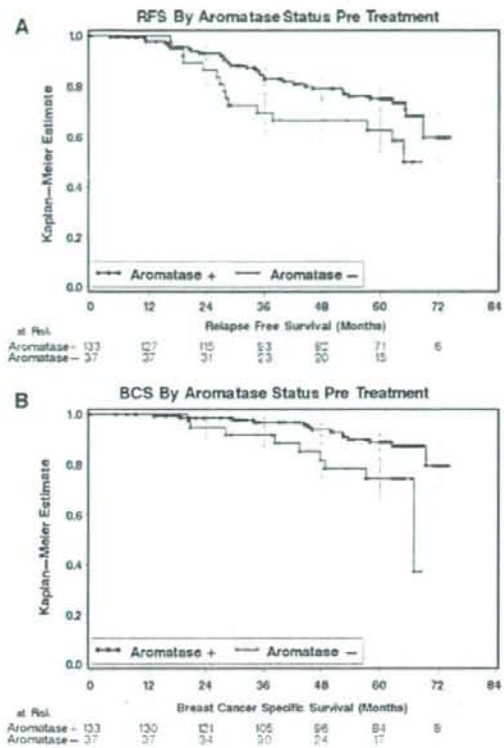


Fig. 2 Kaplan–Meier curves for relapse-free and breast cancer-specific survival by aromatase protein expression status in cancer cells. (**a**) Relapse-free survival (RFS) for patients with aromatase protein expression positive (green or upper curve) versus negative (red or lower curve) in cancer cells; (**b**) Breast cancer-specific survival (BCSS) for patients with aromatase protein expression positive (green or upper curve) versus negative (red or lower curve) in cancer cells; Censorship observations are marked with open circles; log rank tests were used to estimate the difference between Kaplan–Meier curves for RFS and BCSS. 95% confidence intervals are provided on each curve

prediction of neoadjuvant response to endocrine therapy, but may help identify ER positive tumors with favorable long-term outcomes.

References

1. Reed MJ (1994) The role of aromatase in breast tumors. Breast Cancer Res Treat 30(1):7–17. doi:10.1007/BF00682737
2. Miller WR (2006) Aromatase inhibitors and breast cancer. Minerva Endocrinol 31(1):27–46
3. Miller WR, Hawkins RA, Forrest AP (1982) Significance of aromatase activity in human breast cancer. Cancer Res 42(8 Suppl):3365s–3368s
4. Miller WR, Anderson TJ, Jack WJ (1990) Relationship between tumour aromatase activity, tumour characteristics and response to therapy. J Steroid Biochem Mol Biol 37(6):1055–1059. doi:10.1016/0960-0760(90)90465-W
5. Miller WR (1991) Aromatase activity in breast tissue. J Steroid Biochem Mol Biol 39(5B):783–790. doi:10.1016/0960-0760(91)90026-2
6. Lipton A, Santen RJ, Santner SJ, Harvey HA, Sanders SI, Matthews YL (1992) Prognostic value of breast cancer aromatase. Cancer 70(7):1951–1955. doi :10.1002/1097-0142(19921001)70:7<1951::AID-CNCR2820700723>3.0.CO;2-#
7. Esteban JM, Warsi Z, Haniu M, Hall P, Shively JE, Chen S (1992) Detection of intratumoral aromatase in breast carcinomas. An immunohistochemical study with clinicopathologic correlation. Am J Pathol 140(2):337–343
8. Miki Y, Suzuki T, Sasano H (2007) Controversies of aromatase localization in human breast cancer-stromal versus parenchymal cells. J Steroid Biochem Mol Biol 106(1–5):97–101
9. Sasano H, Anderson TJ, Silverberg SG, Santen RJ, Conway M, Edwards DP, Krause A, Bhatnagar AS, Evans DB, Miller WR (2005) The validation of new aromatase monoclonal antibodies for immunohistochemistry-a correlation with biochemical activities in 46 cases of breast cancer. J Steroid Biochem Mol Biol 95(1–5):35–39
10. Miki Y, Suzuki T, Tazawa C, Yamaguchi Y, Kitada K, Honma S et al (2007) Aromatase localization in human breast cancer tissues: possible interactions between intratumoral stromal and parenchymal cells. Cancer Res 67(8):3945–3954. doi:10.1158/0008-5472.CAN-06-3105
11. Eiermann W, Paepke S, Appfelstaedt J, Llombart-Cussac A, Eremin J, Vinholes J et al (2001) Preoperative treatment of postmenopausal breast cancer patients with letrozole: a randomized double-blind multicenter study. Ann Oncol 12(11):1527–1532. doi:10.1023/A:1013128213451
12. Ellis MJ, Coop A, Singh B, Mauriac L, Llombert-Cussac A, Janicke F et al (2001) Letrozole is more effective neoadjuvant endocrine therapy than tamoxifen for ErbB-1- and/or ErbB-2-positive, estrogen receptor-positive primary breast cancer: evidence from a phase III randomized trial. J Clin Oncol 19(18):3808–3816
13. Ellis MJ, Coop A, Singh B, Tao Y, Llombart-Cussac A, Janicke F et al (2003) Letrozole inhibits tumor proliferation more effectively than tamoxifen independent of HER1/2 expression status. Cancer Res 63(19):6523–6531
14. Ellis MJ, Tao Y, Luo J, A'Hern R, Evans DB, Bhatnagar AJ et al (2008) Outcome prediction for estrogen receptor-positive breast cancer based on post neoadjuvant endocrine therapy tumor characteristics. J Natl Cancer Inst 100(19): (in press)
15. Miller WR, Forrest AP (1974) Oestradiol synthesis by a human breast carcinoma. Lancet 2(7885):866–868. doi:10.1016/S0140-6736(74)91202-1
16. Reed MJ, Aherne GW, Ghilchik MW, Patel S, Chakraborty J (1991) Concentrations of oestrone and 4-hydroxyandrostenedione in malignant and normal breast tissues. Int J Cancer 49(4):562–565. doi:10.1002/ijc.2910490415
17. Miller WR (1997) Uptake and synthesis of steroid hormones by the breast. Endocr Relat Cancer 4:307–311
18. Bezwoda WR, Mansoor N, Dansey R, Esser JD (1987) Aromatisation of androstenedione by human breast cancer tissue: correlation with hormone receptor activity and possible biologic significance. Oncology 44(1):30–33
19. Miller WR, Anderson TJ, Evans DB, Krause A, Hampton G, Dixon JM (2003) An integrated view of aromatase and its inhibition. J Steroid Biochem Mol Biol 86(3–5):413–421. doi:10.1016/S0960-0760(03)00352-2
20. Miller WR, Dixon JM (2002) Endocrine and clinical endpoints of exemestane as neoadjuvant therapy. Cancer Control 9(2 Suppl):9–15

CME

What Causes Discrepancies in HER2 Testing for Breast Cancer?

A Japanese Ring Study in Conjunction With the Global Standard

Shinobu Umemura, MD, PhD,[1] R. Yoshiyuki Osamura, MD, PhD,[1] Futoshi Akiyama, MD, PhD,[2] Keiichi Honma, MD, PhD,[3] Masafumi Kurosumi, MD, PhD,[4] Hironobu Sasano, MD, PhD,[5] Satoshi Toyoshima, MD, PhD,[6] Hitoshi Tsuda, MD, PhD,[7] Josef Rüschoff, MD,[8] and Goi Sakamoto, MD, PhD[9]

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

DOI: 10.1309/AJCP5UUMFMA5ZKII

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.

The ASCP is accredited by the Accreditation Council for Continuing Medical Education to provide continuing medical education for physicians. The ASCP designates this educational activity for a maximum of 1 AMA PRA Category 1 Credit ™ per article.
The authors of this article and the planning committee members and staff have no relevant financial relationships with commercial interests to disclose.
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,

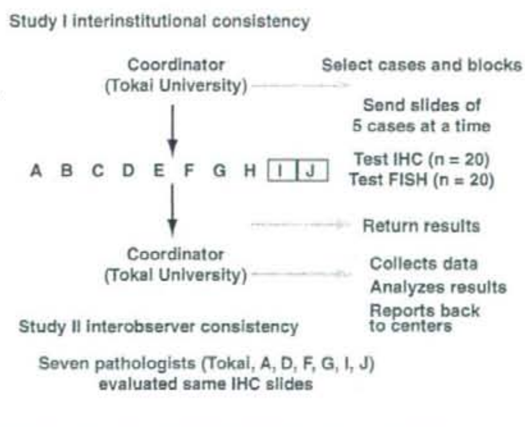


Figure 1 Study designs for studies I and II. Study I examined interinstitutional consistency, and study II examined interobserver consistency of HER2 testing. Institutions A-H participated in immunohistochemical analysis (IHC) and fluorescence in situ hybridization (FISH) analysis, and institutions I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

DOI: 10.1309/AJCP5UUMFMA5ZKII

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

DOI: 10.1309/AJCP5UUMFMA5ZKII

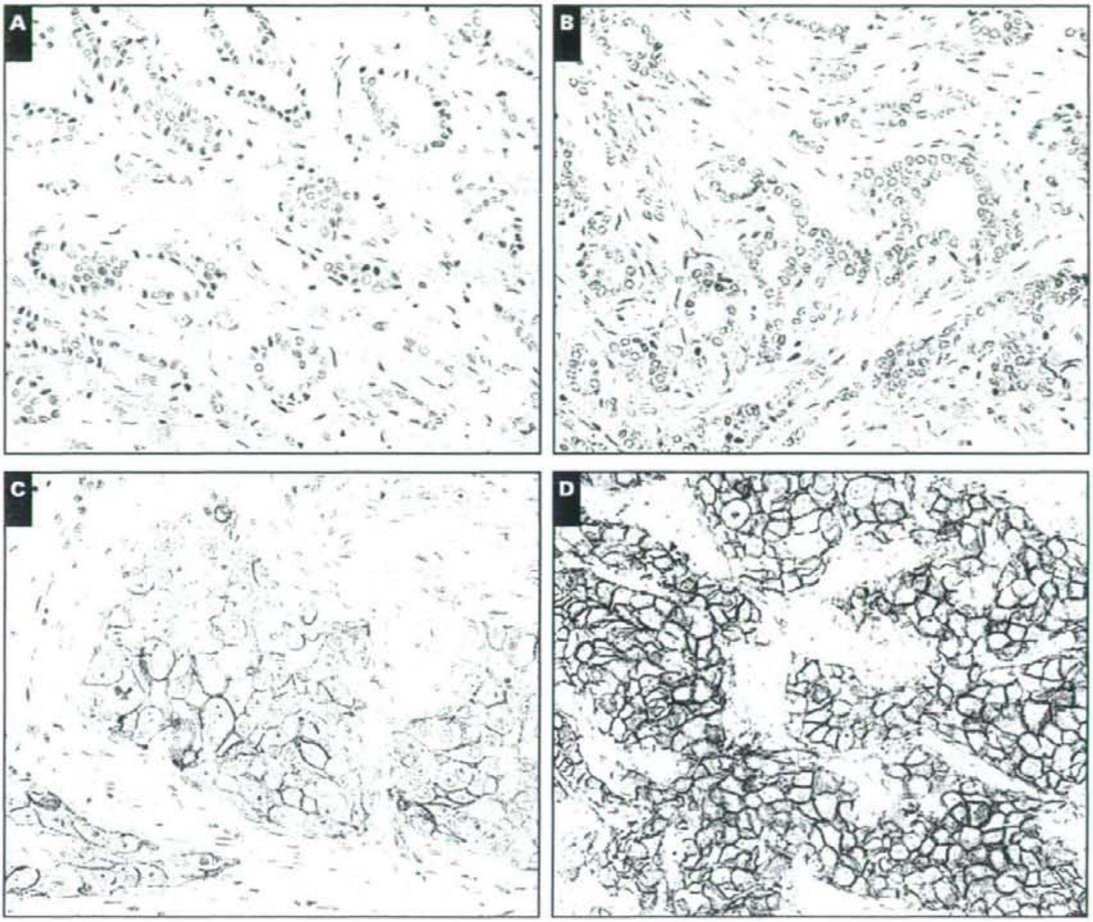


Image 1 Scoring criteria for immunohistochemical examination. **A**, Carcinoma cells lack positive reactivity for HER2 protein (score 0; ×100). **B**, A weakly positive reaction is found, but they are not completely bound to the cell membrane (score 1+; ×100). **C**, Weak to moderate, complete membrane 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

DOI: 10.1309/AJCP5UJMFMA5ZKII

Table 1
Analysis of Interinstitutional Concordance by Immunohistochemical Scoring Results*

		Institution										
Case No.	Coordinator	A	B	C	D	E	F	G	H	I	J	Concordance (%)
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*

		Institution								
Case No.	Coordinator	A	B	C	D	E	F	G	H	Concordance (%)
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).

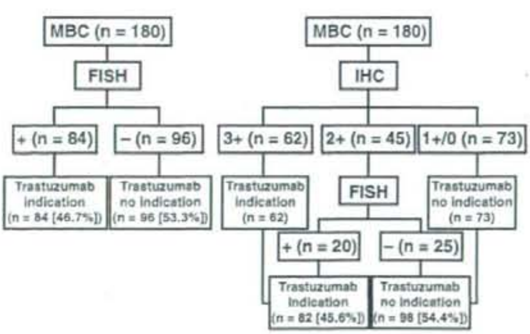


Figure 2 Simulation analysis for retesting fluorescence in situ hybridization (FISH) samples for immunohistochemical analysis (IHC) scores of 2+ according to the HER2 testing algorithm. MBC, metastatic breast cancer.

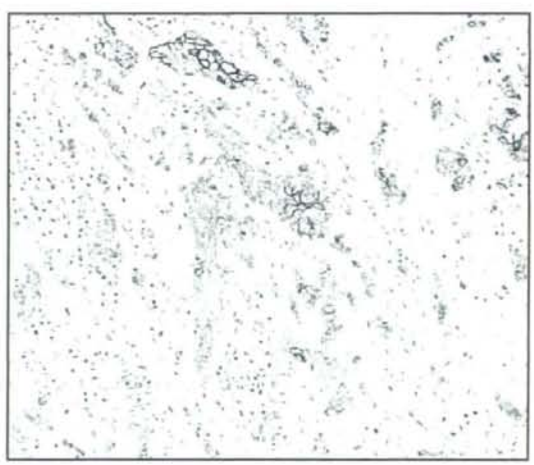


Image 2 (Case 4) Immunohistochemical features. Tumor cells with varied intensity for HER2 protein are heterogeneously distributed (×100).

DOI: 10.1309/AJCP5UUMFMA5ZKII

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.

DOI: 10.1309/AJCPSUUMFMA5ZKII

From the [1]Department of Pathology, Tokai University School of Medicine, Isehara, Japan; [2]Division of Pathology, The Cancer Institute Japanese Foundation for Cancer Research, Koto; [3]Division of Pathology, Niigata Cancer Center Hospital, Niigata, Japan; [4]Department of Pathology, Saitama Cancer Center, Kita-adachi, Japan; [5]Department of Pathology, Tohoku University School of Medicine, Sendai, Japan; [6]Division of Pathology, Kitakyushu Municipal Medical Center, Kitakyushu, Japan; [7]Department of Basic Pathology, National Defense Medical College, Tokorozawa, Japan; [8]Department of Pathology, Institute of Pathology Nordhessen, Kassel, Germany; and [9]Department of Pathology, Sakamoto Memorial Clinic Academy of Breast Pathology, Shinjuku, Japan.

Supported by Chugai Pharmaceutical, Chuo, Japan; DakoCytomation, Bunkyo, Japan; Abbott Japan, Minato; and SRL, Tachikawa, Japan.

Address reprint requests to Dr Umemura: Dept of Pathology, Tokai University School of Medicine, Shimokasuya 143, Isehara city, Kanagawa prefecture, Japan 259-1193.

Acknowledgments: We thank Oliver Stoss, PhD, Institut für Pathologie, Klinikum Kassel, Targos Molecular Pathology, Kassel, Germany, for support from the central laboratory of the Herceptin adjuvant trials; Yutaka Tokuda, MD, Department of Surgery, and Yukiko Kirimura, Department of Pathology, Tokai University School of Medicine, for support from the coordinator institution; Toru Watanabe, MD, Hamamatsu Oncology Center, Hamamatsu; and Masakazu Toi, MD, Department of Breast Surgery, Kyoto University Graduate School of Medicine, for their clinical advice.

References

1. Akiyama T, Sudo C, Ogawara H, et al. The product of the human c-erbB-2 gene: a 185-kilodalton glycoprotein with tyrosine kinase activity. *Science.* 1986;232:1644-1646.
2. Slamon DJ, Clark GM, Wong SG, et al. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. *Science.* 1987;235:177-182.
3. Press MF, Bernstein L, Thomas PA, et al. HER-2/*neu* gene amplification characterized by fluorescence in situ hybridization: poor prognosis in node-negative breast carcinomas. *J Clin Oncol.* 1997;15:2894-2904.
4. Seshadri R, Firgaira FA, Horsfall DJ, et al. Clinical significance of HER-2/*neu* oncogene amplification in primary breast cancer. The South Australian Breast Cancer Study Group. *J Clin Oncol.* 1993;11:1936-1942.
5. Leitzel K, Teramoto Y, Konrad K, et al. Elevated serum c-erbB-2 antigen levels and decreased response to hormone therapy of breast cancer. *J Clin Oncol.* 1995;13:1129-1135.
6. Yamauchi H, O'Neill A, Gelman R, et al. Prediction of response to antiestrogen therapy in advanced breast cancer patients by pretreatment circulating levels of extracellular domain of the HER-2/c-neu protein. *J Clin Oncol.* 1997;15:2518-2525.
7. Paik S, Bryant J, Park C, et al. erbB-2 and response to doxorubicin in patients with axillary lymph node-positive, hormone receptor-negative breast cancer. *J Natl Cancer Inst.* 1998;90:1361-1370.
8. Thor AD, Berry DA, Budman DR, et al. erbB-2, p53, and efficacy of adjuvant therapy in lymph node-positive breast cancer. *J Natl Cancer Inst.* 1998;90:1346-1360.
9. Konecny GE, Thomssen C, Luck HJ, et al. HER-2/*neu* gene amplification and response to paclitaxel in patients with metastatic breast cancer. *J Natl Cancer Inst.* 2004;96:1141-1151.
10. Goldhirsch A, Coates AS, Gelber RD, et al. First: select the target: better choice of adjuvant treatments for breast cancer patients. *Ann Oncol.* 2006;17:1772-1776.
11. Ellis MJ, Coop A, Singh B, et al. Letrozole is more effective neoadjuvant endocrine therapy than tamoxifen for erbB-1– and/or erbB-2–positive, estrogen receptor–positive primary breast cancer: evidence from a phase III randomized trial. *J Clin Oncol.* 2001;19:3808-3816.
12. Slamon D, Eiermann W, Robert N, et al. Phase III randomized trial comparing doxorubicin and cyclophosphamide followed by docetaxel (ACT) with doxorubicin and cyclophosphamide followed by docetaxel and trastuzumab (ACTH) with docetaxel, carboplatin and trastuzumab (TCH) in HER2 positive early breast cancer patients: BCIRG 006 study [abstract]. *Breast Cancer Res Treat.* 2005;94(suppl 1):S5. Abstract 1.
13. Joensuu H, Kellokumpu-Lehtinen PL, Bono P, et al. Adjuvant docetaxel or vinorelbine with or without trastuzumab for breast cancer. *N Engl J Med.* 2006;354:809-820.
14. Romond EH, Perez EA, Bryant J, et al. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *N Engl J Med.* 2005;353:1673-1684.
15. Smith I, Procter M, Gelber RD, et al. 2-year follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer: a randomised controlled trial. *Lancet.* 2007;369:29-36.
16. Dybdal N, Leiberman G, Anderson S, et al. Determination of *HER2* gene amplification by fluorescence in situ hybridization and concordance with the clinical trials immunohistochemical assay in women with metastatic breast cancer evaluated for treatment with trastuzumab. *Breast Cancer Res Treat.* 2005;93:3-11.
17. Vogel CL, Cobleigh MA, Tripathy D, et al. Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. *J Clin Oncol.* 2002;20:719-726.
18. Dowsett M, Bartlett J, Ellis IO, et al. Correlation between immunohistochemistry (HercepTest) and fluorescence in situ hybridization (FISH) for HER-2 in 426 breast carcinomas from 37 centers. *J Pathol.* 2003;199:418-423.
19. Yaziji H, Goldstein LC, Barry TS, et al. HER-2 testing in breast cancer using parallel tissue-based methods. *JAMA.* 2004;291:1972-1977.
20. Dowsett M, Hanna WM, Kockx M, et al. Standardisation of HER2 testing: results of an international proficiency-testing ring study. *Mod Pathol.* 2007;20:584-591.
21. Wolff AC, Hammond ME, Schwartz JN, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *Arch Pathol Lab Med.* 2007;131:19-26.
22. Umemura S, Sakamoto G, Sasano H, et al. Evaluation of HER2 status: for the treatment of metastatic breast cancers by humanized anti-HER2 monoclonal antibody (trastuzumab). Pathological Committee for Optimal Use of Trastuzumab. *Breast Cancer.* 2001;8:316-320.

DOI: 10.1309/AJCPBUUMFMA5ZKII

Vasohibin-1 in human breast carcinoma: A potential negative feedback regulator of angiogenesis

Kentaro Tamaki,[1,4,6] Takuya Moriya,[2] Yasufumi Sato,[3] Takanori Ishida,[1] Yohei Maruo,[4] Kousuke Yoshinaga,[5] Noriaki Ohuchi[1] and Hironobu Sasano[4]

[1]Department of Surgical Oncology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574; [2]Department of Pathology 2, Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama 701-0192; [3]Department of Vascular Biology, Institute of Department, Aging, and Cancer, Tohoku University, 4-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574; [4]Department of Pathology, Tohoku University Hospital, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574; [5]Department of Gynecology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574

(Received August 11, 2008/Revised September 21, 2008/Accepted September 22, 2008/Online publication November 25, 2008)

Vasohibin-1 is a recently identified negative feedback inhibitor or suppressor of angiogenesis induced by vascular endothelial growth factor (VEGF)-A. The status of vasohibin-1 in human breast carcinoma has not been examined. We examined 151 breast specimens including 98 cases of invasive ductal carcinoma (IDC), 12 of ductal carcinoma *in situ* (DCIS), 16 of fibroadenoma (FA), six of inflammatory lesion, nine of fibrocystic change and seven of non-pathological breast tissue. We immunolocalized vasohibin-1 and compared its immunoreactivity to that of VEGF-A, basic fibroblastic growth factor (bFGF), VEGF receptor 2 (Flk-1), CD31, CD34 and Ki-67/MIB-1. The correlation of vasohibin-1 immunoreactivity with overall survival (OS), and disease-free survival (DFS) of the patients with breast carcinoma was also evaluated. In addition, we evaluated Ki-67 and CD31, and Ki-67 and vasohibin-1 double-immunostaining for further characterization of neovascularization. Vasohibin-1 was detected in endothelial cells of human breast and its immunodensity was significantly higher in IDC and inflammatory lesions than the other types ($P < 0.001$). In addition, a significant positive correlation was detected between vasohibin-1 and VEGF-A, bFGF or Flk-1 ($P < 0.001$). There was also positive associations between vasohibin-1 and OS ($P = 0.004$) and between vasohibin-1 and DFS ($P \leq 0.001$) in carcinoma cases. Results of double-immunostaining demonstrated the ratio of Ki-67-positive cells among vasohibin-1-positive endothelial cells (46.5%) was significantly higher than those among CD31-positive cells (23.5%). This is the first study demonstrating the status of vasohibin-1 in human breast lesions, which indicates that vasohibin-1 is associated with neovascularization and may especially play important roles in the regulation of intratumoral angiogenesis in human breast cancer. (*Cancer Sci* 2009; 100: 88–94)

Angiogenesis or the formation of new blood vessel networks, not only plays a pivotal role in human normal development, but also in pathophysiological conditions such as inflammatory diseases and neoplasms. Angiogenesis is generally regulated by an *in situ* balance between stimulatory and inhibitory factors of angiogenesis.[1,2] However, this "angiogenic homeostasis" may be disrupted in pathological conditions such as cancer and dysregulated or excessive production and/or secretion of angiogenesis inducers result in excessive formation of abnormal blood vessels. In general, various biological phenomena in physiological conditions are under stringent control by numerous negative feedback systems as seen in endocrine mechanisms the including hypothalamic–pituitary–adrenal system to maintain their homeostasis. However, little has been known about such negative feedback mechanisms of angiogenesis in both physiological and pathological conditions.

Vasohibin-1 has been very recently identified as one of the first established negative feedback regulators of angiogenesis.[2–5] This interesting factor was identified as one of vascular endothelial growth factor (VEGF)-induced genes with anti-angiogenic properties in endothelial cells (EC) using cDNA microarray analysis.[3,4,6] Vasohibin-1 was subsequently demonstrated to be specifically expressed in EC in response to angiogenic stimulators such as VEGF and basic fibroblastic growth factor (bFGF).[3,6] Vasohibin-1 is also abundantly present in human placenta and fetus[2,3,5] in which angiogenic events markedly occur *in vivo*. VEGF-A is the most potent factor for angiogenesis among known VEGF family members, stimulating protease synthesis, migration and proliferation of EC.[7] In addition, the great majority of VEGF-A-mediated signals are transduced via VEGF receptor 2 (Flk-1)[8] and protein kinase Cδ (PKCδ), one of the signals located in important downstream intrasignaling pathway of Flk-1, and they also induced vasohibin-1 expression markedly.[4] Yoshinaga *et al.* demonstrated that the VEGF-A-mediated induction of vasohibin-1 was preferentially mediated via the Flk-1 signaling pathway in human endometrial carcinoma.[9] However, the status of vasohibin-1 in other human malignancies has not been examined in detail.

Therefore, in this study, we first immunolocalized vasohibin-1 in human breast disorders including breast cancer in order to examine whether this factor is expressed in endothelial cells or not in human breast tissues. We then correlated the findings with various clinicopathological factors of the cases including microvessel density (MVD)[10,11] in order to correlate the status of vasohibin-1 with vascularity of the lesions. We also correlated vasohibin-1 immunoreactivity with neovascularization or proliferating endothelial cells using double immunostaining of Ki-67 in order to further characterize vasohibin-1 expression and its clinical and/or biological significance in human breast disorders.

Materials and Methods

Breast tissue specimens. We retrieved 151 Japanese female cases of breast tissues from surgical pathology files of Tohoku University Hospital (Sendai, Japan). These subjects were operated on between 1995 and 1998 at the Department of Surgery, Tohoku University Hospital. The median age of the patients was 48 years (range, 15–81). The protocol for this study was approved by the Ethics Committee at Tohoku University School of Medicine (Sendai, Japan). The relevant clinicopathological information including age, histological type, stage classification, histological grade for invasive ductal carcinoma (IDC), grading scheme for ductal carcinoma *in situ* (DCIS) (van Nuys classifications[12] for DCIS and T1mic) are summarized in Table 1. Histological findings were 98 cases of IDC including eight cases of T1mic, 12 of DCIS, 16 fibroadenoma (FA), six of

[6]To whom correspondence should be addressed.
E-mail: nahanisikenta@yahoo.co.jp

doi: 10.1111/j.1349-7006.2008.01015.x

Table 1. Clinicopathological characters of examined cases

Histological type	
IDC	98
(T1mic)	8
DCIS	12
FA	16
Inflammatory lesion	6
Fibrocystic change	9
Non-pathological breast tissue	7
Age, years (range)	
All cases	48 years (15–81)
IDC	53 years (28–81)
DCIS	47 years (40–81)
FA	39 years (15–52)
Inflammatory lesion	49 years (35–70)
Fibrocystic change	47 years (39–48)
Non-pathological breast tissue	40 years (34–48)
UICC stage grouping	
Stage 0	12
Stage I	38
Stage II	36
II A	23
II B	13
Stage III	18
III A	8
III B	6
III C	4
Stage IV	6
Histological grade (for IDC)	
G 1	34
G 2	42
G 3	14
Van Nuys scheme (for DCIS and T1mic)	
Group 1	8
Group 2	12

DCIS, ductal carcinoma *in situ*; FA, fibroadenoma; IDC, invasive ductal carcinoma; UICC, International Union Against Cancer staging.

inflammatory lesion, nine of fibrocystic change and seven of non-pathological breast tissue taken from the lumpectomy specimen for breast cancer operation. In the nine cases of fibrocystic change, we evaluated vessels in the areas adjacent to adenosis or ductal hyperplasia. Stage grouping was based on *TNM Classification of Malignant Tumors Sixth Edition* by the International Union Against Cancer (UICC).[13] The tumor grade was determined according to the criteria of Elston and Ellis.[14]

Immunohistochemistry. We performed immunohistochemical staining for vasohibin-1, Flk-1, CD31, Ki-67, VEGF-A and FGF-2. The specimens had been fixed in 10% formalin, embedded in paraffin, cut into 4-µm thick sections and placed on glue-coated glass slides. Sections were deparaffinized in xylene, and hydrated with graded alcohols and distilled water. Endogenous peroxidase activity was blocked by 3% hydrogen peroxidase for 10 min at room temperature. Antigen retrieval was performed using Autoclave (TOMY SX-500 HIGH PRESSURE STEAM STERILIZER, TOMY SEIKO CO., LTD, Tokyo, Japan) in 10 nmol ethylene diamine tetra acetate (EDTA; pH 8) for vasohibin-1 and in citrate buffer for Flk-1, CD31, Ki-67 and FGF-2, heated at 121°C for 5 min, and for VEGF-A using microwave in citrate buffer for 15 min. Sections were subsequently incubated for 30 min at room temperature (RT) in a blocking solution of 10% rabbit serum (Nichirei Biosciences, Tokyo, Japan) for vasohibin-1, Flk-1, CD31, CD34 and Ki-67, and a blocking solution of 10% goat serum (Nichirei Bioscience) for VEGF-A and FGF-2, and then immunostained for 16 h at 4°C with primary antibodies. The primary antibodies of vasohibin-1, Flk-1, CD31, Ki-67, VEGF-1 were mouse monoclonal antibodies, whereas the primary antibody against FGF-2 was a rabbit polyclonal antibody, and were used as follows: antihuman vasohibin-1 monoclonal antibody[9,15] diluted at 1:3200; anti-VEGFR-2 (Flk-1; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted at 1:3200; anti-CD31 (Dako, Copenhagen, Denmark) diluted at 1:40; anti-CD34 (Nichirei Bioscience) diluted at 1:100, Ki-67 (Dako) diluted 1:300; anti-VEGF-A (Laboratory Vision, Fremont, CA, USA) diluted at 1:50; and anti-FGF-2 (Santa Cruz Biotechnology) diluted at 1:100. Antihuman vasohibin-1 monoclonal antibody (mAb) was raised against the synthetic fragment (Gly286-Arg299) of human vasohibin-1 as described by Watanabe *et al.*[3] The specificity and sensitivity of this mAb was confirmed by both western blotting and immunohistochemical analysis.[3] For vasohibin-1, Flk-1, CD31, CD34 and Ki-67 immunohistochemistry, secondary antibody reactions were performed using biotinylated rabbit antimouse antibody (Nichirei Bioscience) at a dilution of 1:100 for 30 min at RT and peroxidase-conjugated avidin (Nichirei Bioscience) was used according to the manufacturer's instructions. Envision (Dako) was used for immunostaining of VEGF-A and FGF-2. Reacted sections were visualized using 3,3′-diaminobenzidine-tetrachloride (DAB)/30% H_2O_2 in 0.05 mol/L Tris buffer (pH 7.6) and counterstained with hematoxylin–eosin (HE) for nuclear staining.

Double staining procedure. For the quantification of proliferating endothelial cells, Ki-67/CD31 and Ki-67/vasohibin-1 double-labeling immunohistochemical staining was performed. A mAb directed against Ki-67 (Dako) was diluted at 1:300 following antigen retrieval using Autoclave in a citrate buffer, and incubated for 30 min at RT in a blocking solution of 10% rabbit serum (Nichirei Bioscience). A secondary antibody reaction was performed using biotinylated rabbit antimouse antibody (Nichirei Bioscience) at a distribution of 1:100 for 30 min at RT. Peroxidase-conjugated avidin (Nichirei Bioscience) was subsequently used in this study. DAB was used to visualize the binding of the first antibody. Antigen retrieval was then performed using a microwave for 15 min in 10 nmol EDTA (pH 8) for vasohibin-1 and in a citrate buffer for CD31. The reacted sections were then incubated for 30 min with antibodies against vasohibin-1 diluted at 1:3200 and CD31 (Dako) diluted at 1:40. Following the reaction with biotinylated rabbit antimouse antibody (Nichirei Bioscience) diluted at 1:100 as a secondary antibody and alkaline phosphatase-conjugated avidin (Nichirei Bioscience), an alkaline phosphatase substrate kit III (Vector Laboratories, Burlingame, CA, USA) was employed.[16,17]

Immunohistochemical analysis. Two of the authors (K. T. and Y. M.) independently evaluated the immunohistochemical staining of the tissue sections. They were blinded to the clinical course of the patients and the average of numbers counted by the two investigators was used for subsequent analysis. We used Olympus (Tokyo, Japan) BX50 and 20X objectives for the analysis.

The number of microvessels was counted within the tumor of IDC and FA, whereas in DCIS, the number of vessels in the stroma among intraductal components was evaluated. In inflammatory lesions, fibrocystic change and non-pathological breast tissues, the greatest number of vessels in the tissue sections was determined as MVD.[10,11,18–20] Microvessels were identified based on the architecture, lumen lined by endothelial cells, complemented by positivity of the endothelial cells with anti-CD31 after scanning the immunostained section at low magnification (×40 and ×100).[10,11] The areas with the greatest number of distinctly highlighted microvessels were selected, and counted at one higher power (×200).[10,11] Any immunostained endothelial cells or clusters separated from adjacent vessels were counted as a single microvessel, even in the absence of vessel lumen. Each single count was defined as the highest number of microvessels identified at the "hot spot". Vasohibin-1- and Flk-1-positive signals

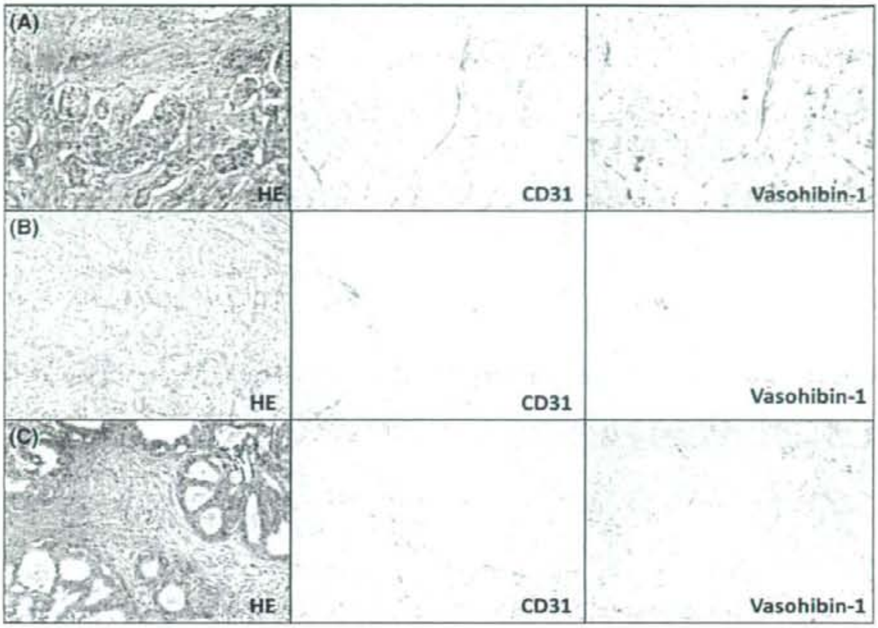


Fig. 1. Representative illustrations of histological and immunohistochemical findings of breast carcinoma cases examined. (A,B) Two invasive ductal carcinoma (IDC) cases stained positively for CD31 and vasohibin-1, whereas (C) a ductal carcinoma *in situ* (DCIS) case stained positive only for CD31 and not for vasohibin-1. (Original magnification, ×200.)

were counted in the hot spot in which the highest number of anti-CD31-positive vessels was identified. We also counted the average of vasohibin-1-positive vessels in 10 representative fields per case (×200). We defined vasohibin-1-positive ratio as the number divided by the number of vasohibin-1-positive vessels by that of CD31-positive vessels in the hot spot. An evaluation of Ki-67 immunoreactivity was performed at high power field (×400) and used as a marker of cell proliferation. More than 500 tumor cells from each of three different representative fields were evaluated and the labeling index was subsequently obtained. VEGF-A immunoreactivity was evaluated using grading, interpreting both relative immunointensity and the proportion of tumor cells associated with an unequivocal positive reaction.[21,22] Relative immunointensity was graded 0 (no staining) to 3 (strong staining), percentage of cells staining positive as 0 (no tumor cells positive), 1 (positive staining in <10% of the tumor cells), 2 (positive staining in 10–50% of the tumor cells) and 3 (positive staining in >50% of the tumor cells).[20,21] A semiquantitative method was used to evaluate the degrees of FGF-2 immunostaining ranging from 0 (no expression), 1 (weak), 2 (moderate) to 3 (highest level of expression).[23] The proportion of proliferating endothelial cells (CD31 and vasohibin-1-positive vessels) was defined as the number of endothelial cells with Ki-67-stained nuclei divided by the total number of endothelial cells.

Analyzes of OS and disease-free survival (DFS) curves were performed by employing the Kaplan–Meyer method. The segregation point of the parameter at 21 for vasohibin-1-positive vessels was determined by the Cox proportional hazards regression model. The values of survival rates represented estimated survival rates. Factors independently associated with OS and DFS – vasohibin-1, MVD, VEGF-A and Ki-67 – were identified by multivariate analyses using multiple regression analysis.

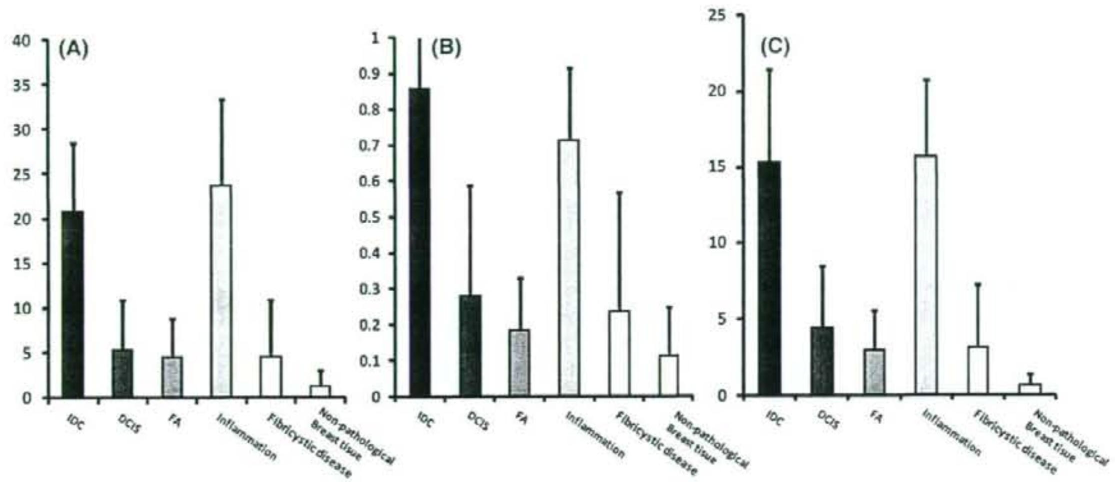
Statistical analysis, such as the one-factor ANOVA and simple regression analysis, were performed using StatMate III for Windows ver. 3.18 (ATMS, Tokyo, Japan). The results were considered significant at $P < 0.05$.

Results

MVD. The representative findings of immunostaining for HE, CD31 and vasohibin-1 are illustrated in Fig. 1. The average number of microvessels detected by CD31 was 24.6 ± 8.3 in IDC, 21.7 ± 11.7 in DCIS, 26.3 ± 15.7 in FA, 34.2 ± 15.4 in inflammatory lesions, 20.6 ± 14.4 in fibrocystic change and 13.6 ± 10.3 in non-pathological breast tissue, respectively. Statistically significant differences of MVD among the lesions were detected only between IDC and non-pathological breast tissue ($P = 0.001$).

Vasohibin-1 immunohistochemistry. Vasohibin-1 immunoreactivity was detected only in endothelial cells (Fig. 1). Vasohibin-1-positive microvessels in the hot spot were 20.9 ± 7.7 in IDC, 5.3 ± 5.5 in DCIS, 4.6 ± 4.1 in FA, 23.7 ± 9.7 in inflammatory lesions, 4.6 ± 6.3 in fibrocystic change and 1.3 ± 1.8 in non-pathological breast tissue. There were statistically significant differences between IDC and four other histological types of breast tissues examined (DCIS, FA, fibrocystic change and non-pathological breast tissue; $P < 0.001$) (Fig. 2A). The ratio of vasohibin-1/CD31[4] was 0.857 ± 0.193 in IDC, 0.279 ± 0.308 in DCIS, 0.183 ± 0.146 in FA, 0.713 ± 0.200 in inflammatory lesions, 0.237 ± 0.332 in fibrocystic change and 0.112 ± 0.136 in non-pathological breast tissue. There were significant differences between IDC and all other histological types ($P < 0.001$) (Fig. 2B). The average number of vasohibin-1-positive vessels per 10 fields (×200) were 15.3 ± 6.1 in IDC, 4.4 ± 4.1 in

doi: 10.1111/j.1349-7006.2008.01015.x



IDC	20.9 ± 7.7
DCIS	5.3 ± 5.5
FA	4.6 ± 4.1
Inflammation	23.7 ± 9.7
Fibrocystic disease	4.6 ± 4.1
Non-pathological breast tissue	1.3 ± 1.8

IDC	0.857 ± 0.193
DCIS	0.279 ± 0.308
FA	0.183 ± 0.146
Inflammation	0.713 ± 0.200
Fibrocystic disease	0.237 ± 0.332
Non-pathological breast tissue	0.112 ± 0.136

IDC	15.3 ± 6.1
DCIS	4.4 ± 4.1
FA	2.9 ± 2.6
Inflammation	15.7 ± 5.0
Fibrocystic disease	3.1 ± 4.1
Non-pathological breast tissue	0.7 ± 0.7

Fig. 2. Analysis of vasohibin-1 immunohistochemistry according to histological subtypes. (A) Number of vasohibin-1-positive vessels in the 'hotspot'. (B) Vasohibin-1-positive ratio defined as the vasohibin-1-positive vessels/CD31-positive vessels. (C) Average of vasohibin-1-positive vessels in 10 different fields. The lower boxes are the statistical analysis compared with invasive ductal carcinoma (IDC) cases.

DCIS, 2.9 ± 2.6 in FA, 15.7 ± 5.0 in inflammatory lesions, 3.1 ± 4.1 in fibrocystic change and 0.7 ± 0.7 in non-pathological breast tissue. There were also statistically significant differences between IDC and four histological types (DCIS, FA, fibrocystic change and non-pathological breast tissue, $P < 0.001$). No significant differences were detected between IDC and inflammatory lesions ($P = 0.781$) (Fig. 2C).

Correlation between vasohibin-1-positive vessels and Ki-67 labeling index in carcinoma cells. A significant positive correlation was detected between the number of vasohibin-1-positive vessels and Ki-67 labeling index in breast tumor cells ($P < 0.001$).

Correlation between vasohibin-1-positive vessels and VEGF-A status in carcinoma cells. The number of vasohibin-1-positive vessels was 5.8 ± 5.5 in VEGF-A of score 0, 11.0 ± 9.4 of score 2, 15.1 ± 10.0 of score 3, 17.5 ± 10.1 of score 4, 22.1 ± 8.9 of score 5 and 22.7 ± 5.7 of score 6. There was a statistically significant association between vasohibin-1 in the vessels and VEGF-A scores in carcinoma cells ($P < 0.001$) (Fig. 3A).

Correlation between vasohibin-1-positive vessels and FGF-2 in carcinoma cells. The number of vasohibin-1-positive vessels was 6.3 ± 6.1 in FGF-2 of score 0, 19.1 ± 6.5 of score 1, 21.9 ± 7.2 of score 2 and 26.8 ± 8.4 of score 3. A statistically significant association was detected between vasohibin-1 immunoreactivity in the vessels and FGF-2 scores in carcinoma cells ($P < 0.001$) (Fig. 3B).

Correlation between vasohibin-1 and Flk-1 in microvessels in breast carcinoma. A significantly positive correlation was detected between vasohibin-1 and Flk-1 positive ratios in microvessels ($P < 0.001$) (Fig. 3C).

Correlation between vasohibin-1 and clinical stage of breast carcinoma cases. The number of vasohibin-1-positive vessels was 5.3 ± 5.5 in TNM Stage 0, 19.6 ± 6.7 in Stage I, 18.7 ± 8.6 in Stage II A, 22.1 ± 8.3 in Stage II B, 23.8 ± 5.8 in Stage III A, 28.7 ± 7.5 in Stage III B, 23.0 ± 7.5 in Stage III C and 21.2 ± 5.6 in Stage IV. Statistically significant differences were detected only between IDC and DCIS ($P < 0.001$) with no significant differences among the different stages of IDC.

Correlation between vasohibin-1 and histological grades of breast carcinoma cells. The number of vasohibin-1-positive vessels among different groups of carcinoma cases and histological grade was 18.4 ± 7.5 in grade I, 20.8 ± 7.0 in grade II and 28.0 ± 8.0 in grade III. There were statistically significant differences of vasohibin-1 density between grade I and III, and grade II and III cases ($P < 0.001$) with no significant difference between grade I and II cases ($P = 0.14684$).

Correlation between vasohibin-1 and overall survival or DFS in breast carcinoma patients. Patients were tentatively classified into two different groups according to the number of vasohibin-1-positive vessels: 0–20 and 21 or more. The 10-year overall survival rates were 0.932203 and 0.72549 among these two groups, respectively. (The total 10-year overall survival rate in this cohort of patients was 0.838836.) Statistically significant differences in the 0–20 and 21 or more groups was $P = 0.004$ (Fig. 4A). The 10-year DFS were 0.92736 and 0.708333, respectively, in these two groups. Statistically significant differences were also detected in the 0–20 and 21 or more groups was at $P \leq 0.001$. (The total 10-year DFS rate was 0.81777; Fig. 4B.) The following variables were included in the multivariate analysis of OS: vasohibin-1, MVD, VEGF-A and Ki-67. This multivariate analysis demonstrated that vasohibin-1 was associated with VEGF-A ($P = 0.038$) and Ki-67 ($P < 0.001$), but was not associated with MVD ($P = 0.083$). The multivariate analysis of

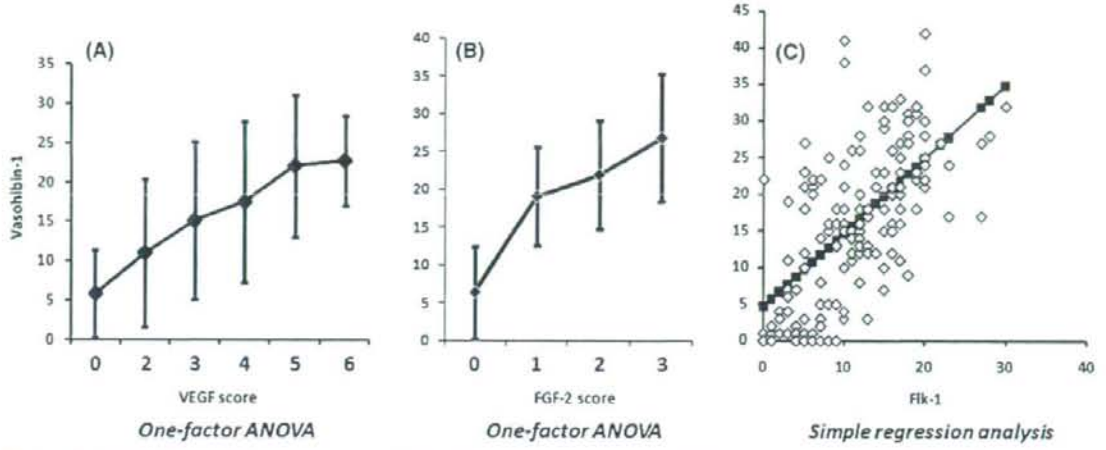
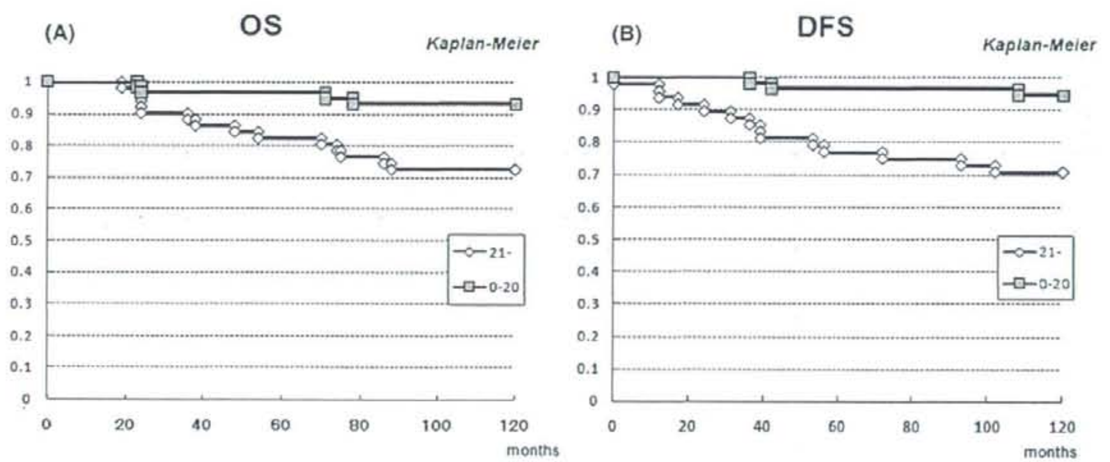


Fig. 3. (A) Result of the correlation between vasohibin-1-positive vessels and vascular endothelial growth factor (VEGF)-A expression in the tumor cells. (B) Result of the correlation between vasohibin-1-positive vessels and fibroblastic growth factor (FGF)-2 expression in the tumor cells. (C) Correlation between vasohibin-1 and Flk-1 in the 'hot spot'.



Vasohibin-1 positive vessels	10 year OS
0-20	0.932203
21-	0.72549

Vasohibin-1 positive vessels	10 year DFS
0-20	0.927356
21-	0.708333

Fig. 4. Summary of analysis of (A) overall survival and (B) disease free survival in relation to the status of vasohibin-1 expression. Patients were tentatively classified into two different groups according to the number of vasohibin-1-positive vessels: 0–20 and 21 or more.

DFS also revealed that vasohibin-1 was associated with VEGF-A ($P = 0.004$) and Ki-67 ($P < 0.001$), but was not associated with MVD ($P = 0.081$).

Double immunostaining with Ki-67 in microvessels. Ki-67/vasohibin-1 double immunostaining analysis demonstrated that Ki-67 labeling index of vasohibin-1-positive vessels was 46.5% (33.3–62.5%), whereas that of CD31-positive vessels was 23.5% (12.7–37.5%) (Fig. 5A,B).

Discussion

One of the most important functions of vasculature in general is to supply nutrients the distal organs. Three major types of regulation occur in the maintenance of vasculature: (i) vasodilation; (ii) changes in capillary permeability; and (iii) growth and development of new vessels, also known as angiogenesis.[24–26] Angiogenesis is a pivotal event in various biological processes

doi: 10.1111/j.1349-7006.2008.01015.x

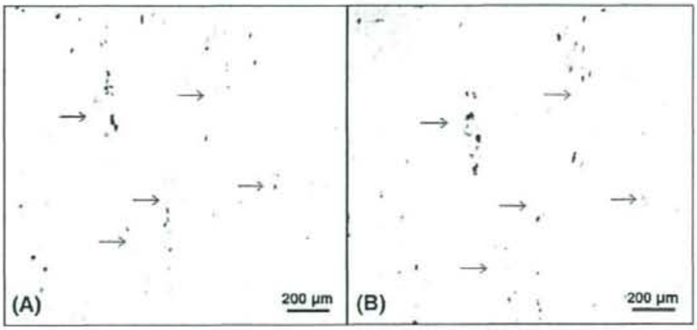


Fig. 5. Representative illustrations of double immunostaining for determining proliferating endothelial cells. (A) CD31/Ki-67 double staining; (B) vasohibin-1/Ki-67 double staining (arrow). (A) CD31 and (B) vasohibin-1 were colored blue, and Ki-67 was colored brown.

under both physiological and pathological conditions. Physiological conditions include embryonic development, reproduction and wound healing, and pathological conditions include cancers and inflammatory conditions.[2] *In situ* balance between angiogenesis stimulators such as VEGF and bFGF and inhibitors such as thrombospondin-1 (TSP-1) and pigment epithelium derived factor (PEDF) is generally considered to regulate the process of angiogenesis.[1] Negative feedback regulation is considered one of the most important physiological mechanisms with which bodies are endowed, and has been demonstrated to be involved in a wide range of biological phenomena.[27] This regulation is most effectively performed through the factors produced in endothelial cells but the endothelium-derived negative feedback regulators of angiogenesis have not been elucidated. Vasohibin-1 is therefore the first secretory anti-angiogenic factor from endothelial cells themselves induced by VEGF in EC.[2–4,28] The other anti-angiogenic regulator has been very recently identified and termed vasohibin-2 but this factor lacks the property of VEGF-A or bFGF inducibility in contrast to vasohibin-1.[28] Vasohibin-1 immunoreactivity was exclusively detected in endothelial cells in the present study, which is also consistent with results of previous studies of endometrial carcinoma[9] in lung carcinoma[3] and ischemic retina.[29] This is the first study to examine the status of vasohibin-1 in human breast disease in which angiogenesis also plays important roles in both physiological and pathological conditions.

Breast cancer has also been considered an angiogenic-dependent disease as in other human malignancies and angiogenesis has been demonstrated to play an essential role in breast cancer development, invasion and metastasis.[30–32] MVD assessed by CD31, CD34 and Factor VIII is generally considered as a gold-standard surrogate marker of tumor angiogenesis and has been also proposed by some investigators to identify patients at high risk of recurrence more precisely than classical indicators.[10,11]

In this study, we first examined how the vasohibin-1 expression was correlated to the MVD status. Vasohibin-1 immunodensity tended to be concordant with MVD in human breast tissues but they were not always parallel. The vasohibin-1 immunodensity was significantly higher in IDC than in DCIS but there was no difference of MVD between these two lesions. In addition, results of double immunostaining analysis which could simultaneously demonstrate two different proteins in the same cells, demonstrated the significant positive correlation between Ki-67-positive proliferating vascular endothelial cells, which may represent neovascular formation[16,17] and vasohibin-1-positive endothelial cells. Indeed, the Ki-67 labeling index among vasohibin-1-positive endothelial cells was significantly higher than Ki-67 in all CD31-positive endothelial cells. These results will clearly indicate that vasohibin-1 is considered a more appropriate biomarker for intratumoral neovascularization compared to CD31, which may detect all the vasculature including both resting and proliferating endothelial cells.

Results of our study also demonstrated the positive correlation between vasohibin-1 and VEGF-A or bFGF in carcinoma cells or Flk-1 in intratumoral endothelial cells, which also suggest that the vasohibin-1 in vasculature in human breast carcinoma is induced by VEGF-A, bFGF/Flk-1 signaling pathway. PKCδ was reported to play an important role in an induction of vasohibin-1 in endothelial cells.[4] Therefore, vasohibin-1 is supposed to be induced in the downstream of VEGF-A, bFGF/Flk-1 signaling pathway. Further investigations are necessary to reach the final conclusion.

The expression of vasohibin-1 in EC was proposed to be regulated either positively or negatively by certain factors at the transcriptional level, and this may influence the process of angiogenesis.[4] Another *in vivo* study also demonstrated the significantly positive correlation between vasohibin-1 and Flk-1 expression in vasculature of human endometrial carcinoma.[9] Significantly higher vasohibin-1 immunodensity in IDC than DCIS in our present study of human breast also indicate that the anti-angiogenic compensatory mechanism may be operational in invasive breast carcinoma, possibly in response to induction of angiogenesis by various factors related to carcinoma invasion into the surrounding stroma.

Results of several recent studies demonstrated the possible correlation between VEGF status in carcinoma cells and clinical outcome in breast cancer patients. VEGF was proposed to be correlated with worse DFS and overall survival rates especially in the patients with early-stage breast cancer.[33] VEGF expression in carcinoma cells was also reported as an independent prognostic marker in both node-positive and node-negative breast cancers.[34] Many previous immunohistochemical studies of MVD assessed by CD31, CD34 or Factor VIII antigen in human breast cancer demonstrated that high MVD in invasive ductal carcinoma is usually correlated with a greater likelihood of metastatic disease,[10] shorter relapse-free intervals and reduced overall survival in patients with node-negative breast cancer.[11] We therefore examined whether vasohibin-1 immunoreactivity is correlated with OS and DFS of the patients. Results of our study demonstrated that the cases with a higher number of vasohibin-1-positive vessels tended to be associated with better and statistically significant OS. In addition, a statistically negative or inverse correlation was detected between vasohibin-1 immunodensity and DFS. These results all suggest that an evaluation of the number of vasohibin-1-positive vessels may become one of the prognostic markers for metastasis and prognosis but it awaits further investigations to establish this approach as a surrogate marker such as MVD.

Recently, newer targeted therapies toward the control of tumor neovascularization such as anti-VEGF therapy have been developed in phase II and III clinical trials and demonstrated the clinical effects such as reduction of tumor angiogenesis and inhibition of solid tumors proliferation, either alone or in combination with chemotherapy.[35–38] In our present study, vasohibin-1 immunohistochemical staining was demonstrated to reasonably reflect the status of angiogenesis, and vasohibin-1 itself may be considered for anti-VEGF and anti-angiogenesis drugs to control tumor angiogenesis in future.

Acknowledgments

We thank Yayoi Takahashi, MT, for her excellent technical assistance. This work was partly supported by the grants from the Japanese Ministry of Health, Labor and Welfare for Researches on Intractable Diseases, Risk Analysis Research on Food and Pharmaceuticals, and Development of Multidisciplinary Treatment Algorithm with Biomarkers and Modeling of the Decision-making Process with Artificial Intelligence for Primary Breast Cancer. This work was also partly supported by a Grant-in-Aid for Scientific Research (no. 18390109) from the Japanese Ministry of Education, Culture, Sports, Science and Technology, and the Yasuda Medical Foundation.

References

1 Folkman J. Angiogenesis in cancers, vascular, rheumatoid and other disease. *Nat Med* 1995; **1**: 27–31.
2 Sato Y, Sonoda H. The Vasohibin Family: a negative regulatory system of angiogenesis genetically programmed in endothelial cells. *Arterioscl Thromb Vasc Biol* 2007; **27**: 37–41.
3 Watanabe K, Hasegawa Y, Yamashita H *et al.* Vasohibin as an endothelium-derived negative feedback regulator of angiogenesis. *J Clin Invest* 2004; **114**: 898–907.
4 Shimizu K, Watanabe K, Yamashita H *et al.* Gene regulation of novel angiogenesis inhibitor, vasohibin, in endothelial cells. *Biochem Biophys Res Commun* 2005; **327**: 700–6.
5 Kerbel RS. Vasohibin. The feedback on a new inhibitor of angiogenesis. *J Clin Invest* 2004; **114**: 884–6.
6 Sonoda H, Ohta H, Watanabe K *et al.* Multiple processing forms and their biological activities of a novel angiogenesis inhibitor vasohibin. *Biochem Biophys Res Commun* 2006; **342**: 640–6.
7 Abe M, Sato Y. cDNA microarray analysis of the gene expression profile of VEGF-activated human umbilical vein endothelial cells. *Angiogenesis* 2001; **4**: 289–98.
8 Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev* 2004; **25**: 581–611.
9 Yoshinaga K, Ito K, Moriya T *et al.* Expression of vasohibin as a novel endothelium-derived angiogenesis inhibitor in endometrial cancer. *Cancer Sci* 2008; **99**: 914–9.
10 Weidner N, Semple JP, Welch WR *et al.* Tumor angiogenesis and metastasis-correlation in invasive breast carcinoma. *N Engl J Med* 1991; **324**: 1–8.
11 Weidner N, Folkman J, Pozza F *et al.* Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. *J Natl Cancer Inst* 1992; **84**: 1875–87.
12 Silverstein MJ. Prognostic classification of breast ductal carcinoma *in situ*. *Lancet* 1995; **345**: 1154–7.
13 Sobin LH, Wittekind C. *TNM Classification of Malignant Tumours* 131–41.
14 Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a larger study with long-term follow-up. *Histopathology* 1991; **19**: 403–10.
15 Yamashita H, Abe M, Watanabe K *et al.* Vasohibin prevents arterial neointimal formation through angiogenesis inhibition. *Biochem Biophys Res Commun* 2006; **345**: 919–25.
16 Nijsten T, Copaert CG, Vermeulen PB *et al.* Cyclooxygenase-2 expression and angiogenesis in squamous cell carcinoma of the skin and its precursors: a paired immunohistochemical study of 36 cases. *Br J Dermatol* 2004; **151**: 837–45.
17 Hoskin PJ, Sibtain A, Daley FM *et al.* The immunohistochemical assessment of hypoxia, vascularity and proliferation in bladder carcinoma. *Radiother Oncol* 2004; **72**: 159–68.
18 Uzzan B, Nicolas P, Cucherat M *et al.* Microvessel density as a prognostic factor in women with breast cancer: a systematic review of the literature and meta-analysis. *Cancer Res* 2004; **64**: 2941–55.
19 Guidi AJ, Schnitt SJ, Fischer L *et al.* Vascular permeability factor (vascular endothelial growth factor) expression and angiogenesis in patients with ductal carcinoma *in situ* of the breast. *Cancer* 1997; **80**: 1945–53.
20 Cao Y, Paner GP, Kahn LB *et al.* Non-invasive carcinoma of the breast: angiogenesis and cell proliferation. *Arch Pathol Lab Med* 2004; **128**: 893–6.
21 Aas T, Borresen AL, Geisler S *et al.* Specific P53 mutations are associated with de novo resistance to doxorubicin in breast cancer patients. *Nat Med* 1996; **2**: 811–14.
22 Toi M, Hoshina S, Takayanagi T *et al.* Association of vascular endothelial growth factor expression with angiogenesis and with early relapse in primary breast cancer. *Jpn J Cancer Res* 1994; **85**: 1045–9.
23 Begum S, Zhang Y, Shintani T *et al.* Immunohistochemical expression of heparin-binding protein 17/fibroblast growth factor-binding protein-1 (HBp17/FGFBP-1) as an angiogenic factor in head and neck tumorigenesis. *Oncol Report* 2007 Mar; **17** (3): 591–6.
24 Folkman J. Fundamental concepts of the angiogenic process. *Curr Mol Med* 2003; **3**: 643–51.
25 Risau W. Mechanism of angiogenesis. *Nature* 1997; **386**: 671–4.
26 Carmeliet P. Angiogenesis in health and disease. *Nat Med* 2003; **9**: 653–60.
27 Lord BI. Feedback regulators in normal and tumor tissues. *J Cell Sci Suppl* 1988; **10**: 231–42.
28 Shibuya T, Watanabe K, Yamashita H *et al.* Isolation of vasohibin-2 as a sole homologue of VEGF-inducible endothelium-derived angiogenesis inhibitor vasohibin: a comparative study on their expressions. *Arterioscl Thromb Vasc Biol* 2006; **26**: 1051–7.
29 Shen JK, Yang XR, Sato Y *et al.* Vasohibin is up-regulated by VEGF in the retina and suppress VEGF receptor 2 and retinal neovascularization. *FASEB J* 2006; **20**: 723–5.
30 Lichtenbeld HC, Barendsz-Janson AF, ven Essen H *et al.* Angiogenic potential of malignant and non-malignant human breast tissue in an *in vivo* angiogenesis model. *Int J Cancer* 1998; **77**: 455–9.
31 Dabrosin C, Palmer K, Muller WJ *et al.* Estradiol promotes growth and angiogenesis in polyoma middle T transgenic mouse mammary tumor explants. *Breast Cancer Res Treat* 2003; **78**: 1–6.
32 Scneider BP, Miller KD. Angiogenesis of breast cancer. *J Clin Oncol* 2005; **22**: 609–14.
33 Sledge GW Jr. Vascular endothelial growth factor in breast cancer: biologic and therapeutic aspects. *Semin Oncol* 2002; **29** (Suppl 11): 104–10.
34 Linderholm B, Lindh B, Beckman L *et al.* The prognostic value of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) and associations to first metastasis site in 1307 patients with primary breast cancer. *Proc Am Soc Clin Oncol* 2001; **20**: 4a.
35 Rugo HS. Bevacizumab in the treatment of breast cancer: rationale and current data. *The Oncologist* 2004; **9** (Suppl 1): 43–9.
36 Fox SB, Generali DG, Harris AL. Breast tumour angiogenesis. *Breast Cancer Res* 2007; **9**: 216.
37 Miller KD, Chap LI, Holmes FA *et al.* Randomized phase III trial of capecitabine compared with bevacizumab plus capecitabine in patients with previously treated metastatic breast cancer. *J Clin Oncol* 2005; **23**: 792–9.
38 Wedam SB, Low JA, Yang SX *et al.* Antiangiogenic and antitumor effects of bevacizumab in patients with inflammatory and locally advanced breast cancer. *J Clin Oncol* 2006; **24**: 769–777.

doi: 10.1111/j.1349-7006.2008.01015.x

Research Article

Steroid Sulfatase and Estrogen Sulfotransferase in Colon Carcinoma: Regulators of Intratumoral Estrogen Concentrations and Potent Prognostic Factors

Ryuichiro Sato,[1,2] Takashi Suzuki,[2,3] Yu Katayose,[1] Koh Miura,[1] Kenichi Shiiba,[4] Hiroo Tateno,[5] Yasuhiro Miki,[2] Junichi Akahira,[2] Yukiko Kamogawa,[2] Shuji Nagasaki,[2] Kuniharu Yamamoto,[1] Takayuki Ii,[1] Shinichi Egawa,[1] Dean B. Evans,[6] Michiaki Unno,[1] and Hironobu Sasano[2]

Departments of [1]Surgery and [2]Pathology, Tohoku University School of Medicine; [3]Department of Pathology, Tohoku University School of Health Sciences, Sendai, Japan; Departments of [4]Surgery and [5]Pathology, Miyagi Cancer Center, Natori, Japan; and [6]Novartis Institutes for BioMedical Research Basel, Oncology Research, Basel, Switzerland

Abstract

Previous epidemiologic and *in vitro* studies have indicated a potential involvement of estrogens in the pathogenesis of human colon carcinoma, but the precise roles of estrogens have remained largely unknown. Therefore, in this study, we first measured intratumoral concentrations of estrogens in 53 colon carcinomas using liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS). Tissue concentrations of total estrogen [estrone (E_1) + estradiol] and E_1 were significantly (2.0- and 2.4-fold, respectively) higher in colon carcinoma tissues than in nonneoplastic colonic mucosa (n = 31), and higher intratumoral concentrations of total estrogen and E_1 were significantly associated with adverse clinical outcome. Intratumoral concentration of total estrogen was significantly associated with the combined status of steroid sulfatase (STS) and estrogen sulfotransferase (EST), but not with that of aromatase. Thus, we subsequently examined the STS/EST status in 328 colon carcinomas using immunohistochemistry. Immunoreactivities for STS and EST were detected in 61% and 44% of the cases, respectively. The −/+ group of the STS/EST status was inversely associated with Dukes' stage, depth of invasion, lymph node metastasis, and distant metastasis and positively correlated with Ki-67 labeling index of the carcinomas. In addition, this −/+ group had significantly longer survival, and a multivariate analysis revealed the STS/EST status as an independent prognostic factor. Results from our present study showed that the STS/EST status of carcinoma tissue determined intratumoral estrogen levels and could be a significant prognostic factor in colon carcinoma, suggesting that estrogens are locally produced mainly through the sulfatase pathway and play important roles in the progression of the disease. [Cancer Res 2009;69(3):914–22]

Introduction

Colon cancer is the third leading cause of cancer-related deaths in both men and women in the United States (1). Although the recent advances in chemotherapy have prolonged the survival of patients with advanced disease (2), the results are still unsatisfactory and further researches are required to understand the disease and to improve the outcome. A number of observational studies (3–5) and a randomized trial (6, 7) have shown that hormone replacement therapy affects the incidence of colon carcinoma and the recurrence of colorectal adenoma (8) in postmenopausal women. In addition, the great majority of colon carcinomas express estrogen receptor β (ERβ; ref. 9), and some colon carcinoma cells are responsive to estrogens (10–12). These findings all suggest a possible involvement of estrogens in the pathogenesis of human colon carcinoma.

It is well known that estrogens are locally produced from circulating inactive steroids in estrogen-dependent tumors, such as breast carcinoma, through sulfatase and aromatase pathways (ref. 13; Supplementary Fig. S1). The sulfatase route synthesizes estrone (E_1) from circulating estrone sulfate (E_1-S) by steroid sulfatase (STS) or inversely inactivates E_1 into E_1-S by estrogen sulfotransferase (EST). E_1 is subsequently converted to a potent estrogen, estradiol (E_2), by reductive 17β-hydroxysteroid dehydrogenases (17βHSD). In the aromatase pathway, aromatase produces E_1 and E_2 from circulating androstenedione and testosterone, respectively (13, 14). In breast carcinoma, STS activity is 50 to 200 times greater than aromatase activity (15), and expressions of STS and EST are both reported as significant prognostic factors (16) whereas aromatase is not (17).

In colon carcinoma, expression of 17βHSDs has been previously examined, and colon carcinoma mainly expresses 17βHSD type 2 and type 4, which metabolize E_2 to E_1 (18, 19). It has been also shown that colon carcinoma tissue has sulfatase (20, 21) and aromatase activities (18). However, to the best of our knowledge, intratumoral concentrations of estrogens and their clinical significance have not been reported. Therefore in this study, we first measured tissue concentrations of estrogens in 53 colon carcinoma tissues and correlated these findings with immunoreactivities for STS, EST, and aromatase. The result showed a strong association between the intratumoral estrogen concentrations and STS/EST status, so we subsequently examined the STS/EST status in 328 colon carcinomas to obtain better understanding of the role and significance of estrogenic actions in colon carcinoma.

Materials and Methods

Patients and tissues. Fifty-three specimens (28 men and 25 postmenopausal women) of colon carcinoma tissues were obtained from patients who underwent surgery from 2000 to 2008 in the Department of Surgery at Tohoku University Hospital, Sendai, Japan (mean age, 70.3 y; range, 45–93 y).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Takashi Suzuki, Department of Pathology, Tohoku University School of Health Sciences, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan. Phone: 81-22-717-8050; Fax: 81-22-717-8051; E-mail: t-suzuki@patholo2.med.tohoku.ac.jp.

doi:10.1158/0008-5472.CAN-08-0906