Comparing Fluorescence *In Situ* Hybridization and Chromogenic *In Situ* Hybridization Methods to Determine the *HER2/neu* Status in Primary Breast Carcinoma using Tissue Microarray

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Identification of *HER2/neu* status is important for predicting response to specific chemotherapy in breast carcinoma. Chromogenic *in situ* hybridization was performed using tissue microarray technology on 188 primary breast carcinomas. To validate the reliability of novel chromogenic *in situ* hybridization technology, the results of chromogenic *in situ* hybridization were correlated with the results of two-color fluorescence *in situ* hybridization done with the same tumors. On tissue microarray panels containing 188 breast carcinoma tissues, fluorescence *in situ* hybridization and chromogenic *in situ* hybridization were conducted simultaneously. *HER2/neu* amplification was detected in 46 tumors (24.5%) by fluorescence *in situ* hybridization and in 43 tumors (22.9%) by chromogenic *in situ* hybridization. Results of each method agreed with each other in 177 tumors (concordance: 94.1%). *HER2/neu* amplification by fluorescence *in situ* hybridization was associated with nuclear pleomorphism (P = .021), and *HER2/neu* amplification by chromogenic *in situ* hybridization was associated with poor nuclear grade (P = .037). High concordance between fluorescence *in situ* hybridization and chromogenic *in situ* hybridization indicated that chromogenic *in situ* hybridization can be a tempting alternative to fluorescence *in situ* hybridization for the detection of *HER2/neu* amplification in breast carcinoma because of its accuracy and relative low cost. *HER2/neu* appeared to have a prognostic implication because its amplification was associated with aggressive biologic features of the breast carcinoma. Integration of tissue microarray technology enabled high-throughput determination of *HER2/neu* amplification profile with rapidity and accuracy in large cohorts of the breast carcinoma.

KEY WORDS: Breast carcinoma, Chromogenic *in situ* hybridization, Fluorescence *in situ* hybridization, *HER2/neu*, Tissue microarray.

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Among the numerous oncogenes and their products, *HER2/neu* is the most widely exploited one in clinical oncology. *HER2/neu* has moved from a laboratory-based prognostic factor to a target for the specific therapy, trastuzumab (Herceptin; Genentech, Inc., South San Francisco, CA), which binds to *HER2/neu* protein. The *HER2/neu* is a 185-kDa transmembrane tyrosine kinase, and overexpression of *HER2/neu* protein arises from *HER2/neu* gene amplification, resulting in increased gene number (1). *HER2/neu* overexpression has been shown in 20–40% of human breast carcinomas and is associated with poor clinical outcome, even with systemic chemotherapy (2–4). Recent studies suggest that *HER2/neu* is a useful determinant of response to hormonal or cytotoxic chemotherapy. Data from Cancer and Leukemia Group B 8869 and the National Surgical Adjuvant Breast and Bowel Project protocol B-11 suggest that patients whose tumors overexpress *HER2/neu* may derive a preferential benefit from treatment with doxorubicin (5, 6). With recent introduction of trastuzumab therapy, it is now reasonable to test all new cases of breast carcinomas, both early and late stage, for *HER2/neu* overexpression. However, there is no consensus about which is the most optimal and accurate testing strategy.

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A vast majority of *HER2/neu* studies has been performed using immunohistochemistry that detects the *HER2/neu* protein overexpression. Measurement of *HER2/neu* gene amplification is more accurate because protein overexpression is the result of gene amplification. Introduction of fluorescence *in situ* hybridization (FISH) allows assessment of the level of gene amplification with information about distribution of gene copies in histologic sections (7). A number of reports have verified its accuracy and apparent superiority over immunohistochemistry in a prediction of response to trastuzumab in metastatic breast carcinoma patients (8, 9). The main difficulty for adopting FISH in a clinical setting is the need of additional equipment for analysis such as fluorescence microscopy and multiband fluorescence filters. Recently, novel technology to detect DNA probe has been developed. Chromogenic *in situ* hybridization uses a simple immunohistochemistry-like peroxidase reaction (10). Chromogenic *in situ* hybridization is a tempting technology to overcome the practical limitations of FISH, although its standardization has not been validated.

Completion of the human genome sequence has provided the basic structural information on all human genes. Functional technique such as cDNA microarrays enables analysis of expression levels of thousands of genes and proteins at once (11). Compared with the high-throughput techniques of genomics and proteomics, most tissue-based molecular analyses have been tedious and require extensive manual interaction. Tissue microarray is a novel technology of harvesting small disks of tissue from individual donor paraffin-embedded tissue blocks and placing them in a recipient block with defined array coordinates (12). Tissue microarray technology allows high-throughput molecular profiling of cancer from DNA to protein level by enabling the simultaneous analysis of hundreds of tissue specimens (13). This technology provides maximal use of limited tissue resources and renders the advantage of generating gene expression profiles of cells as they occur in actual neoplastic tissues *in vivo*. Tissue microarray technology has the potential to significantly accelerate molecular studies and has become one of the most promising tools in cancer research fields.

In this report, we summarize the results of simultaneous analyses for *HER2/neu* amplification in 188 human breast carcinomas using tissue microarray technology. Chromogenic *in situ* hybridization appeared as a reasonable alternative to FISH in the current study, and genetic analyses on the archival cancer tissues were successful with novel technologies.

MATERIALS AND METHODS

Materials

One hundred eighty-eight primary breast carcinomas were collected at Inje University Sanggye Paik Hospital, Seoul, Korea. Histopathologic classification and determination of tumor collecting regions were done on hematoxylin and eosin–stained slides. The invasive ductal carcinoma was graded I, II, or III with the Nottingham histologic grading system (14) in ascending degree of malignancy and was graded I, II, or III with the Black's nuclear grading system (15) in descending degree of malignancy.

Tissue Microarray Block

Recipient blocks were made with purified agar in 3.8 × 2.2–cm frames. Holes with 2 mm in each size were made on the recipient blocks by core needle, and agar core was discarded. Donor blocks were prepared after through evaluation of hematoxylin and eosin slides. Representative cancer portions caught from matching donor blocks were transplanted to the recipient blocks using a 2-mm core needle. Recipient blocks were framed in the mold that is used to frame conventional paraffin block, and then paraffin was added to the frame. Consecutive sections in 3.5-μm thickness were cut from the recipient blocks using an adhesive-coated slide system (Instrumedics Inc., New Jersey) supporting the cohesion of the 2-mm array elements on the glass (Fig. 1).

Fluorescence *In Situ* Hybridization

Two-color FISH was done on a 3.5-μm-thick consecutive microarray sections from the same paraffin blocks with chromogenic *in situ* hybridization. Before hybridization, the sections were deparaffinized, air dried, and dehydrated in 100% ethanol after incubation at 56° C for 24 hours. Microarray slides were treated in wash buffer (Vysis Inc., Downers Grove, IL) for 3 minutes after treatment with 0.2 N HCl for 20 minutes. Pretreatment solution (Vysis) at 80° C was applied for 30 minutes, and the slides were washed with purified water. Slides were treated with wash buffer twice for 5 minutes serially. Immersed slides in protease solution (Vysis) at 37° C was applied for 10 minutes, and the slides were washed with wash buffer at 45–50° C and air dried. Slides were fixed in 10% buffered formalin for 10 minutes and were washed with wash buffer at 45–50° C. For denaturation, slides were immersed in denaturation solution (Vysis) for 5 minutes at 72° C, followed by dehydration with 70%, 85%, 100% ethanol serially at 45–50° C. For hybridization, 20 μL of LSI *HER-2*/CEP17 probe

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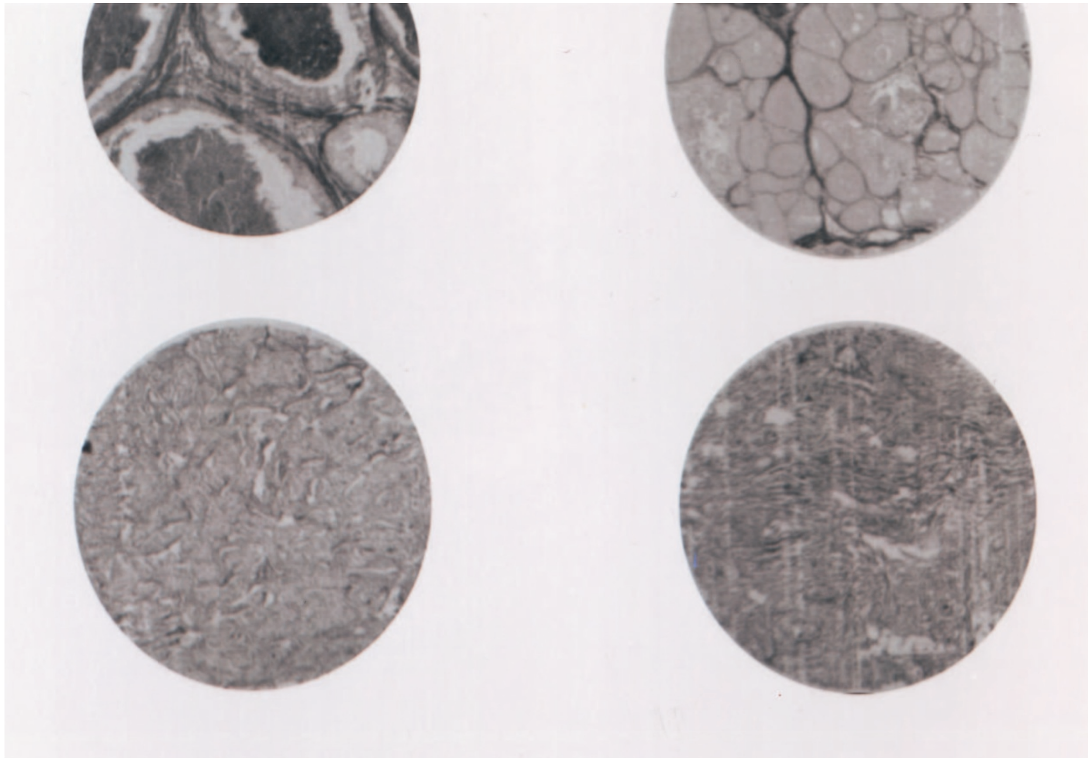

FIGURE 1. A portion of tissue microarray section of breast carcinoma (H & E, 10×).

(PathVysion™, Vysis) was applied, and a coverslip was applied over the probe. After overnight hybridization at 37° C in a humidified chamber, the slides were washed with 72° C posthybridization wash buffer (Vysis) for 2 minutes. Nuclei were counterstained with 20 μL 4,6-diamino-2-phenylindole (DAPI) (Vysis). The centromere 17 (CEP) and *HER2/neu* copy numbers were estimated for the predominant tumor cell population.

Hybridization signals were enumerated by the ratio of orange signals for *HER2/neu* to green signals for CEP in morphologically intact and nonoverlapping nuclei. At least two times more *HER2/neu* signals than CEP17 signals in the tumor cells was considered as the criterion for *HER2/neu* amplification.

Chromogenic *In Situ* Hybridization

Chromogenic *in situ* hybridization was done on 3.5-μm-thick tissue microarray sections. Tissue microarray slides were deparaffinized and were incubated in a SPOT-Light Heat Pretreatment buffer (Zymed Inc., South San Francisco, CA) at 92–100° C for 15 minutes. After washing with phosphate buffer saline, 100 μL of SPOT-Light Tissue Pretreatment Enzyme (Zymed) was applied at 37° C for 5 minutes. Microarray slides were washed with phosphate buffered saline and were dehydrated with graded ethanols. A coverslip was applied on microarray slide after application of 15 μL of digoxigenin-labeled *HER2/neu* probe. The slides were treated on a 95° C hot plate for 5–10 minutes and were incubated at 37° C for 16–24 hours. After incubation, microarray slides were treated in 0.5× sodium chloride citrate for 5 minutes and were washed with phosphate-buffered saline/Tween solution. The slides were treated with 3% hydrogen peroxide for 10 minutes. One hundred μL of FITC-sheep anti-digoxigenin (Zymed) was applied for 30–60 minutes after application of 100 μL of CAS-Block (Zymed) application for 10 minutes. After washing with phosphate buffered saline, 100 μL of HRP-goat anti-FITC (Zymed) was applied for 30–60 minutes. After washing with phosphate buffered saline, 150 μL of 3,3-diaminobenzidine tetrahydrochloride was applied for 20–30 minutes. Microarray slides were counterstained with hematoxylin and eosin after washing with purified water and dehydration with ethanol and xylene.

Amplification of *HER2/neu* was defined when gene copy number was more than four or when a large gene copy cluster was seen in >50% of cancer cell nuclei.

Data Analysis

Results from chromogenic *in situ* hybridization and FISH were merged and analyzed. The χ^2 test was used for data analysis, and correlation between the results was estimated by Spearman's correlation coefficient (κ). A κ value of 1 denotes complete

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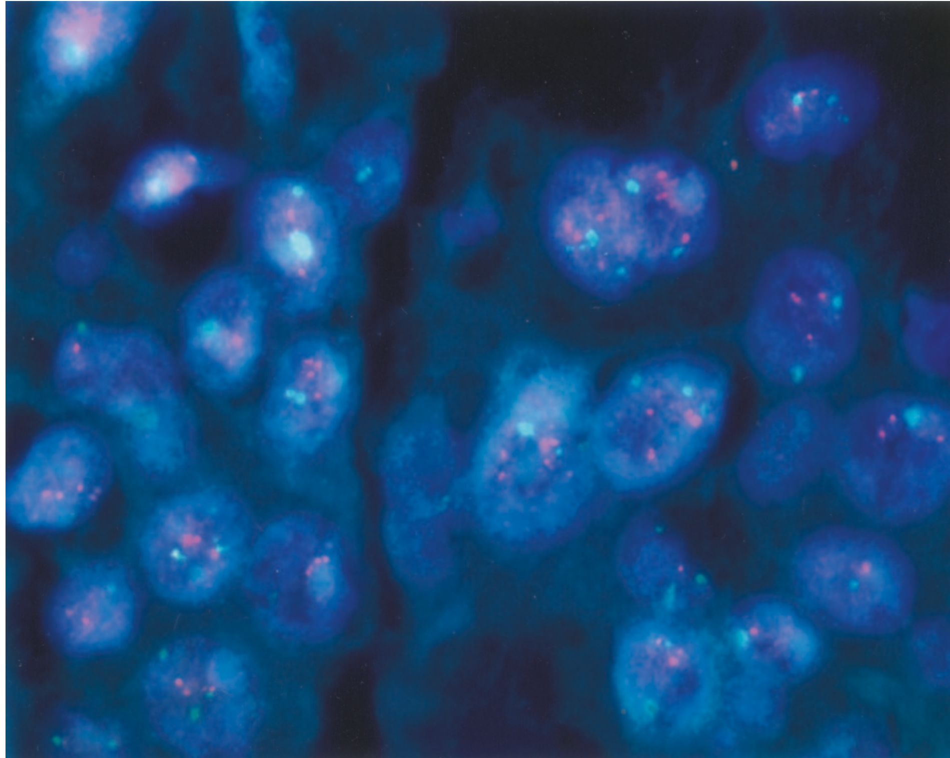

FIGURE 2. Fluorescence *in situ* hybridization shows increased HER2/neu gene copy number in breast cancer tissue (**orange: HER2/neu,** green: CEP17 control).

agreement, values of 0.75 denote excellent agreement, values between 0.4 and 0.75 denote fairly good agreement, and values of <0.4 denote poor agreement.

RESULTS

We performed FISH and chromogenic *in situ* hybridization simultaneously for the validation of fea-

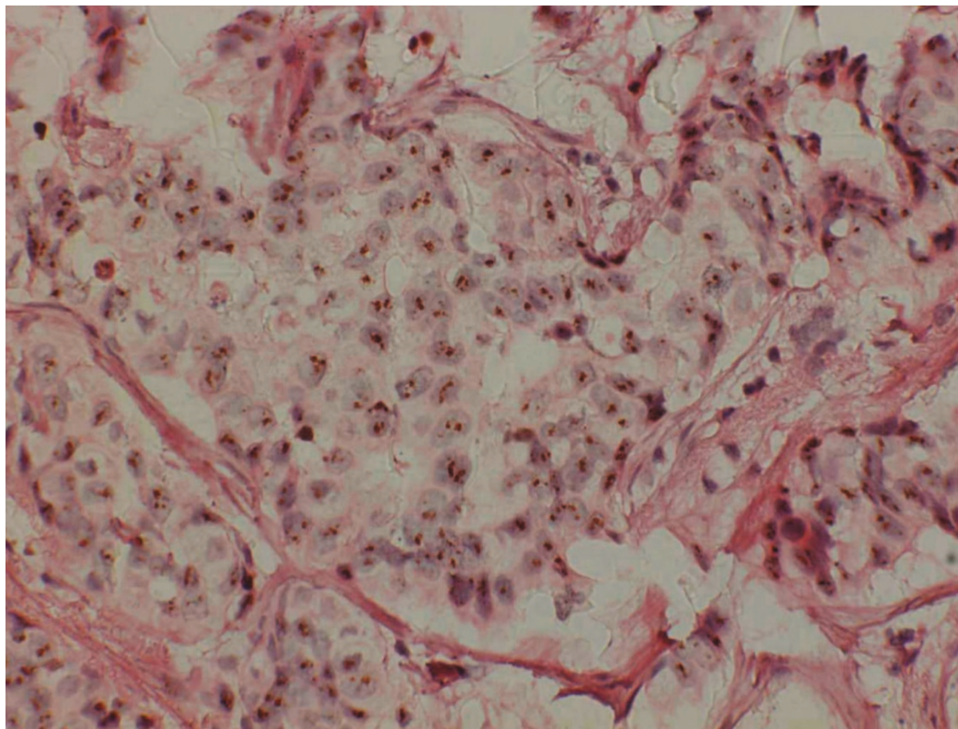

FIGURE 3. Chromogenic *in situ* hybridization with amplification of HER2/neu gene.

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sibility of chromogenic *in situ* hybridization in *HER2/neu* amplification assay. *HER2/neu* amplification was detected in 46 cases (24.5%) by FISH and in 43 (22.9%) of 188 by chromogenic *in situ* hybridization (Fig. 2 and Fig. 3). Results of each method agreed with each other in 177 cases (94.1%), whereas results of 11 cases were different from each other. *HER2/neu* amplification was detected by chromogenic *in situ* hybridization in 4 cases, although the amplification signals were not observed by FISH in these cases. Results of another 7 cases were vice versa (Table 1). Between the two methods, κ value was 0.838. The results of the study indicated that efficiency of the two methods was equivalent to each other.

Among the 178 invasive ductal carcinoma, 22 (12.4%) were histologic Grade I, and 83 (46.6%) and 73 (41.0%) were Grades II and III, respectively. Seventy-seven (43.3%) were nuclear Grade I, and 84 (47.2%) and 17 (9.6%) were Grades II and III, respectively. *HER2/neu* amplification by FISH and chromogenic *in situ* hybridization was associated with poor nuclear grade (P = .043 and P = .037). However, it was not associated with histologic grade. One of the histologic characteristics, nuclear pleomorphism, was associated with *HER2/neu* amplification by FISH (P = .021). However, it was not associated with *HER2/neu* amplification by chromogenic *in situ* hybridization (P = .064). No significant association was found between tubule formation or mitotic counts and *HER2/neu* amplification (Table 2 and 3).

DISCUSSION

This is the first study that investigated the correlation between chromogenic *in situ* hybridization and FISH using tissue microarray technology. A study that was conducted using conventional histologic blocks demonstrated that the results of chromogenic *in situ* hybridization correlated well with those of FISH in archival breast carcinoma samples (10). A significant correlation between chromogenic *in situ* hybridization and FISH was affirmed in the current study with high concordance rate. High concordance between chromogenic *in situ* hybridization and FISH for the detection of *HER2/neu* status in the current study seems to be partly influenced by the advantage of tissue microarray technology. Problems of tissue heterogeneity could be minimized because three assay methods were performed on the consecutive sections of microarray blocks that contain almost the same cancer cells in their biologic states.

The current study indicated that tissue microarray technology was feasible for assaying gene amplification with a limited tissue volume. We used a 2-mm-sized needle for collecting microarray panels. A tissue microarray panel originally was developed that is as small as 0.6 mm in diameter (12).

TABLE 1. Comparison between FISH and CISH for the Detection of HER2 Amplification in 188 Breast Carcinomas

FISH	CISH		Total (%)
	No Amplification	Amplification	
No amplification	138 (97.2)	4 (2.8)*	142
Amplification	7 (15.2)	39 (84.8)	46

FISH = fluorescence in situ hybridization; CISH = chromogenic in situ hybridization. Concordance: 94.1% (177/188, kappa = 0.838).

* Two cases : aneusomy.

TABLE 2. Relationships between HER2/neu Amplification by FISH and Pathological Parameters

	HER2/neu (%)		P Value
	No Amplification	Amplification	
Histologic grade			.152
I	20 (90.9)	2 (9.1)	
II	63 (75.9)	20 (24.1)	
III	53 (72.6)	20 (27.4)	
Tubule formation			.644
1	9 (81.8)	2 (18.2)	
2	25 (78.1)	7 (21.9)	
3	102 (75.6)	33 (24.4)	
Nuclear pleomorphism			.021
1	5 (100.0)	0 (0.0)	
2	31 (88.6)	4 (11.4)	
3	100 (72.5)	38 (27.5)	
Mitotic counts			.118
1	72 (81.8)	16 (18.2)	
2	28 (73.7)	10 (26.3)	
3	36 (69.2)	16 (30.8)	
Nuclear grade			.043
I	54 (70.1)	23 (29.9)	
II	66 (78.6)	18 (21.4)	
III	16 (94.1)	1 (5.9)	

FISH = fluorescence in situ hybridization.

TABLE 3. Relationships between HER2/neu Amplification by CISH and Pathological Parameters

	HER2/neu (%)		
	No Amplification	Amplification	
Histologic grade			.140
I	19 (86.4)	3 (13.6)	
II	66 (79.5)	17 (20.5)	
III	53 (72.6)	20 (27.4)	
Tubule formation			.725
1	9 (81.8)	2 (18.2)	
2	25 (78.1)	7 (21.9)	
3	104 (77.0)	31 (23.0)	
Nuclear pleomorphism			.064
1	5 (100.0)	0 (0.0)	
2	30 (85.7)	5 (14.3)	
3	103 (74.6)	35 (25.4)	
Mitotic counts			.093
1	73 (83.0)	15 (17.0)	
2	28 (73.7)	10 (26.3)	
3	37 (71.2)	15 (28.8)	
Nuclear grade			.037
I	54 (70.1)	23 (29.9)	
II	69 (82.1)	15 (17.9)	
III	15 (88.2)	2 (11.8)	

CISH = chromogenic in situ hybridization.

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Heterogeneity of the breast carcinoma sometimes makes it difficult to accurately analyze the biologic properties of individual cancers, especially in antigens with heterogeneous staining patterns. Some investigators recommend that punching multiple small cores from different regions captures the heterogeneity of the tumors more effectively (13). We applied a large needle of 2-mm size in collecting the microarray panels to minimize the inadvertent variation in results from tumor heterogeneity. By the time of this writing, three studies have compared biomarker expression using tissue microarrays and conventional histologic sections of the same breast carcinomas (16–18). Three studies uniformly reported high concordance (90–95%) for expression of biomarkers such as estrogen receptor, progesterone receptor, and *HER2/neu*. Although the tissue microarray analysis did not represent specific tumor biology completely, tissue microarray analysis had a merit: it could be performed in consecutive sections that had the same cancer tissues in the same coordinate positions as the others.

A main obstacle to the popularization of FISH analysis has been the need to use special fluorescence microscopy with multi-bandpass fluorescence filters that makes it difficult for most institutes to integrate FISH in routine clinical diagnostics. The results of the current study indicated the practical superiority of chromogenic *in situ* hybridization over FISH in the assessment of gene amplification. Chromogenic *in situ* hybridization does not require equipment that does not already exist in routine pathologic laboratories. Moreover, most pathologists are familiar with peroxidase-based immunostaining. Another advantage of chromogenic *in situ* hybridization over FISH in routine practice is that simultaneous verification of histology can be done with chromogenic *in situ* hybridization. In FISH, sufficient histopathologic evaluation of the individual cells is impossible because of nuclear DAPI staining. The current study indicated that chromogenic *in situ* hybridization can be used instead of FISH in the screening of *HER2/neu* amplification in the primary breast carcinomas with feasibility and relative cost-effectiveness.

A commonly expressed concern is whether analysis of molecular targets on tissue microarray may result in lower prevalence than findings obtained from conventional histologic sections. In the results of the current study, prevalence of *HER2/neu* amplification was 22.9% by chromogenic *in situ* hybridization and 24.5% by FISH, which well coincides with the results of other studies (7–10, 19). The validity of tissue microarray analysis has been shown by comparisons with whole-section analysis in breast carcinoma (16–18). These results together with the current study indicate the feasibility of tissue microarray samples.

The main purpose in analyzing *HER2/neu* status is to provide the most effective therapeutic regimens for breast cancer patients. Most studies have reported a considerable disagreement in *HER2/neu* status between FISH and immunohistochemistry analysis (9, 20–22). Recently, an interesting report has been published (9). The investigators performed FISH and RNA-RNA *in situ* hybridization for *HER2/neu* on the same cancer tissues. Results of that study indicated that mRNA expression was highly concordant with FISH and that most cases of immunohistochemistry positive without gene amplification in FISH were devoid of mRNA expression. Hence, those investigators suggested that such cases were most likely false positive and nonspecific. Results from most studies comparing FISH and immunohistochemistry have indicated that FISH was superior to all other methodologies in assessing formalin-fixed, paraffin-embedded material for *HER2/neu* amplification (10–19).

Data from the current study indicate that tissue microarray analysis is a feasible and reliable method for assessing *HER2/neu* amplification with rapidity in a large number of tissues. High concordance of chromogenic *in situ* hybridization data with those of FISH indicates that chromogenic *in situ* hybridization can be a tempting alternative to FISH because of its accuracy and relative low cost. Chromogenic *in situ* hybridization with tissue microarray technology enables high-throughput determination of *HER2/ne* expression profile and its abnormalities in large cohorts of breast carcinoma. Integration of two novel technologies can provide a rapid validation of identified predictive markers in other cancer research fields.

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