Original Article

Breast Cancers Presenting Luminal B Subtype Features Show Higher Discordant Human Epidermal Growth Factor Receptor 2 Results Between Immunohistochemistry and Fluorescence In Situ Hybridization

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BACKGROUND: The aims of this study were to compare human epidermal growth factor receptor 2 (HER2) results between immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH), and to investigate the clinicopathological characteristics and outcomes according to their results. **METHODS:** Using consecutive tissue microarrays, IHC and FISH were performed as guidelines in 950 invasive breast cancers treated between November 1999 and August 2005. Characteristics and outcomes were retrospectively analyzed using a chi-square test, the Kaplan-Meier method, and Cox's model. **RESULTS:** FISH-positivity was observed in 2.6%, 4.8%, 28.1%, and 93.8% of IHC 0, 1+, 2+, and 3+, respectively, and the concordance rate between the 2 assays was 95.5%. IHC-positive or FISH-positive cases were associated with poorer differentiation, negative expression of hormone receptors, and higher proliferative index. Among IHC-equivocal or IHC-negative patients, positive FISH was negatively associated with survival in univariate and multivariate analyses. Among IHC-negative patients, tumors showing luminal B subtype features such as estrogen receptor (ER)-positive, grade II/III, and high Ki-67 presented discordantly high FISH-positivity. Among IHC-positive cases, FISH was not related to outcomes. **CONCLUSIONS:** The result of FISH is significantly related to prognosis of patients with IHC-negative or IHC-equivocal result. Therefore, FISH should be performed in IHC-equivocal cases. FISH assay might be considered for a selected group of patients with IHC-negative tumors showing luminal B subtype features of ER-positive, grade II/III, and high Ki-67 expression. ***Cancer*** **2012;118:914-23.** © *2011 American Cancer Society.*

KEYWORDS: breast cancer, fluorescence in situ hybridization, human epidermal growth factor receptor 2, immunohistochemistry, luminal B subtype.

Human epidermal growth factor receptor 2 (HER2) protein overexpression or gene amplification is found in 20% to 30% of breast cancers and is known to play important roles in the carcinogenesis and the prediction of clinical outcomes and therapeutic responses.[1-5] Among current several testing methods, immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) are approved by the U.S. Food and Drug Administration and are the most commonly used for assessing HER2 at many institutions. Both methods have complementary advantages and disadvantages.[6,7] The advantages of IHC are widely available, relatively inexpensive, quickly performed, easily preserved, and interpretable using light microscope. FISH has the advantages of more objective and quantitative scoring system, being less influenced by preanalytic factors, and a built-in internal control.

Accurate determination of HER2 is critically relevant in the management of breast cancer patients, but clinical situations are more complex. Although FISH has shown high concordance rates with IHC because tumors without amplified HER2 gene consistently did not present protein overexpression, a significant number of patients have demonstrated

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discrepant results between the 2 assays.[6,8-12] The most discordant cases are those with IHC score of 2+, and they are now considered as equivocal result. When IHC is used for the primary assessment of HER2, the current American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines recommend IHC-equivocal result be validated by FISH.[9,13] FISH is not mandatory for IHC-negative or IHC-positive cases.

Actually, HER2 protein, not the gene, is a definite target for trastuzumab. Accurate prediction of response to anti-HER2 therapy is one of clinical usages of HER2. Pivotal clinical trials using trastuzumab included metastatic breast cancer patients with IHC-equivocal or IHC-positive result, and retrospective analyses demonstrated that FISH is superior to IHC in selecting patients appropriate for trastuzumab therapy.[9,14] Even a small number of IHC-negative or IHC-positive patients have been reported to amplify HER2 gene or not by FISH, respectively.[8,11] Several questions regarding discordancies between IHC and FISH remain to be determined. In this study, we investigated the correlation between IHC and FISH performed by current guidelines. If 2 assays of our study are highly concordant as shown in previous studies, it is assumed that discrepant results might be associated with heterogeneous internal tumor-related features. The aims of this study were to determine clinicopathological characteristics and survival outcomes according to IHC and FISH, and to explore whether FISH provides any additional information in IHC-negative or IHC-positive patients, and if so, to characterize patients who are likely to benefit from FISH assay.

MATERIALS AND METHODS

Patient Selection

We had collected tumor specimens between November 1999 and August 2005. All 1153 samples were formalin-fixed and paraffin-embedded. All archival hematoxylin and eosin (H&E)-stained slides were reviewed by 2 pathologists. IHC and FISH were interpreted respectively in a blinded fashion, without any information regarding clinical parameters or outcomes. Among 1153 initial study population, 203 cases were excluded for analyses; pure in situ carcinoma (n = 38), recurrent or metastatic tumor (n = 10), invasive tumors that did not present invasive focus in the review of archival H&E-stained slides (n = 89), FISH-equivocal results (n = 15), and unreadable HER2 assays due to insufficient invasive foci on the tissue microarray (TMA) slides, overlapping nuclei, or lacking signals (n = 51). As a result, 950 invasive breast cancer patients were finally enrolled for analyses. Because most excluded cases were in situ carcinomas and small tumors with extensive intraductal components, excluded patients showed earlier stage and well-differentiated tumor (data not shown). This study was reviewed and approved by the institutional review board of Severance Hospital, Yonsei University Health System (4-2010-0136).

Data regarding patient characteristics and survival were retrospectively obtained from medical records. Patients were treated with either mastectomy or breast-conserving surgery and sentinel lymph node biopsy or axillary node dissection. After surgery, local radiotherapy or adjuvant therapy was administered if patients were able to tolerate it. Clinical follow-up included history taking, physical examination, laboratory tests, and radiologic imaging tests every 6-12 months for detection of relapse. Tumor stage was based on the 6th American Joint Committee on Cancer criteria. Histologic grade was assessed by the modified Bloom-Richardson classification. Disease-free survival (DFS) was measured from the date of the first curative surgery to the date of the first locoregional or systemic recurrence, or death without any type of relapse. Distant relapse-free survival (DRFS) was calculated from the date of the first operation to the date of distant metastasis or death without any type of relapse. Overall survival (OS) was measured from the date of the first surgery to the date of the last follow-up or death from any cause.

Tissue Microarray

Formalin-fixed, paraffin-embedded tumor blocks were arrayed using a tissue-arraying instrument (AccuMax Array, Petagen, Inc., Seoul, Korea). On each H&E slide of tumor block, invasive component was selected and a corresponding area was marked on the surface of tumor block. The designated zone of each donor block was punched with a tissue cylinder and a core sample of 3 mm in diameter with 3 to 5 mm in length was transferred to a 6×5 recipient block in a grid pattern. Normal breast tissues entrapped within the block. Each core was assigned with a unique TMA location number that was linked to a database containing clinicopathological parameters.

Immunohistochemistry

TMA blocks were subjected to immunohistochemical staining. Briefly, 5 μm-thick sections were obtained, deparaffinized, and rehydrated. After treatment with 3% hydrogen peroxide solution for 10 minutes to block

endogenous peroxidase, the sections were pretreated in 10 mM citrate buffer for antigen retrieval in a microwave oven for 20 minutes. After incubation with primary antibodies against estrogen receptor (ER; SP1, 1:100; Thermo Scientific, Fremont, Calif), progesterone receptor (PR; PgR 636, 1:50; DAKO, Glostrup, Denmark), HER2 (polyclonal, 1:1,500; DAKO), and Ki-67 (MIB-1, 1:100; DAKO), immunodetection was performed with biotinylated antimouse/rabbit immunoglobulin, followed by peroxidase-labeled streptavidin using a labeled streptavidin biotin kit with 3,3′-diaminobenzidine chromogen as a substrate. Slides were counterstained with hematoxylin.

Tumors with ≥1% nuclear-stained cells were considered positive for ER and PR as the ASCO/CAP guidelines.[15] The optimal cutoff for Ki-67 expression has not been determined.[16] We used an arbitrary value of 10% for Ki-67 because the mean ± standard deviation (SD) of Ki-67 was 9.4 ± 11.1 among our whole study population. HER2 staining was scored by counting the number of cells positively stained on the membrane and expressed as a percentage of total tumor cells according to the ASCO/CAP guidelines[13] using the following categories: 0, no immunostaining; 1+, weak incomplete membranous staining in any proportion of tumor cells; 2+, complete membranous staining, either nonuniform or weak in at least 10% of tumor cells; and 3+, uniform intense membranous staining in >30% of tumor cells. Tumors scored as 0 or 1+ were considered to be IHC-negative, those with 2+ were defined as IHC-equivocal, and cases scoring 3+ were considered IHC-positive.

FISH

FISH using PathVysion HER2 DNA Probe Kit (Abott, Abott Park, III) was manually performed in all patients. Briefly, consecutive sections from TMA blocks were mounted on ProbeOn Plus microscope slides (Fisher Scientific, Pittsburgh, PA), deparaffinized, and rehydrated. Afterward, they were boiled for 10 minutes in pretreatment solution, incubated with pepsin solution for 10 minutes, dehydrated in ethanol for 6 minutes, and finally air dried. For hybridization, the buffered probe (HER2/*neu* and centromere 17) was placed on the slide and protected by a coverslip that was sealed with rubber cement. For denaturation, slides were heated to 82°C and incubated overnight at 45°C in a dark humidified chamber. The rubber cement and coverslip were then removed, and the slides were transferred to stringent wash buffer for 10 minutes at 65°C. Then, the slides were dehydrated in ethanol for 6 minutes and air dried. Finally, they were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Evaluation of signals was performed using an epifluorescence microscope (Olympus, Tokyo, Japan) equipped with a fluorescein, Cy3, and DAPI filter set and a 100-W mercury lamp. Counting was performed according to the manufacturer's manual. Signals were counted in at least 60 invasive tumor nuclei each TMA cores. As the ASCO/CAP guidelines,[13] an absolute HER2 gene copy number <4 or HER2 gene/chromosome 17 copy number ratio (HER2/Chr 17 ratio) of <1.8 was considered FISH-negative; an absolute HER2 gene copy number between 4 and 6 or HER2/Chr 17 ratio between 1.8 and 2.2 was considered FISH-equivocal; and an absolute HER2 gene copy number >6 or HER2/Chr 17 ratio >2.2 was considered FISH-positive. Lymphocytes, fibroblasts, and normal ductal epithelial cells were used as internal controls.

Statistical Analysis

The differences between the groups were evaluated by an independent samples *t*-test in cases of continuous variables and by a chi-square test in cases of categorical variables. Fisher's exact test was used when appropriate. Survival curves were plotted using the Kaplan-Meier method and group differences in survival time were investigated by a log-rank test. A Cox hazards model was used to identify variables that were independently associated with survival. Logistic regression analysis was employed to investigate parameters associated with FISH-positivity in IHC-negative patients. All statistical tests were 2 sided and $P < .05$ was considered significant. SPSS version 17.0 (SPSS Inc., Chicago, IL) was used for all statistical analyses.

RESULTS

Patient Characteristics and Comparison of HER2 Results

The mean age at diagnosis and follow-up duration of 950 study population was 49.0 years (SD = 10.4) and 71.2 months (SD = 23.6), respectively. Patient characteristics are presented in Table 1. Only 1 patient received weekly adjuvant trastuzumab for 3 months due to financial burden, and 12 (1.3%) received anti-HER2 therapy in the metastatic setting regardless of HER2 results of this study.

IHC and FISH findings are summarized in Table 2. Patients with IHC scoring 0, 1+, 2+, and 3+ were 20.4%, 41.8%, 20.9%, and 16.8%, respectively. FISH-positivity was demonstrated in 24.2% of all cases. IHC and FISH results matched in 97.4% of patients with IHC

Table 1. Patient Characteristics

Factors	Number	%
Age (y)		
Mean ± SD	49.0 ± 10.4	
Median (range)	47.0 (20-87)	
Type		
Ductal	858	90.3
Lobular	25	2.6
Specified	67	7.1
Tumor stage		
T1	450	47.4
T2	476	50.1
T3-4	24	2.5
Node stage		
N0	508	53.5
N1	257	27.1
N2	116	12.2
N3	69	7.3
Grade		
I	174	18.3
II	511	53.8
III	265	27.9
Estrogen receptor		
Negative	257	27.1
Positive	693	72.9
Progesterone receptor		
Negative	353	37.2
Positive	597	62.8
Ki-67		
<10%	534	56.2
≥10%	415	43.7
Unknown	1	0.1
Type of surgery		
Breast-conserving surgery	268	28.2
Total mastectomy	682	71.8
Radiotherapy		
Not done	517	54.4
Done	433	45.6
Chemotherapy		
Not done	152	16.0
Done	798	84.0
Endocrine therapy		
Not done	316	33.3
Done	634	66.7
Anti-HER2 therapy		
Not done	937	98.6
Done	13	1.4

Abbreviations: HER2, human epidermal growth factor receptor 2; SD, standard deviation.

0, 95.2% of IHC 1+, and 93.8% of IHC 3+. Discrepancies between IHC and FISH were observed in 2.6% of patients with IHC 0, 4.8% of IHC 1+, and 6.3% of IHC 3+. When considering 751 IHC-negative or IHC-positive patients, overall concordance and discordance rates between the 2 assays were 95.5% and 4.5%, respectively. In patients with IHC 2+, 71.9% did not amplify the HER2 gene and 28.1% showed FISH-positivity.

Table 2. Comparison of HER2 Results Between Immunohistochemistry and Fluorescence In Situ Hybridization

IHC Scoring	FISH		Total (n = 950, %)
	Negative (n = 720, %)	Positive (n = 230, %)	
0	189 (97.4)	5 (2.6)	194 (20.4)
1+	378 (95.2)	19 (4.8)	397 (41.8)
2+	143 (71.9)	56 (28.1)	199 (20.9)
3+	10 (6.3)	150 (93.8)	160 (16.8)

Abbreviations: FISH, fluorescence in situ hybridization; IHC, immunohistochemistry.

Characteristics and Outcomes According to Results of IHC and FISH

By analyses of characteristics according to IHC (data not shown), IHC-positive patients were significantly associated with poorly differentiated tumors ($P < .001$), ER-negativity ($P < .001$), PR-negativity ($P < .001$), and higher proliferative index ($P < .001$). When cases were divided into 2 groups as IHC-negative or IHC-equivocal versus IHC-positive results, these statistical associations were maintained similarly to analyses of 3 IHC subgroups. DFS, DRFS, and OS according to IHC are demonstrated in Figure 1A-C. IHC-positive patients showed a trend toward poorer survival without statistical significance. When patients were dichotomized into 2 groups as IHC 0, 1+, or 2+ versus IHC 3+, DFS of IHC 3+ patients was significantly poorer than that of IHC 0, 1+, or 2+ cases ($P = .041$). However, DRFS and OS between IHC-positive patients and IHC-equivocal or IHC-negative cases were not different ($P = .171$ for DRFS, $P = .343$ for OS).

Analyses of clinicopathological features according to FISH determined that FISH-positive patients were significantly associated with poorly differentiated tumors ($P < .001$), ER-negativity ($P < .001$), PR-negativity ($P < .001$), and higher proliferative index ($P < .001$), as shown in analyses of IHC (data not shown). DFS, DRFS, and OS according to FISH are shown in Figure 1D-F. FISH-positive patients were significantly related to poorer survival.

Characteristics and Survival According to FISH Stratified by IHC Results

Patient characteristics according to FISH stratified by IHC results are shown in Table 3. In IHC-negative

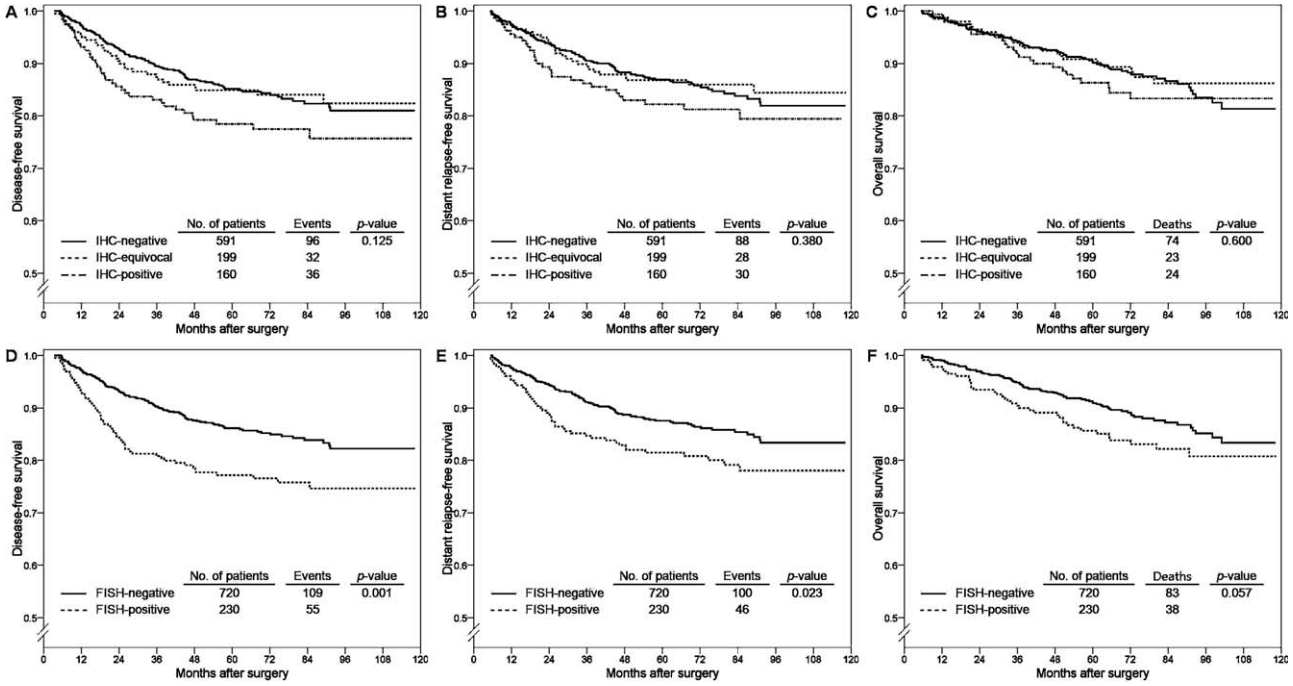

Figure 1. Disease-free (A, D), distant relapse-free (B, E), and overall (C, F) survival according to results of human epidermal growth factor receptor 2 (HER2) immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) are shown.

patients, no difference in characteristics was observed between FISH-negative and FISH-positive subgroups. In IHC-equivocal patients, FISH-positive cases were significantly associated with ER-negativity and PR-negativity. In IHC-positive patients, FISH-positive subgroup was significantly related to ER-negativity, PR-negativity, and higher Ki-67 proliferative index. The mean ± SD of percentage of ER, PR, and Ki-67 expression was 63.0 ± 26.5, 50.0 ± 29.4, and 5.3± 3.7 in IHC-positive/FISH-negative cases and 32.0± 34.8, 18.6 ± 29.4, and 10.9 ± 7.6 in IHC-positive/FISH-positive cases, respectively ($P = .005$, .001, and .023, respectively; *t*-test).

Survival according to FISH in patients with IHC-negative, IHC-equivocal, and IHC-positive result is presented in Figures 2, 3, and 4, respectively. Among IHC-negative patients, FISH-positive cases showed poorer DFS, DRFS, and OS with borderline significance (Fig. 2). Among IHC-equivocal patients, FISH-positive subgroups were significantly associated with poorer DFS, DRFS, and OS (Fig. 3). However, among IHC-positive patients, there was no difference between FISH-negative and FISH-positive cases (Fig. 4).

Multivariate analyses adjusted for FISH, age at diagnosis, tumor and nodal stages, grade, and ER in IHC-negative, IHC-equivocal, and IHC-positive patients are shown in Tables 4, 5, and 6, respectively. In IHC-negative patients, FISH-positivity was a significant factor for DFS, but was not associated with DRFS and OS (Table 4). In IHC-equivocal patients, positive FISH showed a negative relationship with DFS, DRFS, and OS by Cox models (Table 5). In IHC-positive patients, however, FISH was not associated with outcomes (Table 6).

Factors Associated with Discordant HER2 Amplification in IHC-Negative Patients

Logistic regression model was applied to explore which subgroup with IHC-negative result was associated with HER2 amplification (Table 7). Of 591 IHC-negative cases, 590 patients who had all parameters available were analyzed with this model. Tumors with ER-positive, grade II/III, and higher Ki-67 proliferative index had a higher risk for discordant HER2 amplification. Age at diagnosis, tumor and node stages, and PR expression did not increase the risk of FISH-positive results. Among our dataset of IHC-negative patients, 8 (9.3%) of 86 patients with ER-positive, grade II/III and Ki-67 ≥10% demonstrated discordantly FISH-positive results compared with 3 (2.4%) of 124 patients with ER-negative, grade II/III, and Ki-67 ≥10% ($P = .054$, Fisher's exact test) or 16 (3.2%) of 504 patients without having all these risk factors ($P = .015$, Fisher's exact test).

Table 3. Characteristics According to Fluorescence In Situ Hybridization Stratified by Immunohistochemical Results

Factors	IHC-Negative			IHC-Equivocal			IHC-Positive		
	FISH-Negative (n = 567)	FISH-Positive (n = 24)	P[a]	FISH-Negative (n = 143)	FISH-Positive (n = 56)	P[a]	FISH-Negative (n = 10)	FISH-Positive (n = 150)	P[b]
Age (y)			>.999[b]			.845			.486
≤35	38 (6.7)	1 (4.2)		14 (9.8)	6 (10.7)		1 (10.0)	9 (6.0)	
>35	529 (93.3)	23 (95.8)		129 (90.2)	50 (89.3)		9 (90.0)	141 (94.0)	
Tumor stage			.966			.875			>.999
T1	281 (49.6)	12 (50.0)		57 (39.9)	23 (41.1)		5 (50.0)	72 (48.0)	
T2-4	286 (50.4)	12 (50.0)		86 (60.1)	33 (58.9)		5 (50.0)	78 (52.0)	
Node stage			.331			.303			.751
N0	317 (55.9)	11 (45.8)		65 (45.5)	30 (53.6)		6 (60.0)	79 (52.7)	
N1-3	250 (44.1)	13 (54.2)		78 (54.5)	26 (46.4)		4 (40.0)	71 (47.3)	
Grade			.768			.076			.320
I/II	417 (73.5)	17 (70.8)		114 (79.7)	38 (67.9)		8 (80.0)	91 (60.7)	
III	150 (26.5)	7 (29.2)		29 (20.3)	18 (32.1)		2 (20.0)	59 (39.3)	
Estrogen receptor			.152			.017			.005
Negative	144 (25.4)	3 (12.5)		24 (16.8)	18 (32.1)		0 (0.0)	68 (45.3)	
Positive	423 (74.6)	21 (87.5)		119 (83.2)	38 (67.9)		10 (100)	82 (54.7)	
Progesterone receptor			.697			<.001			.002
Negative	187 (33.0)	7 (29.2)		36 (25.2)	29 (51.8)		1 (10.0)	93 (62.0)	
Positive	380 (67.0)	17 (70.8)		107 (74.8)	27 (48.2)		9 (90.0)	57 (38.0)	
Ki-67[c]			.208			.193			.016
<10%	355 (62.7)	12 (50.0)		86 (60.1)	28 (50.0)		7 (70.0)	46 (30.7)	
≥10%	211 (37.3)	12 (50.0)		57 (39.9)	28 (50.0)		3 (30.0)	104 (69.3)	

Abbreviations: FISH, fluorescence in situ hybridization; IHC, immunohistochemistry.
[a] Chi-square test.
[b] Fisher's exact test.
[c] Of 591 patients with IHC-negative result, 590 were available for Ki-67 expression.

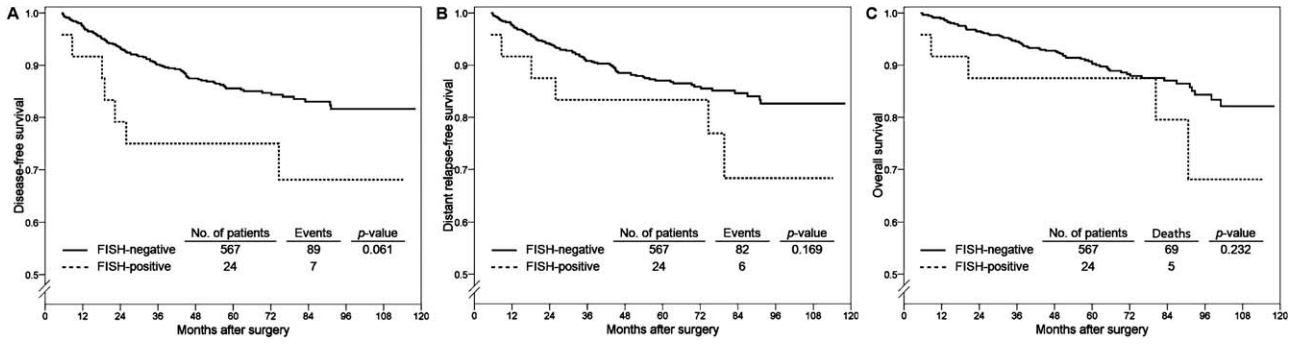

Figure 2. Disease-free (A), distant relapse-free (B), and overall (C) survival according to fluorescence in situ hybridization (FISH) in immunohistochemistry-negative patients are shown.

DISCUSSION

In real clinical situations, discordant results between IHC and FISH have been demonstrated, and such cases may be problematic. Unnecessary overtreatment may increase side effects and costs without clinical benefits, whereas undertreatment may affect patient's outcomes. In previous reports, HER2 amplification was identified in 2%-11% of patients with IHC 0 or 1+ and in 15%-48% of those with IHC 2+, and no amplification was observed in 5%-24% of cases with IHC 3+.[8-12] If HER2-positivity is

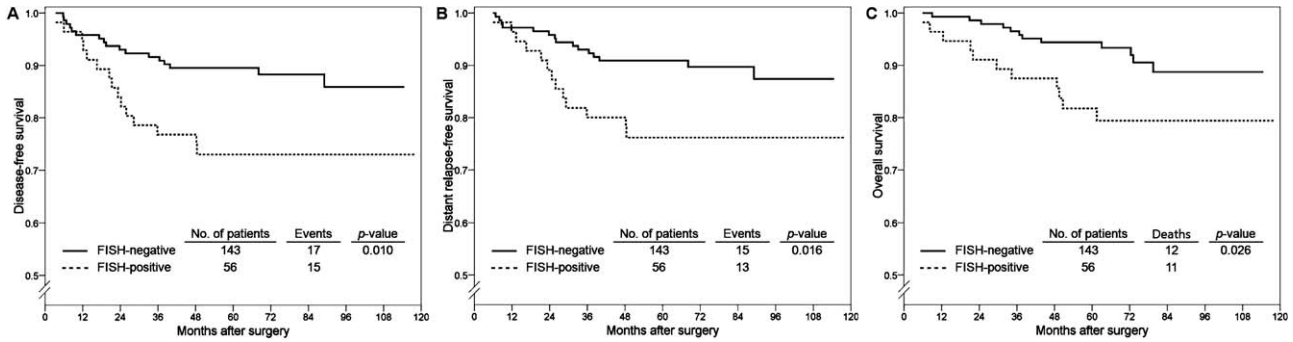

Figure 3. Disease-free (A), distant relapse-free (B), and overall (C) survival according to fluorescence in situ hybridization (FISH) in immunohistochemistry-equivocal patients are shown.

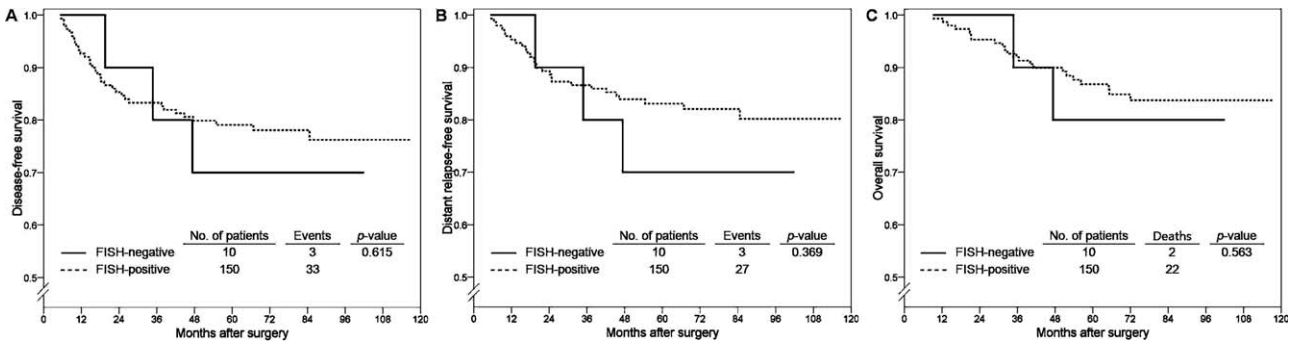

Figure 4. Disease-free (A), distant relapse-free (B), and overall (C) survival according to fluorescence in situ hybridization (FISH) in immunohistochemistry-positive patients are shown.

defined as the ASCO/CAP guidelines (Table 2), 216 (22.7%) patients in our study were determined to be HER2-positive, which is consistent with previous reports.[3] When excluding IHC-equivocal cases, overall concordance rates in our study were 95.5% between the 2 assays. These results were not significantly different from earlier reports,[8,10,11] confirming high concordance between IHC and FISH.

Characteristics of IHC-positive patients were not different from those of FISH-positive cases in the present study. These features were poorly differentiation, ER-negativity, PR-negativity, and higher proliferative index as widely known features of HER2-positive tumors.[17] However, regarding prediction of prognosis, FISH more clearly split survival outcomes with significance than IHC interpretations classified into both 2 (0, 1+, 2+ vs 3+) and 3 (0, 1+ vs 2+ vs 3+) groups. These findings were consistent with a previous study by Pauletti et al.[18] Although our study was different from the 1 by Pauletti et al[18] with respect to patient characteristics, genetics of study cohort (Asian vs South Australian), methodology including used commercial antibody, and interpretation criteria, taken together, FISH better predicts adverse outcomes and is considered as ideal method for assessing HER2.

It has not been clearly understood why protein overexpression without gene amplification is documented or gene amplification not being able to yield protein overexpression is possible. Several explanations have been suggested mainly in the aspect of methodology and external validation because IHC-positive/FISH-negative cases are the majority of discordance.[9,10,19] However, patients with discrepancies between IHC and FISH may be associated with potential biologic causes. These are chromosome 17 polysomy,[20] dysregulated transcriptional activity,[21] alternative splicing, which produces inhibitory isoforms,[22] gene rearrangements affecting a part of HER2 epitope,[12] altered stability of HER2 protein in tumor cells,[23] and a real intratumoral heterogeneity of HER2.[24]

Although equivocal IHC result should be validated by FISH, it is still controversial whether FISH provides additional information in IHC-negative or IHC-positive cases. After stratification by IHC findings, we further explored outcomes according to FISH. Among

Table 4. Multivariate Analyses in Immunohistochemistry-Negative Patients

Factors	Disease-Free Survival			Distant Relapse-Free Survival			Overall survival		
	Hazard Ratio	95% CI	P^a	Hazard Ratio	95% CI	P^a	Hazard ratio	95% CI	P^a
FISH (negative vs positive)	2.203	1.011-4.803	.047	1.789	0.774-4.136	.174	1.815	0.724 - 4.552	0.204
Age (≤35 vs >35)	0.566	0.300-1.069	.080	0.569	0.292-1.109	.098	0.609	0.301 - 1.232	0.167
Tumor stage (T1 vs T2-4)	1.870	1.170-2.988	.009	1.835	1.120-3.007	.016	1.816	1.045 - 3.155	0.034
Node stage (N0 vs N1-3)	2.608	1.659-4.100	<.001	2.930	1.806-4.754	<.001	2.687	1.594 - 4.531	<.001
Grade (I/II vs III)	1.108	0.671-1.829	.689	1.120	0.666-1.884	.668	1.059	0.608 - 1.845	0.838
ER (negative vs positive)	0.651	0.386-1.096	.106	0.658	0.383-1.130	.129	0.547	0.309 - 0.967	0.038

Abbreviations: CI, confidence interval; ER, estrogen receptor; FISH, fluorescence in situ hybridization.
[a] Log-rank test.

Table 5. Multivariate Analyses in Immunohistochemistry-Equivocal Patients

Factors	Disease-Free Survival			Distant Relapse-Free Survival			Overall survival		
	Hazard Ratio	95% CI	P^a	Hazard Ratio	95% CI	P^a	Hazard ratio	95% CI	P^a
FISH (negative vs. positive)	3.011	1.372-6.610	.006	3.011	1.296-6.991	.010	2.566	1.008-6.532	.048
Age (≤35 vs >35)	0.324	0.135-0.777	.012	0.343	0.133-0.887	.027	0.292	0.109-0.781	.014
Tumor stage (T1 vs T2-4)	1.561	0.697-3.498	.279	1.187	0.517-2.726	.686	1.572	0.585-4.223	.369
Node stage (N0 vs N1-3)	2.540	1.102-5.855	.029	3.464	1.350-8.885	.010	3.739	1.229-11.377	.020
Grade (I/II vs III)	0.374	0.136-1.029	.057	0.335	0.109-1.024	.055	0.441	0.142-1.369	.157
ER (negative vs positive)	0.808	0.348-1.879	.621	0.807	0.325-2.008	.645	0.436	0.171-1.111	.082

Abbreviations: CI, confidence interval; ER, estrogen receptor; FISH, fluorescence in situ hybridization.
[a] Log-rank test.

Table 6. Multivariate Analyses in Immunohistochemistry-Positive Patients

Factors	Disease-Free Survival			Distant Relapse-Free Survival			Overall survival		
	Hazard Ratio	95% CI	P^a	Hazard Ratio	95% CI	P^a	Hazard ratio	95% CI	P^a
FISH (negative vs positive)	0.757	0.221-2.592	.658	0.608	0.174-2.125	.436	0.624	0.136 - 2.855	.543
Age (≤35 vs >35)	1.263	0.298-5.358	.751	1.061	0.247-4.550	.936	1.623	0.215 - 12.235	.639
Tumor stage (T1 vs T2-4)	2.253	1.013-5.012	.046	3.105	1.238-7.789	.016	5.097	1.625 - 15.981	.005
Node stage (N0 vs N1-3)	1.497	0.717-3.123	.283	1.353	0.610-3.001	.457	1.217	0.508 - 2.912	.660
Grade (I/II vs III)	0.627	0.291-1.353	.234	0.727	0.319-1.658	.448	0.991	0.416 - 2.359	.984
ER (negative vs positive)	1.085	0.534-2.207	.821	1.207	0.550-2.650	.639	1.087	0.460 - 2.570	.849

Abbreviations: CI, confidence interval; ER, estrogen receptor; FISH, fluorescence in situ hybridization.
[a] Log-rank test.

IHC-negative patients, FISH-positive cases showed poorer DFS in univariate analyses and FISH positivity was a significantly negative prognostic factor when adjusted for other clinicopathological variables. Our findings suggest that FISH might provide additional clinical implications on the prediction of prognosis and another indication for anti-HER2 therapy in a small minority of IHC-negative subgroup. Actually the predictive significance of HER2 was not able to be determined in the present study because most our study cohort did not receive anti-HER2 therapy. Although it is not yet determined whether trastuzumab therapy works or improves survival in patients with IHC-negative/FISH-positive or not, recent studies suggest FISH identifies patients who are likely to benefit from trastuzumab therapy.[9,14] This hypothesis needs to be confirmed through other dataset or new prospective clinical trials because many trials have excluded IHC-negative patients at the time of enrollment screening.

IHC-positive patients demonstrated no difference in outcomes according to FISH. Nevertheless, these findings did not determine that FISH did not provide any information of IHC-positive cases. Because HER2 protein-overexpressed tumors without gene amplification are associated with better clinicopathological features such as

Table 7. Logistic Regression for HER2 Amplification in Immunohistochemistry-Negative Patients

Factors	Odds Ratio	95% CI	*P*[a]
Age (≤35 vs >35)	1.629	0.205-12.946	.644
Tumor stage (T1 vs T2-4)	0.688	0.277-1.705	.419
Node stage (N0 vs N1-3)	1.505	0.609-3.718	.375
Grade (I vs II/III)	7.647	0.988-59.164	.051
ER (negative vs positive)	5.718	1.248-26.192	.025
PR (negative vs positive)	0.764	0.255-2.294	.632
Ki-67 (<10% vs ≥10%)	2.477	0.987-6.216	.053

Abbreviations: CI, confidence interval; ER, estrogen receptor; PR, progesterone receptor.
[a] Logistic regression analysis.

lower grade, higher hormone receptor expression, and lower proliferative index, FISH might suggest a distinct heterogeneity of HER2-positive breast cancers.[21] Implications of FISH on the prediction of outcomes and responsiveness to anti-HER2 therapy in IHC-positive cases remain to be determined.

From the viewpoint of cost-effectiveness, additional FISH test might provide little benefits due to the very low incidence of discrepancy, relatively high costs, and unproven efficacy of anti-HER2 therapy in IHC-negative/FISH-positive patients.[25,26] However, if a subgroup is determined as having a higher probability of FISH-positivity among IHC-negative patients, additional FISH may provide clinical benefits to those subgroups. Our IHC-negative subgroup analyses showed that ER-positivity, grade II/III, and higher Ki-67 proliferative index were associated with discordantly higher risk for HER2 gene amplification. It was unexpected that ER-positivity was associated with FISH-positivity because HER2 amplification was significantly related to negative expression of steroid hormone receptors. However, if considering FISH result as the gold standard for determining HER2, undifferentiation, and higher proliferation, our findings may present undetermined heterogeneous characteristics of luminal B molecular subtype. A clear immunohistochemical definition of luminal B subtype is still being debated. In addition to ER-positivity and/or PR-positivity, various definitions such as HER2-positive,[27] high grade,[28] and high Ki-67 proliferative index[29] have been included in the criteria for luminal B subtype. It is not clear whether aberrant HER2 signal pathways at transcriptional or translational levels induce luminal B subtype to amplify the HER2 gene without protein overexpression. Further basic or clinical research is necessary, and cost-effectiveness of additional FISH test in IHC-negative cases should be determined because economic burden of HER2 assays and anti-HER2 therapy is different among nations.

Limitations of our study were TMA results could not verify the identical results from whole sections, which is the standard material for HER2 assays. Because 1 core sample of tumor block was used, that may have impacted interpretation of HER2 assays. Furthermore, the retrospective nature and different characteristics between present study cohort and other population make our tentative findings should be validated and replicated independently.

In summary, IHC is highly concordant with FISH in the assessment of HER2 status, and FISH better predicts prognosis of patients with invasive breast cancer. However, a small number of discrepancies between the 2 assays are still reported and show different clinicopathological features and survival outcomes. As current clinical guidelines, FISH assay should be recommended to IHC-equivocal cases. Higher discordant HER2 gene amplification is demonstrated in IHC-negative tumors showing luminal B molecular subtype features of ER-positivity, undifferentiation and higher Ki-67 proliferative index. FISH test might be considered for a selected subgroup of patients with IHC-negative result and additional clinical benefits of FISH assay including the predictive significance should be determined in those patients for improving outcomes.

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