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Dual-color silver-enhanced *in situ* hybridization for assessing *HER2* gene amplification in breast cancer

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Amplification of the *human epidermal growth factor receptor 2* (*HER2*) gene occurs in 20–25% of breast cancers, and is recognized as a prognostic and predictive marker. *HER2* gene amplification, evaluated as a change in protein expression or gene copy number, can be identified by a number of methods. Fluorescence *in situ* hybridization (FISH) is considered the gold standard for *HER2* gene copy number determination; however, a number of impediments prevent its wider use in a clinical setting. The aims of our study were to compare dual-color silver-enhanced *in situ* hybridization (SISH) with single-color SISH and FISH on formalin-fixed, paraffin-embedded sections, and to validate its use as a routine method for assessing *HER2* status in breast cancers. A total of 146 invasive breast carcinoma cases were assessed for *HER2* gene amplification by FISH and dual-color SISH. Dual-color SISH and FISH results exhibited a concordance rate of 97% ($\kappa = 0.912$). A comparison of the single-color SISH method with dual-color SISH showed that 142 of 146 cases were in agreement (97%, $\kappa = 0.930$). Our results showed that dual-color SISH is a viable alternative to FISH that offers a number of advantages in a clinical setting.
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Amplification of the *human epidermal growth factor receptor 2* (*HER2*) gene occurs in 20–25% of breast cancers, and is recognized as a prognostic and predictive marker.[1] The use of humanized mouse monoclonal antibody-based therapy targeting the *HER2* oncogene is now expanding to adjuvant therapy.[2,3] The biological effects of monoclonal antibody therapy include inhibition of HER2 dimerization, decreased cellular proliferation, induction of apoptosis, and modulation of signal transduction pathways.[4]

HER2 gene amplification, evaluated either as a change in protein expression or gene copy number, can be identified by a number of methods, the most common of which are immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH). Globally, the algorithm for HER2 testing to determine patient eligibility for therapy starts with the performance of IHC to assess HER2 protein overexpression.[5] Next, cases showing weak immunopositivity (2+) are assessed for amplification of the *HER2* gene using a method that can detect gene copy number. The use of a centromere 17 (CEP17) probe has been recommended for accurately assessing *HER2* gene copy number so as not to misinterpret chromosome 17 polysomy as *HER2* amplification.[4]

The gold standard for *HER2* gene copy number evaluation is considered to be FISH, which provides simultaneous viewing of both signals without an obscured CEP17 probe in cases with *HER2* amplification.[6] However, FISH is technically demanding, expensive, requires fluorescence microscopy, and its fluorescence signal fades over time.[5] Collectively, these limitations create impediments for the widespread use of FISH in a clinical setting.

Silver-enhanced *in situ* hybridization (SISH), which has been introduced as an alternative to FISH, combines the accuracy of FISH with the use of

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light opaque silver instead of fluorescent spot-like signals. As SISH can be performed more rapidly than FISH and requires only a conventional light microscope, it would be more appropriate for routine use in pathology laboratories. Our previous study using single-color SISH showed a 98% concordance rate between SISH and FISH methods.[7]

Nitta *et al*[8] published the development of dual-color SISH technique for assessing *HER2* gene status. Recently, Fritzsche *et al*[9] reported a correlation study between dual-color SISH and FISH. They used cytology specimens for assessing dual-color SISH, and showed a high concordance rate with FISH. Dual-color SISH is based on the use of two separate probes in a single slide: a HER2 probe, resulting in a black signal, and a CEP17 probe, resulting in a red signal. This method enables calculation of HER2/CEP17 ratios and detection of chromosome 17 polysomy on a single slide. The aim of this study was to confirm the good correlation among this new dual-color SISH method and single-color SISH and FDA-approved FISH for assessing *HER2* gene status on formalin-fixed, paraffin-embedded sections.

Materials and methods

Single-color SISH, FISH, and IHC data obtained from a previously reported study were used.[7]

Case Selection

A total of 201 consecutive invasive breast cancer cases diagnosed and treated surgically during 2003 and 2004 at the Asan Medical Center, Seoul, were selected. Written informed consent was obtained from each patient at the time of surgery. In all cases, samples were formalin-fixed, paraffin-embedded, and processed in a pathology laboratory according to standardized institutional protocols.

Construction of Tissue Microarray Blocks

Formalin-fixed, paraffin-embedded tissue samples were arrayed using a tissue-arraying instrument (AccuMax Array, Seoul, Korea). In brief, representative areas of the invasive tumor portion were selected and marked on a hematoxylin and eosin-stained slide, and its corresponding tissue block was sampled. The designated zone of each donor block was punched with a tissue cylinder 1 mm in diameter, and the sample was transferred to a recipient block. Each sample was arrayed in duplicate to minimize tissue loss and to overcome tumor heterogeneity.

Fluorescence *In Situ* Hybridization

Consecutive sections from microarray blocks were cut at 5 μm thickness and mounted on SuperFrost + / + slides. Deparaffinizing, pretreatment, and protease digestion procedures followed the Abbott PathVysion *HER2* DNA Probe Kit protocol (Abbott Laboratories, Abbott Park, Des Plaines, USA), with additional monitoring for the progress of proteolytic digestion by propidium iodide staining. Probe mixes were hybridized at 37°C between 14 and 18 h. After hybridizations, slides were washed in 2 × SSC/0.3% NP-40 at 72°C for 30 min, air dried, and counterstained with DAPI.

Single-Color SISH

For single-color SISH, 5 μm-thick sections from the microarray block were prepared. Automated SISH of slides were performed according to the manufacturer's protocols for INFORM *HER2* DNA and chromosome 17 probes (INFORM *HER2* DNA probe and ultraView SISH Detection Kit, Ventana Medical Systems, Tucson, USA).[10] Both probes were labeled with dinitrophenol (DNP) and optimally formulated for use with the ultraView SISH Detection Kit and accessory reagents from Ventana's Benchmark series of automated slide stainers. The *HER2* DNA probe was denatured at 95°C for 12 min and hybridization was performed at 52°C for 2 h. After hybridization, appropriate stringency washes (3 times at 72°C) were performed. The chromosome 17 probe was denatured at 95°C for 12 min and hybridization was performed at 44°C for 2 h in a separate slide. After hybridization, appropriate stringency washes (3 times at 59°C) were performed. *HER2* and chromosome 17 DNP-labeled probes were visualized using the rabbit anti-DNP primary antibody and the ultraView SISH Detection Kit, which contains a goat anti-rabbit antibody conjugated to horseradish peroxidase used as the chromogenic enzyme. Silver precipitation is deposited in the nuclei after the sequential addition of silver acetate, hydroquinone, and H_2O_2 and a single copy of the *HER2* gene is visualized as a black dot. The specimen is then counterstained with Harris hematoxylin.

Dual-Color SISH

For dual-color SISH, 5-μm-thick sections from the microarray block were prepared. Dual-color SISH slides were also processed using an automated system that followed the manufacturer's protocols for INFORM *HER2* DNA and chromosome 17 probes.[8] Both probes were sequentially hybridized in one slide. A single copy of the *HER2* gene is visualized as a black dot. A red dot for chromosome 17 appears following the reaction with fast red and naphthol phosphate. The specimen is then counterstained with Harris hematoxylin.

Scoring Criteria

We interpreted only invasive carcinoma areas in TMA slides. We excluded cases that failed to

demonstrate one or both of the two signals. Normal HER2 or CEP17 signals served as the internal positive control in endothelial cells, stromal fibroblasts, and lymphocytes.

Dual-color SISH and single-color SISH signals were visualized as single copies, multiple copies, and clusters. A discrete dot was counted as a single copy of *HER2* or CEP17. The size of these single dots was used as a reference to determine the relative number of amplified copies in the cancer cell nuclei. In some nuclei, clusters of dots representing many copies of the *HER2* gene were apparent. A small cluster of multiple signals was counted as 6 signals and a large cluster as 12 signals.

We interpreted results using the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines for all three methods.[11] HER2 and CEP17 signals were enumerated in 20 nuclei within a target area, and the HER2/CEP17 ratio was calculated. Cases with a HER2/CEP17 ratio <1.8 were considered negative for *HER2* gene amplification, whereas those with a HER2/CEP17 ratio >2.2 were considered positive for *HER2* gene amplification. If a HER2/CEP17 ratio fell on or between the values of 1.8 and 2.2, we counted the number of signals in an additional 20 nuclei in a second target area. The *HER2*/CEP17 ratio was then calculated from both target areas (40 cells). Polysomy 17 was defined as three or more copy numbers of an average CEP17.[11–14]

Statistics

Only cases that yielded informative results with all techniques were included in the concordance analyses. Concordance rates between different methods were determined and κ-statistics calculated by Cohen's κ. A κ-value of 1 denotes complete agreement, a value of 0.75 denotes excellent agreement; values between 0.4 and 0.75 denote fairly good agreement, and values <0.4 denote poor agreement. Statistical analysis was performed using SPSS, version 18.0.

Results

We performed FISH, single-color SISH, and dual-color SISH to validate the feasibility of using dual-color SISH as a *HER2* amplification assay. Of the 201 invasive breast carcinoma cases, 55 could not be evaluated because only a normal breast tissue was present (15 cases), or because only the ductal carcinoma *in situ* component was present (10 cases), or because the assay failed to demonstrate HER2 or CEP17 signals (18, 14, and 18 cases for FISH, single-color SISH, and dual-color SISH, respectively). Thus, 146 cases for which results were obtained from all three methods were available for the comparative analysis reported in this study. The characteristics of the 146 patients enrolled in this study are described in Table 1.

Signals obtained by dual-color SISH demonstrated obvious black signals for HER2 and red signals for CEP17 (Figure 1). In cases with no amplification or low amplification, both signals could be counted without difficulty. Although interpretation was not difficult in cases with high amplification, the presence of numerous black signals occasionally tended to obscure red signals.

HER2 amplification was identified in 38 cases (26%) by FISH and in 37 cases (25%) by dual-color SISH. In contrast to FISH, which yielded no equivocal cases, two cases were equivocal by dual-color SISH. The dual-color SISH method showed a 97% concordance rate (141 cases) with FISH. There were five discrepancies: two were FISH negative and dual-color SISH equivocal, two were FISH positive and dual-color SISH negative, and one

Table 1 Baseline characteristics of patients ($n=146$)

Characteristics at diagnosis	*No. of patients (%)*
Age, median (range)	
<50	100 (69)
≥ 50	46 (31)
Histological subtype	
Invasive ductal	112 (77)
Invasive lobular	4 (3)
Micropapillary	4 (3)
Mucinous	4 (3)
Metaplastic	2 (1)
Medullary	1 (1)
Mixed	19 (13)
Histological grade	
I	9 (6)
II	70 (48)
III	67 (46)
Estrogen receptor	
Negative	59 (40)
Positive	87 (60)
Progesteron receptor	
Negative	74 (51)
Positive	72 (49)
T stage	
T1	76 (52)
T2	60 (41)
T3	10 (7)
T4	0(0)
N stage	
NX	3 (2)
N0	79 (54)
N1	41 (28)
N2	11 (8)
N3	12 (8)
M stage	
M0	136 (93)
M1	10 (7)

LN, lymph node.

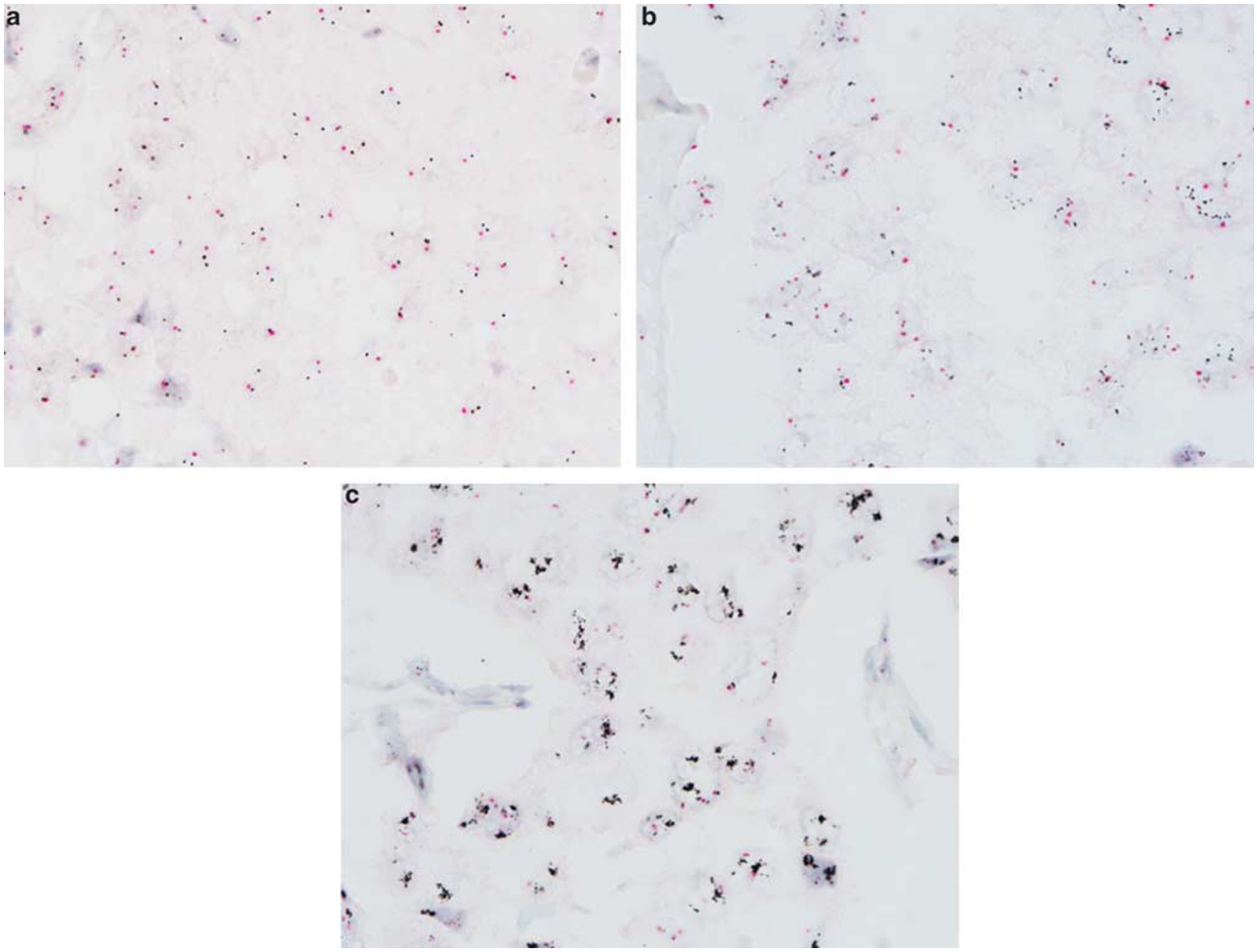

Figure 1 Micrographs demonstrating dual-color SISH of the *HER2* oncogene (black signal) and CEP17 (red signal) in tissues with (**a**) no *HER2* amplification, (**b**) low-level *HER2* amplification, and (**c**) high-level *HER2* amplification ($\times 1000$).

Table 2 Comparison of FISH and dual-color SISH

	Dual-color SISH			
FISH	*No amplification*	*Amplification*	*Equivocal*	*Total*
No amplification	105	1	2	108
Amplification	2	36	0	38
Equivocal	0	0	0	0
Total	107	37	2	146

FISH, fluorescence *in situ* hybridization; SISH, silver *in situ* hybridization.

was dual-color SISH positive and FISH negative (Tables 2 and 3).

A comparison of the single-color SISH method with dual-color SISH showed that 142 of 146 cases were in agreement (97%, $\kappa = 0.930$). Of the four discrepant cases, two showed amplification by dual-color SISH but were negative by single-color SISH, and two were equivocal by one method and negative by the other (Tables 3 and 4).

Polysomy was identified in 25 cases (17%) by single-color SISH and in 20 cases (14%) by dual-color SISH. All 20 polysomy cases that were identified by the dual-color method were included in polysomy cases detected by single-color SISH.

Discussion

Previous investigators have reported overall concordance rates between FISH and dual-color chromogenic *in situ* hybridization (CISH) assays ranging from 91 to 100%,[15,16] establishing dual-color CISH as a viable alternative to FISH. Dietel *et al*[10] reviewed a series of 99 invasive breast carcinomas using automated single-color SISH and FISH, and reported an overall concordance rate of 96%. Numerous other studies comparing FISH and single-color SISH methods, including a previous study from our laboratory, have shown concordance rates from 94 to 99.6%.[4,7] Using dual-color SISH and FISH, Nitta *et al*[8] reported a 95.7% concordance rate on formalin-fixed, paraffin-embedded specimens, and

Table 3 Analysis of discrepant cases

	FISH	*Single-color SISH*	*Dual-color SISH*	*IHC*
Case 1	No amplification	No amplification	Equivocal	Negative
Case 2	No amplification	Equivocal	Equivocal	Negative
Case 3	No amplification	No amplification	Amplification	Positive
Case 4	Amplification	No amplification	No amplification	Positive
Case 5	Amplification	No amplification	No amplification	Positive
Case 6	Amplification	No amplification	Amplification	Positive
Case 7	No amplification	Equivocal	No amplification	Equivocal

FISH, fluorescence *in situ* hybridization; IHC, immunohistochemistry; SISH, silver *in situ* hybridization.

Table 4 Comparison of single-color SISH and dual-color SISH

	Dual-color SISH			
Single-color SISH	*No amplification*	*Amplification*	*Equivocal*	*Total*
No amplification	106	2	1	109
Amplification	0	35	0	35
Equivocal	1	0	1	2
Total	107	37	2	146

FISH, fluorescence *in situ* hybridization; SISH, silver *in situ* hybridization.

Fritzsche *et al*[9] found that the overall concordance was 92.9% on cytological specimens. In this correlation study between FISH and dual-color SISH on formalin-fixed, paraffin-embedded sections, we found a concordance rate of 97%. The concordance rate between single-color SISH and dual-color SISH was also 97%. These results meet the ASCO/CAP requirements for test validation of >95% concordance for amplified *vs* nonamplified cases.[17] Discrepancies observed in our study may be explained by HER2 heterogeneity in the tumor and multiple leveling of TMA block.

Correlation studies of IHC and FISH have shown that *HER2* is not amplified in 6–23% of IHC 3+ cases.[6] Using a single probe for *HER2* in CISH assays, van de Vijver *et al*[18] also reported only a 57% concordance rate in cases in which FISH showed low-level *HER2* amplification. These problems can be explained by the presence of chromosome 17 polysomy.[19] In these equivocal cases, dual-probe methods can detect polysomy by detecting both HER2 and CEP17 signals, preventing the misdiagnosis of such cases as having low-level *HER2* amplification.

CISH has been validated as an acceptable method for evaluating *HER2* gene status.[4,5] Like FISH, CISH determines the actual degree of *HER2* gene amplification, but unlike FISH, positive signals can be identified by CISH using standard laboratory equipment. CISH is based on a peroxidase chromogenic reaction, which can be viewed by light microscopy. Therefore, the invasive tumor and corresponding histological or nuclear grade can be correlated immediately with *HER2* amplification. As its appearance is similar to IHC staining, CISH is also easier to interpret for pathologists who are not trained in fluorescence techniques. However, its signal can be difficult to distinguish from counterstains, and some chromogens are hazardous.

SISH is a type of enzyme metallographic *in situ* hybridization that uses an enzymatic reaction to deposit metal at the target site.[4] In addition to the advantages it shares with CISH, SISH provides higher sensitivity and resolution for both amplified and nonamplified genes, more accurate quantitation, and better visualization of tissue morphology and counterstains.[20] It also requires a shorter hybridization time than CISH (6 h *vs* overnight). In the recently published study using single-color SISH, high interobserver reproducibility was obtained among 10 pathologists.[21] However, the single-color method is inconvenient because the detection of the *HER2* gene and centromere 17 cannot be performed on one slide.

In two-thirds of our cases, the HER2/CEP17 ratio was higher in dual-color SISH than in previous single-color SISH.[7] This is possibly because the black and red signals of the dual-color method are occasionally positioned on top of each other, allowing one signal to conceal the other in bright field microscopy. This issue has also been raised in the context of dual-color CISH,[6] which, unlike standard CISH, uses two separate probes—a HER2 probe and a CEP17 probe—on one slide. Pedersen and Rasmussen[6] reported that a hidden CEP17 signal could account for several *HER2*-amplified cases that showed remarkably larger HER2/CEP17 ratios in dual-color CISH compared with FISH.

However, *HER2* amplification results were not greatly influenced by this phenomenon.

The rate of polysomy was slightly higher in single-color SISH than in dual-color SISH. CEP17 signals were sometimes confused with nonspecific background black dots in single-color SISH. However, dual-color SISH revealed discrete red signals that enable accurate assessment of CEP17 signals.

Our assay failed to demonstrate some HER2 or CEP17 signals for FISH, single-color SISH, and dual-color SISH. Although formalin-based fixatives are considered a best method for fixation, the time between tissue obtaining and fixation and complete fixation are also important factors for tissue-based assay.[20] Some specimens might be delivered to the pathology department a long time after tissue collection. In addition, large specimens cannot be completely fixed and may undergo autolysis, causing loss of hybridization. Shortening of time between tissue collection and fixation and complete fixation will improve the quality of DNA to be hybridized.

The major aims of our study were to compare dual-color SISH with single-color SISH and FISH and validate its use as our routine method for assessing *HER2* status in formalin-fixed, paraffin-embedded sections of breast cancers. Our results showed that dual-color SISH correlated well with FISH and could be used as an alternative to FISH.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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