Reproducibility in the Assessment of *HER2* DISH in Breast Cancer

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Abstract

OBJECTIVE: *HER2* gene status is an important biological marker that determines both the prognosis and response to Trastuzumab in patients with breast cancers. Although FISH assay has long been used to determine the *HER2* gene copy number, DISH is gaining popularity since it can be assessed with a conventional microscope. The DISH test relies on an individual inspection however, so inter-observer variation may be an issue.

MATERIALS AND METHODS: In the current study, reproducibility in the assessment of *HER2* DISH test was evaluated in 69 breast cancer samples with *HER2* IHC score 2+ and 3+. Two independent investigators evaluated the digitally-captured images of DISH, without the knowledge of *HER2* IHC status.

RESULTS: The *HER2/CEP17* ratio obtained from both observers were highly correlated (Rho = 0.883, $p < 0.001$). Based on the ASCO 2013 guideline, agreement of both investigators for *HER2* gene diagnosis was 92.75 % ($\kappa = 0.848$). The *HER2/CEP17* ratio of the discordant cases ranged from 1.6 (non-amplified *HER2*) to 2.6 (low level of *HER2* gene amplification).

CONCLUSION: It is concluded that the DISH method produces good reproducibility between independent observers, and the result also implies that the *HER2* gene copy number is fairly homogenous across an individual breast cancer sample.

Keywords: breast cancer, *HER2*, DISH, FISH, reproducibility

Breast cancer has long been at the top of both incidence and cause of cancer death in women worldwide.[1] Recent studies have shown that, in the US, the incidence of breast cancer death has been overtaken by lung cancer.[2] This is due largely to the effectiveness of Trastuzumab in the treatment of human epidermal growth factor receptor 2 (*HER2*) amplified breast cancer, either at the early or advanced stage of disease.[3,4] Therefore, *HER2* testing is an important part of the pathological examination, and should be performed in all breast cancer patients.[5,6]

Immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) methods have been the mainstay for determining *HER2* status.[5] More recently, the dual in situ hybridization assay (DISH) method has been introduced as an alternative approach to FISH in the assessment of *HER2* gene copy number. Head-to-head comparisons between FISH and DISH have been performed, and both methods showed good correlation and agreement.[7,8] Since the DISH test relies on visual inspection of individuals, inter-observer variation may be an issue. Here, the inter-observer reproducibility in the assessment of *HER2* DISH test was evaluated.

Materials and Methods

Sixty-nine invasive ductal carcinoma specimens that had been submitted to the Department of Pathology, King Chulalongkorn Memorial Hospital for *HER2* DISH assay during 2012-2013 were included in the study. Fifty-eight cases were institutional cases while the remaining eleven cases were specimens received from other institutes.

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The morphological grading was available for 55 institutional cases (6 grade 1, 29 grade 2, and 20 grade 3). The *HER2* IHC score using the ASCO/CAP guidelines[5] was available for all 58 institutional cases (32 cases with 2+, 26 cases with 3+).

The 4 micron-thick sections were prepared from formalin-fixed paraffin-embedded tissue of invasive carcinoma specimens. Sections were then stained with Ventana Benchmark XT system (Roche Diagnostics), using an Inform *HER2* Dual ISH DNA Probe Cocktail (Ventana Medical System). The *HER2* signals were visualized with an UltraView SISH DNP Detection Kit (Ventana Medical Systems) while the chromosome 17 centromere (CEP17) signals were visualized with an UltraView Red ISH DIG Detection kit (Ventana Medical Systems).

One pathologist (SS) inspected the DISH slides and digitally captured 10-20 images of the invasive components at the x600 magnification, using Olympus DP22 digital camera for microscopes (Olympus Optical CO. LTD., Japan). Two investigators (TA and TC), without the knowledge of *HER2* IHC status, independently counted the signals of 20 tumor cells with well-visualized signals, and calculated the *HER2*/*CEP17* ratio and *HER2*/nucleus ratio. Interpretation of the *HER2* gene copy number was made according to the ASCO/CAP guidelines as shown in Table 1.[5] The results were then compared between the two investigators.

Table 1: Interpretation Guideline of *HER2* ISH (ASCO/CAP 2013).

Interpretation Guideline of *HER2* ISH
Positive for *HER2* gene amplification
- *HER2/CEP17* ratio ≥ 2.0 with an average *HER2* copy number ≥ 4.0 signals/cell, or
- *HER2/CEP17* ratio ≥ 2.0 with an average *HER2* copy number < 4.0 signals/cell, or
- *HER2/CEP17* ratio < 2.0 with an average *HER2* copy number ≥ 6.0 signals/cell
Equivocal
- *HER2/CEP17* ratio < 2.0 with an average *HER2* copy number ≥ 4.0 and < 6.0 signals/cell
Negative for *HER2* gene amplification
- *HER2/CEP17* ratio < 2.0 with an average *HER2* copy number < 4.0 signals/cell

Statistical analysis

We constructed a scatter diagram from the *HER2*/*CEP17* ratios of both investigators and made a comparison. The Spearman's correlation coefficient (Rho) was calculated using SPSS statistic 20 (IBM Corporation, New York, USA). The Rho of 0 to 1 indicates a positive correlation and -1 to 0 indicates negative correlation. The strength of correlation coefficients is as follows: 0 and 1 indicate zero and perfect correlation respectively; ±0.1 to ±0.3 indicate weak correlation; ±0.4 to ±0.6 indicate moderate correlation; and ±0.7 to ±0.9 indicate strong correlation. A *p*-value was calculated using *p*-value of < 0.05 as the cut off point for statistical significance.

The agreement of both investigators (amplified vs. non-amplified) was compared using κ statistic. The κ statistic of 0 to 0.4 indicates poor agreement, 0.4 to 0.6 indicates moderate agreement, 0.6 to 0.8 indicates good agreement and 0.8 to 1 indicates excellent agreement.

Results

Two cases were considered uncountable. One example showed only *CEP17* signals without *HER2* signals and was regarded as uncountable by both investigators. Another case showed strong *CEP17* signals with faint and small *HER2* signals in only a small portion of the tumor cells. This case was regarded as uncountable by investigator 1 but countable by the other (*HER2*/*CEP17* = 0.76, non-amplified). These two cases were excluded from analysis. There were 5 disagreed cases as shown in Table 2. All of them are considered non-amplified by investigator 1 and amplified by investigator 2. Table 3 shows the correlation between the IHC score and DISH *HER2* status, excluding the 5 discordant cases and 2 failed DISH cases. The tumors with IHC score 2+ were 14% *HER2* amplified by DISH method while the tumors with IHC score 3+ were 77% *HER2* amplified by DISH method. Agreement of both investigators for *HER2* gene diagnosis was 92.75 % (κ = 0.848), reflecting an excellent agreement. The *HER2*/*CEP17* ratio of investigator 1 and 2 ranged from 0.8 to 13.46 and 0.76 to 11.63 respectively. 26 cases were interpreted as *HER2*-amplified by investigator 1, while investigator 2 interpreted 31 cases as amplified. None of the cases was in the equivocal category or showed heterogeneous *HER2* gene copy number. Representative images of *HER2*-amplified and non-amplified are depicted in Figure 1 and 2, respectively.

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Table 2: Disagreed Cases.

HER2/CEP17 Investigator 1	*HER2/CEP17* Investigator 2
1.77 (non-amplified)	2.03 (amplified)
1.95 (non-amplified)	2.10 (amplified)
1.93 (non-amplified)	2.40 (amplified)
1.62 (non-amplified)	2.54 (amplified)
1.89 (non-amplified)	2.68 (amplified)

Table 3: Correlation of *HER2* IHC score and DISH status of all concordance cases.

IHC and DISH result	Cases	Percentage of *HER2* amplified cases by DISH method
IHC 2+, DISH non-amplified	25	14%
IHC 2+, DISH amplified	4	
IHC 3+, DISH non-amplified	5	77%
IHC 3+, DISH amplified	17	

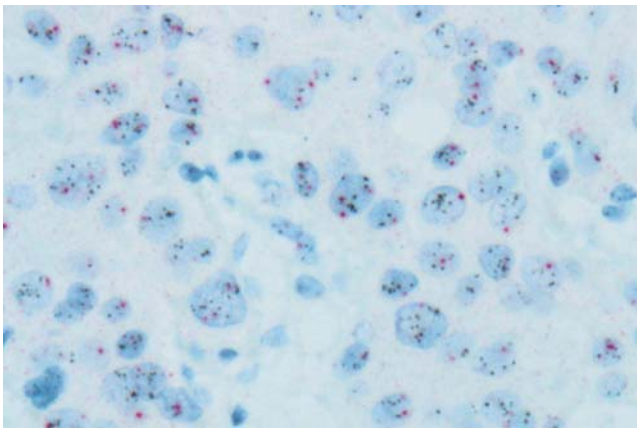

Figure 1: Invasive breast cancer with *HER2* amplified by DISH method.

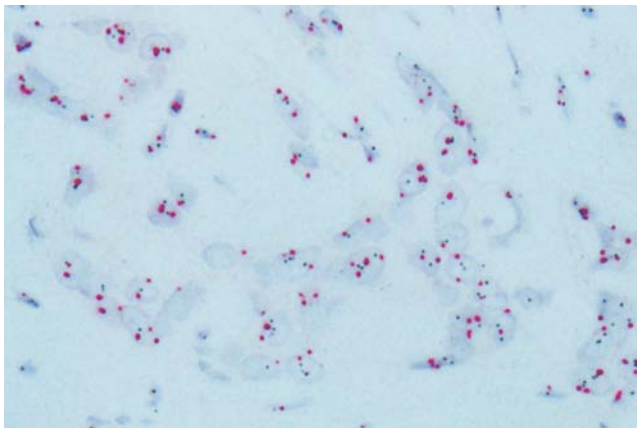

Figure 2: Invasive breast cancer with *HER2* non-amplified by DISH method.

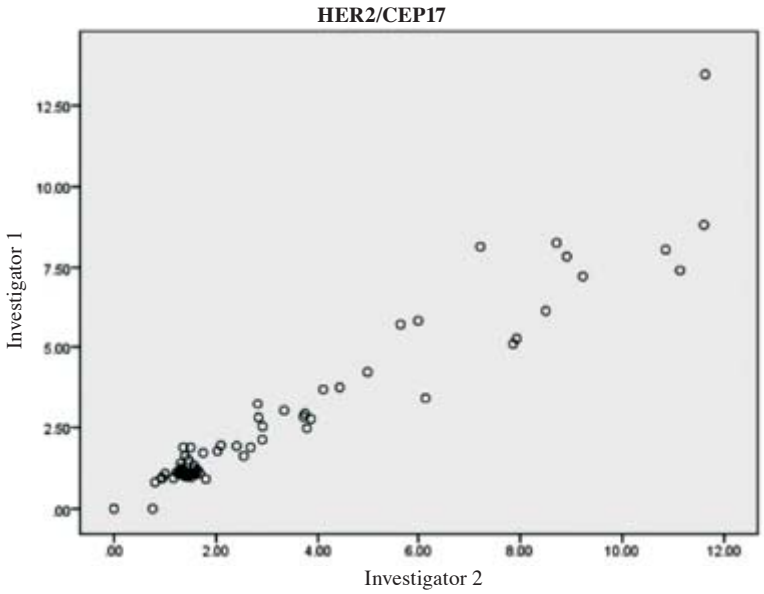

Figure 3

The *HER2/CEP17* ratios obtained from both investigators were compared. The scatter diagrams revealed a strong correlation between the two (Rho = 0.883, $p < 0.001$) (Figure 3). The case by case comparison is shown in (Figure 4). All 5 discordant cases have *HER2/CEP17* ratios of less than 3 and most of them fall in the equivocal range of the previous ASCO guideline.[9] The *HER2* count and *CEP17* count were compared separately. Both *HER2* and *CEP17* counts from two investigators show strong correlation (*HER2* count: Rho = 0.955, $p < 0.001$; *CEP17* count: Rho = 0.779, $p < 0.001$). The scatter diagrams are shown in Figure 5 and 6.

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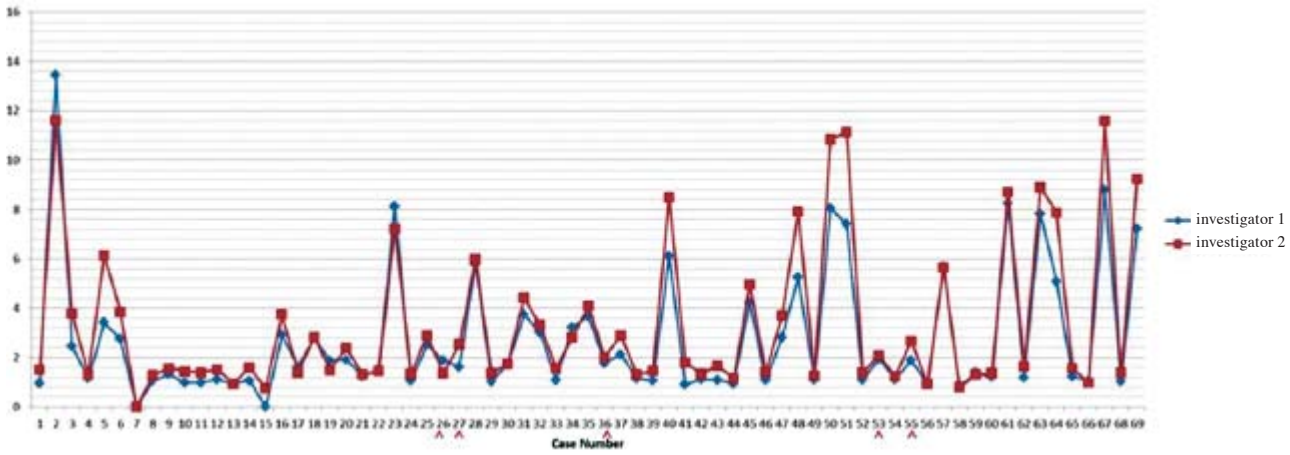

Figure 4: *HER2* DISH results assessed independently by two investigators (disagreed cases marked with red arrow heads).

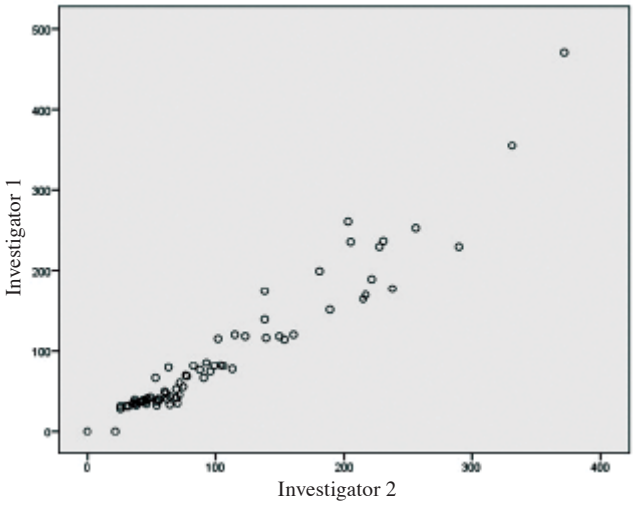

Figure 5: Invasive breast cancer with *HER2* amplified by DISH method.

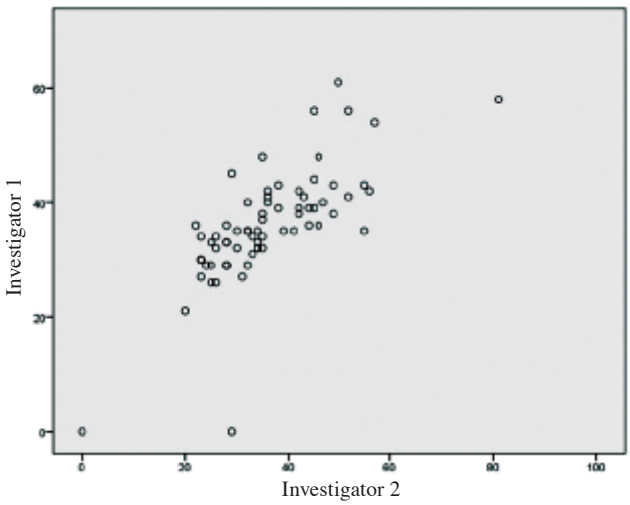

Figure 6: Invasive breast cancer with *HER2* amplified by DISH method.

Discussion

Discovery of *HER2* and its implication in breast cancer is one of the examples of molecular oncology in which fundamental biological knowledge has been transformed into a practical application and has saved lives. To be able to fully appreciate the importance of this knowledge, *HER2* status must be established in virtually all breast cancer cases. Accessibility to the *HER2* tests is dependent on overcoming technical difficulty, logistical issues, interpretation difficulty, and cost. *HER2* immunohistochemistry (IHC) has been used widely at an acceptable cost and has proven to be a great screening tool for determining *HER2* status.[10] When *HER2* IHC status is inconclusive (2+), assessment of *HER2* gene copy number is mandatory and the FISH method has been regarded as the gold standard for this purpose for many years. FISH technique, however, requires the costly fluorescent microscope for visualization. *HER2* DISH assay that can assess the gene copy number under an ordinary microscope has emerged as an alternative tool to FISH. Multiple studies validating the *HER2* DISH assay with FISH have shown promising results.[7,8] Comparison between DISH and FISH methods is shown in Table 4. We can expect the shift from the FISH assay to DISH in the near future.

Determination of the *HER2* gene copy number is typically done by manual counting[5], and interpersonal and even intrapersonal reproducibility may have an impact on the testing result. To address this issue, two observers were asked to independently assess the captured DISH images in this study. Very high correlation was found in the result obtained from the two investigators, and this implies that all tumor samples enrolled were reasonably homogenous in terms of *HER2* status. Previous evaluations of intratumoral heterogeneity in breast cancer have also demonstrated similar results.[11,12] Having said that, we do acknowledge the increasingly recognized cases of breast cancers with heterogeneous *HER2* copy number[13,14] but such an example was not found in our relatively small series of cases.

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Table 4: Comparison between DISH and FISH methods for *HER2* gene assessment.

DISH	FISH
- FDA approved	- FDA approved
- Assessed by ordinary microscopy	- Assessed by fluorescence microscope
- Examined at x 600 magnification	- Examined at x 1000 magnification
- Easy to identify invasive cancer cells	- May be difficult to identify invasive cancer cells, especially in cases with extensive intraductal component
- *HER2*/CEN17 ratio provided	- *HER2*/CEN17 ratio provided
- Slides can be archived for further re-examination	- Slides cannot be archived (degraded fluorescence dye)
- Lower cost	- More expensive

The ASCO 2013 set the cut-off for *HER2/CEP17* ratio at 2.0 for a tumor to be assigned as being '*HER2* amplified'. As expected, cases with discrepancy in the results in our cohort had a *HER2/CEP17* ratio around that level (negative for or low level of amplification). Multiple studies have shown that the response to Trastuzumab therapy correlates highly with the level of amplification, with a significant response noted when *HER2/CEP17* > 6.[6,15] Therefore, the discrepancy cases in our series are unlikely to have a huge impact on the patients' long term survival.

Conclusion

The DISH method for determining the *HER2* gene copy number is highly reliable, with high inter-observer reproducibility. Only a few discrepancy cases fell into the range of negative or low level of *HER2* gene amplification. The high inter-observer reproducibility also indicates that, in terms of *HER2* status, most of the invasive breast carcinomas are fairly homogeneous throughout the lesion.

References

1. Jemal A, Bray F, Center M, et al. Global cancer statistics. *CA Cancer J Clin* 2011;61:69-90.
2. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. *CA Cancer J Clin* 2013;63:11-30.
3. Slamon D, Leyland-Jones B, Shak S, et al. Use of Chemotherapy plus a Monoclonal Antibody against HER2 for Metastatic Breast Cancer That Overexpresses HER2. *N Engl J Med* 2001;344:783-92.
4. Perez EA, Romond EH, Suman VJ, et al. Trastuzumab plus adjuvant chemotherapy for human epidermal growth factor receptor 2-positive breast cancer: planned joint analysis of overall survival from NSABP B-31 and NCCTG N9831. *J Clin Oncol* 2014;32:3744-52.
5. Wolff AC, Hammond ME, Hicks DG, et al. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. *J Clin Oncol* 2013;31:3997-4013.
6. Gullo G, Bettio D, Torri V, et al. Level of HER2/neu gene amplification as a predictive factor of response to trastuzumab-based therapy in patients with HER2-positive metastatic breast cancer. *Invest New Drugs* 2008; 27:179-83.
7. Mansfield A, Sukov W, Eckel-Passow J, et al. Comparison of Fluorescence In Situ Hybridization (FISH) and Dual-ISH (DISH) in the Determination of HER2 Status in Breast Cancer. *Am J Clin Pathol* 2013;139:144-50.
8. Horii R, Matsuura M, Iwase T, et al. Comparison of dual-color in-situ hybridization and fluorescence in-situ hybridization in HER2 gene amplification in breast cancer. *Breast Cancer* 2014;21:598-604.
9. Wolff A, Hammond E, Schwartz J, 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;131:18-43.
10. Cuadros M, Villegas R. Systematic review of HER2 breast cancer testing. *Appl Immunohistochem Mol Morphol*. 2009;17:1-7.
11. Shin SJ, Hyjek E, Early E, Knowles DM. Intratumoral heterogeneity of HER2/neu in invasive mammary carcinomas using fluorescence in-situ hybridization and tissue microarray. *Int J Surg Pathol* 2006;14:279-84.
12. Brunelli M, Manfrin E, Martignoni G, et al. Genotypic intratumoral heterogeneity in breast carcinoma with HER2/neu amplification: evaluation according to ASCO/CAP criteria. *Am J Clin Pathol* 2009;131:678-82.
13. Hanna WM, Rüschoff J, Bilous M, et al. HER2 in situ hybridization in breast cancer: clinical implications of polysomy 17 and genetic heterogeneity. *Mod Pathol* 2014;27:4-18.
14. Shuangshoti S, Sawatdee R, Sakapibunnan A, et al. Heterogeneity of Unilateral Multiple Breast Cancer: Implications for Biomarker Testing. *Bangkok Med J* 2015;8:17-21.
15. Fuchs E, Köstler W, Horvat R, et al. High-level ERBB2 gene amplification is associated with a particularly short time-to-metastasis, but results in a high rate of complete response once trastuzumab-based therapy is offered in the metastatic setting. *Int J Cancer* 2014;135:224-31.