Research Article

A Validation Study of Human Epidermal Growth Factor Receptor 2 Immunohistochemistry Digital Imaging Analysis and its Correlation with Human Epidermal Growth Factor Receptor 2 Fluorescence *In situ* Hybridization Results in Breast Carcinoma

Ramon Hartage[1,#], Aidan C. Li[2,#], Scott Hammond[1], Anil V. Parwani[1]

[1]Department of Pathology, The Ohio State University Wexner Medical Center, Columbus, OH 43210, USA, [2]Department of NA, Jerome High School, Dublin, OH 43017, USA

[#]First two authors contributed equally

Received: 09 September 2019 Accepted: 16 October 2019 Published: 04 February 2020

Abstract

Background: The Visiopharm human epidermal growth factor receptor 2 (HER2) digital imaging analysis (DIA) algorithm assesses digitized HER2 immunohistochemistry (IHC) by measuring cell membrane connectivity. We aimed to validate this algorithm for clinical use by comparing with pathologists' scoring and correlating with HER2 fluorescence *in situ* hybridization (FISH) results. **Materials and Methods:** The study cohort consisted of 612 consecutive invasive breast carcinoma specimens including 395 biopsies and 217 resections. HER2 IHC slides were scanned using Philips IntelliSite Scanners, and the digital images were analyzed using Visiopharm HER2-CONNECT App to obtain the connectivity values (0–1) and scores (0, 1+, 2+, and 3+). HER2 DIA scores were compared with Pathologists' manual scores, and HER2 connectivity values were correlated with *HER2* FISH results. **Results:** The concordance between HER2 DIA scores and pathologists' scores was 87.3% (534/612). All discordant cases (n = 78) were only one-step discordant (negative to equivocal, equivocal to positive, or vice versa). Five cases (0.8%) showed discordant HER2 IHC DIA and *HER2* FISH results, but all these cases had relatively low *HER2* copy numbers (between 4 and 6). HER2 IHC connectivity showed significantly better correlation with *HER2* copy number than *HER2/CEP17* ratio. **Conclusions:** HER2 IHC DIA demonstrates excellent concordance with pathologists' scores and accurately discriminates between *HER2* FISH positive and negative cases. HER2 IHC connectivity has better correlation with *HER2* copy number than *HER2/CEP17* ratio, suggesting *HER2* copy number may be more important in predicting HER2 protein expression, and response to anti-HER2-targeted therapy.

Keywords: Breast carcinoma, digital imaging analysis, fluorescence *in situ* hybridization, human epidermal growth factor receptor 2, immunohistochemistry, Visiopharm

Introduction

Human epidermal growth factor receptor 2 (HER2; ERBB2) gene amplification and/or protein overexpression occurs in approximately up to 20% of breast cancers.[1-4] Anti-HER2 targeted drugs, such as trastuzumab and pertuzumab, are effective in treating HER2-positive breast cancers, but not HER2-negative breast cancers.[5-8] Given anti-HER2 drugs' side effects and significant cost, accurate determination of HER2-positive status is mandatory before offering them to any breast cancer patient.[9]

HER2 status is usually assessed by immunohistochemistry (IHC) for HER2 protein expression and/or by fluorescence *in situ* hybridization (FISH) for *HER2* gene amplification. IHC

Address for correspondence: Dr. Anil V. Parwani, Department of Pathology, The Ohio State University Wexner Medical Center; 410 W, 10th Ave, Columbus, OH 43210, USA. E-mail: anil.parwani@osumc.edu

How to cite this article: Hartage R, Li AC, Hammond S, Parwani AV. A validation study of human epidermal growth factor receptor 2 immunohistochemistry digital imaging analysis and its correlation with human epidermal growth factor receptor 2 fluorescence *in situ* hybridization results in breast carcinoma. J Pathol Inform 2020;11:2.

Available FREE in open access from: http://www.jpathinformatics.org/text.asp?2020/11/1/2/277789

Access this article online

Quick Response Code:

Website: www.jpathinformatics.org

DOI: 10.4103/jpi.jpi_52_19

is used primarily and FISH is used as a reflex test on IHC equivocal cases by most laboratories in the United States.[9] HER2 IHCs are usually evaluated by pathologists in a nonquantitative manner and given a score from 0 to 3+ based on membranous staining of HER2 protein. Although the American Society of Clinical Oncology and the College of American Pathologists (ASCO/CAP) published guidelines on how to assess HER2 IHCs, interobserver variability does occur.[9-11]

Since the wide implementation of whole slide imaging (WSI), digital image analysis (DIA) has emerged as an objective and reproducible scoring method to assess HER2 IHC in a quantitative manner.[12-16] Studies have demonstrated DIA could reduce HER2 IHC equivocal cases.[12,14,17] The ASCO/CAP HER2 guideline has acknowledged DIA as a diagnostic modality for HER2 status assessment,[9] and CAP has created guidelines to facilitate adoption of HER2 DIA into routine pathology workflows.[18]

The Visiopharm HER2 IHC DIA algorithm evaluates cell membrane connectivity and the preliminary data have demonstrated accurate assessment of HER2 IHCs in breast carcinoma and gastric/esophageal adenocarcinoma.[12,19,20] We aimed to validate this DIA algorithm for clinical use by comparing with pathologists' scores and correlating with HER2 FISH results in breast carcinomas.

Materials and Methods

Case selection

This study included 612 consecutive primary invasive breast carcinomas from the Ohio State University Wexner Medical Center between January 01, 2016, and January 31, 2017. The use of human materials was approved by the institutional review board at the Ohio State University.

Immunohistochemistry

HER2 IHC was performed using PATHWAY anti-HER2 (4B5) on Benchmark XT automated slide stainer according to the manufacturer's protocol (Roche Ventana Medical Systems, Tucson, AZ). An automated deparaffinization step was followed by cell conditioning and then rinsed and incubated with the prediluted anti-HER2 rabbit monoclonal primary antibody (clone 4B5) at 37°C. After rinsing, staining was visualized using the ultraView Universal DAB Detection Kit (Roche Ventana Medical Systems, Tucson, AZA). The slides were counterstained, then rinsed, and coverslipped.

Pathologists' scoring

HER2 IHC was manually scored by subspecialized breast pathologists according to ASCO/CAP guidelines: 0 (negative): no staining or faint/barely perceptible, incomplete membrane staining in ≤10% of tumor cells; 1+ (negative): faint/barely perceptible, incomplete membrane staining in >10% of tumor cells; 2+ (equivocal): weak/moderate complete membrane staining in >10% of tumor cells; and 3+ (positive): circumferential complete intense membrane staining in >10% of tumor cells.

Image acquisition and digital imaging analysis

Glass slides were scanned using Philips UltraFast Scanner (Philips, the Netherlands) at ×40 magnification with a single-focus layer. The tissue on slides was detected automatically with focus points to obtain the optimal image. Whole slide images were stored in a centralized server located at The Ohio State University's campus. HER2 IHCs were evaluated using the HER2-CONNECT algorithm in the Visiopharm Integrator System (Visiopharm, Hørsholm, Denmark) and recorded as a value from 0 to 1[12] [Figure 1].

HER2 DIA scores were categorized into four categories with the following cutoff values: (1) 0: connectivity = 0; (2) 1+: 0 <connectivity ≤0.12; (3) 2+: 0.12 <connectivity ≤0.49; and (4) 3+: connectivity >0.49. Similar to the HER2 scores used by pathologists, a HER2 DIA score 0 and 1 + were defined as negative, 2 + as equivocal, and 3 + as positive based on preliminary analysis and previously reported data.

Fluorescence *in situ* hybridization

The FISH analysis with the CEN17 probe was performed at our institution using the dual-color Vysis FDA-approved PathVysion HER2 DNA Probe Kit (Abbott Molecular, Des Plaines, IL). The signals for the HER2 gene and CEN17 were visualized under a fluorescence microscope using appropriate filters. The average numbers of HER2 and CEN17 signals per cell were recorded for at least 50 cells, and the HER2/CEN17 ratio was calculated for each case. The results were interpreted by specialized molecular pathologists and signed out by case pathologists with a specialization in breast pathology.

Statistical analysis

All clinicopathologic data were summarized using percentages and descriptive statistics. The HER2 DIA values were compared with the paired *HER2* copy numbers/ratios to assess correlation (Pearson correlation). Kappa coefficient was

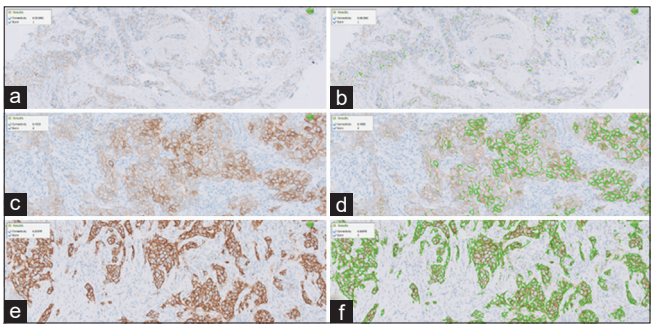

Figure 1: Human epidermal growth factor receptor 2 immunohistochemistry and the connectivity analyzed by Visiopharm human epidermal growth factor receptor 2 immunohistochemistry algorithm. (a and b) One case with human epidermal growth factor receptor 2 immunohistochemistry 1+; (c and d) one case with human epidermal growth factor receptor 2 immunohistochemistry 2+. (e and f) one case with human epidermal growth factor receptor 2 immunohistochemistry 3+. (a, c, and e) Human epidermal growth factor receptor 2 immunohistochemistry; (b, d, and f) human epidermal growth factor receptor 2 connectivity (green color line) detected by Visiopharm

calculated to measure the agreement between DIA scores and pathologists' scores using GraphPad Prism (San Diego, CA). This calculation used linear weights. The kappa result was interpreted as follows: ≤0 as no agreement; 0.01–0.20 as none to slight; 0.21–0.40 as fair; 0.41–0.60 as moderate; 0.61–0.80 as substantial; and 0.81–1.00 as almost perfect agreement. The correlations of HER2 IHC connectivity with *HER2* copy number or *HER2/CEP17* ratio were compared using cocor analysis at cocor website (http://comparingcorrelations.org/).[21] Other statistics were performed using SAS version 9.3 (SAS Institute, Cary, NC, USA). For all results, $P < 0.05$ was considered as statistically significant.

Results

Demographic characteristics of the study cohort

The study cohort was composed of 612 invasive breast carcinomas, including 496 invasive ductal carcinomas, 65 invasive lobular carcinomas, 25 mixed ductal/lobular carcinomas, 7 metaplastic carcinomas, and 19 metastatic carcinomas in axillary lymph nodes [Table 1]. Four hundred and thirty-two cases had HER2 IHC scores of 0 or 1+ (negative), 101 cases had scores of 2+ (equivocal) and 79 cases had scores of 3+ (positive). Among the 101 HER2 2+ cases, 49 were not amplified, 41 were equivocal, and 11 were positive on FISH based on the 2013 HER2 guidelines. Based on the 2018 guidelines,[9] 90 cases were negative for FISH and 11 were positive for FISH.

Table 1: Demographic features of study cohort

	Case number (n=612)/average	Percentage/range
Age (range) (year)	58.5 (26-95)	26-95
Specimen		
Biopsy	395	64.5%
Resection	217	35.5%
Histologic type		
IDC	496	81.0%
ILC	65	10.6%
Mixed IDC/ILC	25	4.1%
Metaplastic carcinoma	7	1.1%
Metastatic carcinoma	19	3.1%
ER		
Positive	380	62.1%
Negative	141	23.0%
Not available	91	14.9%
PR		
Positive	317	51.8%
Negative	204	33.3%
Not available	91	14.9%
HER2 IHC		
Negative (0/1+)	432	70.6%
Equivocal (2+)	101	16.5%
Positive (3+)	79	12.9%

IDC: Invasive ductal carcinoma, ILC: Invasive lobular carcinoma, HER2: Human epidermal growth factor receptor 2, ER: Estrogen receptor, PR: Progesterone receptor, IHC: Immunohistochemistry

The correlation between human epidermal growth factor receptor 2 immunohistochemistry digital imaging analysis scores and pathologists' scores

Each HER2 IHC WSI slide was analyzed using a HER2 membrane algorithm in Visiopharm and the HER2 connectivity value was recorded. Four hundred forty-eight cases were categorized as negative (0/1+), 85 as equivocal (2+), and 79 as positive (3+). The HER2 DIA scores were correlated with pathologists' scores and 78 cases showed discordant results, including 25 pathologist-negative/Visiopharm-equivocal cases, 41 pathologist-equivocal/Visiopharm-negative cases, 6 pathologist-equivocal/Visiopharm-positive cases, and 6 pathologist-positive/Visiopharm-equivocal cases. All discordant cases (n = 78) were only one-step discordant (negative to equivocal/equivocal to positive, or vice versa) [Table 2]. The agreement between HER2 DIA and pathologists' read was "substantial" (kappa = 0.713, weighted kappa = 0.797, 87.3%). HER2 DIA decreased the HER2 IHC equivocal cases from 101 to 85 (16%).

Table 2: The correlation between human epidermal growth factor receptor 2 digital image analysis scores and pathologists' scores

Pathologists	Visiopharm			
	Negative (0/1+)	Equivocal (2+)	Positive (3+)	Total
Negative (0/1+)	407	25	0	432
Equivocal (2+)	41	54	6	101
Positive (3+)	0	6	73	79
Total	448	85	79	612

HER2 DIA scores were categorized into four categories with the following cutoff values: 0: connectivity=0; 1+: 0 <connectivity ≤0.12; 2+: 0.12<connectivity ≤0.49; 3+: Connectivity >0.49. HER2: Human epidermal growth factor receptor 2, DIA: Digital image analysis

The correlation between human epidermal growth factor receptor 2 immunohistochemistry digital imaging analysis values and human epidermal growth factor receptor 2 fluorescence *in situ* hybridization copy numbers/ratios

Next, we analyzed the correlation between HER2 IHC DIA connectivity values with *HER2* FISH copy numbers and ratios using the Pearson correlation in the 442 cases in which FISH results were available. The Pearson correlation coefficient (r) for HER2 IHC DIA connectivity and *HER2* FISH copy number was 0.860594 (n = 442; $P < 0.0001$) with a mean coefficient of determination (R^2) of 0.7245 [Figure 2]. The Pearson correlation coefficient (r) for HER2 IHC DIA connectivity and HER2 FISH copy number was 0.824927 (n = 442; $P < 0.0001$) with a mean coefficient of determination (R^2) of 0.6701 [Figure 3]. The difference between these two correlation coefficients (DIA/copy number vs. DIA/ratio) was 0.0357 and was statistically significant based on cocor analysis using a backtransformed average Fisher's z procedure (z = 3.8429, P = 0.0001).

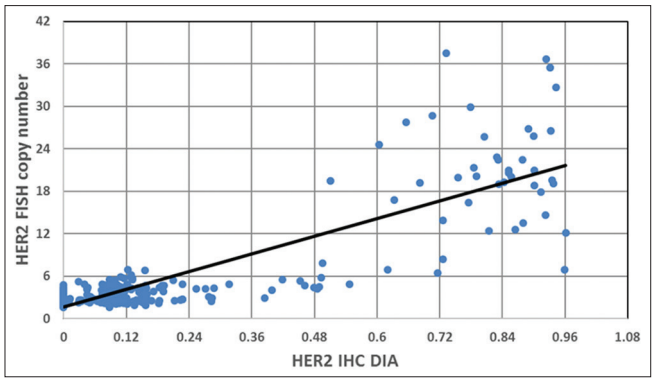

Figure 2: Correlation between human epidermal growth factor receptor 2 immunohistochemistry digital imaging analysis connectivity and human epidermal growth factor receptor 2 fluorescence *in situ* hybridization copy number. The Pearson correlation coefficient (*r*) for human epidermal growth factor receptor 2 immunohistochemistry digital imaging analysis connectivity and human epidermal growth factor receptor 2 fluorescence *in situ* hybridization copy number was 0.860594 ($n = 442$; $P < 0.0001$). ($y = 20.498x + 1.7076$. $R^2 = 0.7245$)

Table 3: The correlation between human epidermal growth factor receptor 2 digital image analysis scores and fluorescence *in situ* hybridization results in 442 cases with fluorescence *in situ* hybridization

	Visiopharm (%)			
	Negative (0/1+)	Equivocal (2+)	Positive (3+)	Total
FISH positive				
Group 1	3 (0.9)	6 (9)	53 (91.4)	62
Group 3	0 (0)	1 (1.5)	3 (4.5)	4
FISH negative				
Group 2	1 (0.3)	0 (0)	0 (0)	1
Group 4	36 (11.4)	24 (35.8)	2 (3.0)	62
Group 5	277 (87.4)	36 (53.7)	0	313
Total	317	67	58	442

HER2 FISH results were categorized into the following 5 groups according to ASCO/CAP HER2 guidelines: Group 1: HER2/CEP17 ratio ≥2.0 and average HER2 copy number ≥4.0 signals/cell; Group 2 HER2/CEP17 ratio ≥2.0 and average HER2 copy number <4.0 signals/cell; Group 3: HER2/CEP17 ratio <2.0 and average HER2 copy number ≥6.0 signals/cell; Group 4: HER2/CEP17 ratio <2.0 and average HER2 copy number ≥4.0 and <6.0 signals/cell; Group 5: HER2/CEP17 ratio <2 and average HER2 copy number <4.0 signals/cell. HER2: Human epidermal growth factor receptor 2, FISH: Fluorescence *in situ* hybridization, ASCO: American Society of Clinical Oncology, CAP: College of American Pathologist

FISH results from all 442 cases were re-interpreted based on the 2018 ASCO/CAP HER2 guidelines to separate into five groups with groups 1 and 3 being FISH-positive, and groups 2, 4 and 5 being FISH-negative. Overall, only five cases (0.8%) showed discordant HER2 IHC DIA and *HER2* FISH results, including 3 cases with negative IHC DIA but positive FISH, and 2 cases with positive IHC DIA but negative FISH [Table 3]. All five cases had HER2 copy numbers between 4 and 6, but the ratio was >2 in three FISH-positive cases (ISH group 1) and <2 in 2 FISH-negative cases (ISH group 4) [Table 4].

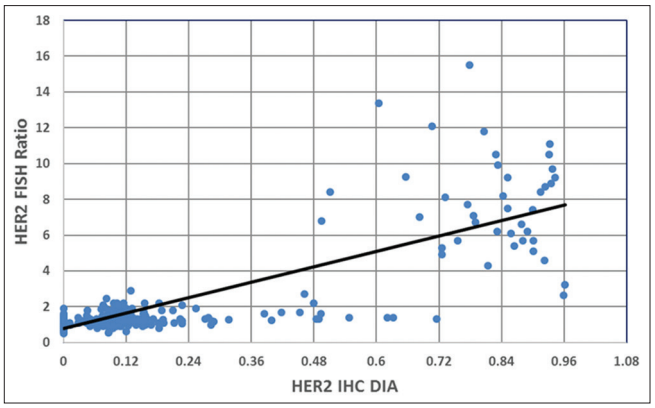

Figure 3: Correlation between human epidermal growth factor receptor 2 immunohistochemistry digital imaging analysis connectivity and human epidermal growth factor receptor 2 fluorescence *in situ* hybridization ratio. The Pearson correlation coefficient (*r*) for human epidermal growth factor receptor 2 immunohistochemistry digital imaging analysis connectivity and human epidermal growth factor receptor 2 fluorescence *in situ* hybridization ratio was 0.824927 ($n = 442$; $P < 0.0001$). ($y = 7.1342x + 0.7798$. $R^2 = 0.6701$)

Four of these cases showed 2+ and 1 case showed 1+ on pathologists' scores. Additional FISH assays with or without alternative probe (D17S122) were performed, and the results were included in Table 4.

Pitfalls in human epidermal growth factor receptor 2 immunohistochemistry digital imaging analysis

In the process of analyzing HER2 IHC using DIA, we had identified several pitfalls and challenges. The causes for false positive DIA included air bubbles, pigments, ductal carcinoma *in situ* (DCIS) components, and inks [Figure 4a-d]. On the other hand, out of focus or rare tumor cells caused false-negative DIA results [Figure 4e and f].

Discussion

To the best of our knowledge, this study, including 612 invasive breast carcinomas, is one of the largest to validate HER2 IHC DIA using whole slide images. Our data have demonstrated that HER2 IHC DIA is a reliable measurement for HER2 protein expression based on HER2 membrane connectivity. HER2 DIA not only shows an excellent concordance with pathologists' manual scoring (87.3%), but also reduces HER2 IHC equivocal case numbers (16% reduction). All discordant cases ($n = 78$, 12.7%) show only one-step discordance (negative to equivocal/equivocal to positive, or vice versa). Our results are consistent with previous studies which have shown high agreement between HER2 DIA and manual scoring in breast cancer specimens, with 87.5%–94.2% agreement rates.[12-15,17,22,23]

Our data also reveal that HER2 IHC DIA can accurately discriminate between *HER2* FISH positive and negative cases interpreted based on 2018 ASCO/CAP HER2 guidelines.[9] Overall, only five cases (0.8%) showed discordant HER2 IHC DIA and *HER2* FISH results, including 3 cases with negative

Table 4: Five cases with discordant Human epidermal growth factor receptor 2 immunohistochemistry digital image analysis/fluorescence *in situ* hybridization results

Case number	HER2 DIA	HER2 DIA value	Original IHC	FISH	FISH copy number	FISH ratio	Additional FISH results
1	1+	0.076643	2+	P	4.80	2.10	D17Z1 (ratio 1.68, copy number: 4.5) D17S122 (ratio 2.25, copy number: 4.6)
2	1+	0.096429	2+	P	4.20	2.20	D17Z1 (ratio 1.54, copy number: 3.70)
3	1+	0.11514	1+	P	5.70	2.20	D17Z1 (ratio 2.4, copy number: 4.4)
4	3+	0.49328	2+	N	5.80	1.60	D17Z1 (ratio 1.47, copy number: 5.0) D17S122 (ratio 1.74, copy number: 5.4)
5	3+	0.54750	2+	N	4.90	1.40	D17Z1 (ratio 1.74, copy number: 4.2) D17S122 (ratio is 1.59, copy number: 4.2)

HER2: Human epidermal growth factor receptor 2, FISH: Fluorescence *in situ* hybridization, DIA: Digital image analysis, IHC: Immunohistochemistry

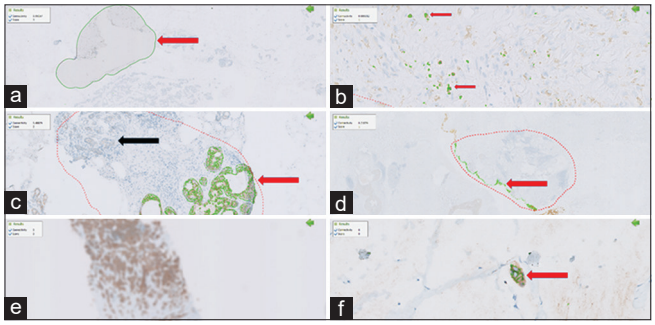

Figure 4: Pitfalls of human epidermal growth factor receptor 2 immunohistochemistry digital imaging analysis. (a). Air bubbles (red arrow); (b) pigments (red arrow); (c) ductal carcinoma *in situ* components (red arrow); invasive carcinoma was pointed with black arrow; (d) ink (red arrow); (e) out of focus; (f) rare tumor cells (red arrow). The human epidermal growth factor receptor 2 connectivity was labeled with green lines

IHC DIA but positive FISH (false-negative), and 2 cases with positive IHC DIA but negative FISH (false-positive). Previous studies also revealed false-negative and/or false-positive cases with variable frequencies.[12-14,17] All five cases had *HER2* copy numbers between 4 and 6, and pathologist's score of 2+ (except one case with 1+), representing the difficult cases with borderline *HER2* gene amplification/protein overexpression and lack of information regarding clinical outcomes.

Since the HER2 DIA used in this study also rendered an absolute value of HER2 connectivity from 0 to 1 for each case, we investigated its correlation with *HER2* FISH copy number and ratio. The analysis reveals HER2 IHC connectivity has better correlation with *HER2* copy number than *HER2*/CEP17 ratio (*r*: 0.860594 vs. 0.824927), suggesting *HER2* copy number may be more accurate to predict HER2 protein expression, and even response to anti-HER2 targeted therapy than *HER2*/CEP17 ratio. The findings are consistent with previous literatures demonstrating breast cancers with ratio ≥2 and copy number <4 are predominantly HER2 IHC-negative and less responsive to HER2-targeted therapy than breast cancers with copy number u4.[24-26]

Lastly, we have identified several pitfalls in the HER2 DIA process, including air bubbles, pigments, DCIS components, and inks, that cause false-positive results and out of focus and rare tumor cells to cause false-negative results. These pitfalls are not infrequent (4%, 25/612), but most of them can be avoided by reprocessing glass slides (air bubble), rescanning (out of focus), and carefully annotating region of interest (excluding pigments, DCIS components, and inks).

Conclusion

In summary, we have demonstrated that HER2 IHC DIA is a feasible and valid tool to determine HER2 status in breast carcinoma with high concordance when compared to pathologists' manual scoring and HER FISH. Our data have also revealed HER2 IHC connectivity has better correlation with HER2 copy number than HER2/CEP17 ratio, suggesting HER2 copy number may be more important to predict HER2 protein expression, and even response to anti-HER2-targeted therapy. Furthermore, HER2 IHC DIA measures HER2 protein in continuous quantitative values, providing useful information for correlating with clinical outcomes.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL, *et al.* Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 1987;235:177-82.
2. Tandon AK, Clark GM, Chamness GC, Ullrich A, McGuire WL. HER-2/neu oncogene protein and prognosis in breast cancer. J Clin Oncol 1989;7:1120-8.
3. Press MF, Pike MC, Chazin VR, Hung G, Udove JA, Markowicz M, *et al.* Her-2/neu expression in node-negative breast cancer: Direct tissue quantitation by computerized image analysis and association of overexpression with increased risk of recurrent disease. Cancer Res 1993;53:4960-70.
4. Park JW, Neve RM, Szollosi J, Benz CC. Unraveling the biologic and clinical complexities of HER2. Clin Breast Cancer 2008;8:392-401.
5. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, *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.
6. Romond EH, Perez EA, Bryant J, Suman VJ, Geyer CE Jr., Davidson NE, *et al.* Trastuzumab plus adjuvant chemotherapy for

operable HER2-positive breast cancer. N Engl J Med 2005;353:1673-84.
7. Baselga J, Cortés J, Kim SB, Im SA, Hegg R, Im YH, *et al.* Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer. N Engl J Med 2012;366:109-19.
8. Vogel CL, Cobleigh MA, Tripathy D, Gutheil JC, Harris LN, Fehrenbacher L, *et al.* Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. J Clin Oncol 2002;20:719-26.
9. Wolff AC, Hammond ME, Allison KH, Harvey BE, Mangu PB, Bartlett JM, *et al.* Human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline focused update. J Clin Oncol 2018;36:2105-22.
10. Gancberg D, Järvinen T, di Leo A, Rouas G, Cardoso F, Paesmans M, *et al.* Evaluation of HER-2/NEU protein expression in breast cancer by immunohistochemistry: An interlaboratory study assessing the reproducibility of HER-2/NEU testing. Breast Cancer Res Treat 2002;74:113-20.
11. Jacobs TW, Gown AM, Yaziji H, Barnes MJ, Schnitt SJ. Comparison of fluorescence *in situ* hybridization and immunohistochemistry for the evaluation of HER-2/neu in breast cancer. J Clin Oncol 1999;17:1974-82.
12. Brügmann A, Eld M, Lelkaitis G, Nielsen S, Grunkin M, Hansen JD, *et al.* Digital image analysis of membrane connectivity is a robust measure of HER2 immunostains. Breast Cancer Res Treat 2012;132:41-9.
13. Dobson L, Conway C, Hanley A, Johnson A, Costello S, O'Grady A, *et al.* Image analysis as an adjunct to manual HER-2 immunohistochemical review: A diagnostic tool to standardize interpretation. Histopathology 2010;57:27-38.
14. Helin HO, Tuominen VJ, Ylinen O, Helin HJ, Isola J. Free digital image analysis software helps to resolve equivocal scores in HER2 immunohistochemistry. Virchows Arch 2016;468:191-8.
15. Laurinaviciene A, Dasevicius D, Ostapenko V, Jarmalaite S, Lazutka J, Laurinavicius A, *et al.* Membrane connectivity estimated by digital image analysis of HER2 immunohistochemistry is concordant with visual scoring and fluorescence *in situ* hybridization results: Algorithm evaluation on breast cancer tissue microarrays. Diagn Pathol 2011;6:87.
16. Skaland I, Øvestad I, Janssen EA, Klos J, Kjellevold KH, Helliesen T, *et al.* Comparing subjective and digital image analysis HER2/neu expression scores with conventional and modified FISH scores in breast cancer. J Clin Pathol 2008;61:68-71.
17. Holten-Rossing H, Møller Talman ML, Kristensson M, Vainer B. Optimizing HER2 assessment in breast cancer: Application of automated image analysis. Breast Cancer Res Treat 2015;152:367-75.
18. Bui MM, Riben MW, Allison KH, Chlipala E, Colasacco C, Kahn AG, *et al.* Quantitative image analysis of human epidermal growth factor receptor 2 immunohistochemistry for breast cancer: Guideline from the college of American pathologists. Arch Pathol Lab Med 2019;143:1180-95.
19. Koopman T, Buikema HJ, Hollema H, de Bock GH, van der Vegt B. What is the added value of digital image analysis of HER2 immunohistochemistry in breast cancer in clinical practice? A study with multiple platforms. Histopathology 2019;74:917-24.
20. Koopman T, de Bock GH, Buikema HJ, Smits MM, Louwen M, Hage M, *et al.* Digital image analysis of HER2 immunohistochemistry in gastric- and oesophageal adenocarcinoma: A validation study on biopsies and surgical specimens. Histopathology 2018;72:191-200.
21. Diedenhofen B, Musch J. Cocor: A comprehensive solution for the statistical comparison of correlations. PLoS One 2015;10:e0121945.
22. Mohammed ZM, McMillan DC, Elsberger B, Going JJ, Orange C, Mallon E, *et al.* Comparison of visual and automated assessment of Ki-67 proliferative activity and their impact on outcome in primary operable invasive ductal breast cancer. Br J Cancer 2012;106:383-8.
23. Tuominen VJ, Tolonen TT, Isola J. ImmunoMembrane: A publicly available web application for digital image analysis of HER2 immunohistochemistry. Histopathology 2012;60:758-67.
24. Dowsett M, Procter M, McCaskill-Stevens W, de Azambuja E, Dafni U, Rueschoff J, *et al.* Disease-free survival according to degree of HER2 amplification for patients treated with adjuvant chemotherapy with or without 1 year of trastuzumab: The HERA trial. J Clin Oncol 2009;27:2962-9.
25. Zare SY, Lin L, Alghamdi AG, Daehne S, Roma AA, Hasteh F, *et al.* Breast cancers with a HER2/CEP17 ratio of 2.0 or greater and an average HER2 copy number of less than 4.0 per cell: Frequency, immunohistochemical correlation, and clinicopathological features. Hum Pathol 2019;83:7-13.
26. Bhargava R, Dabbs DJ. Interpretation of human epidermal growth factor receptor 2 (HER2) *in situ* hybridization assays using 2013 update of American Society of Clinical Oncology/College of American Pathologists HER2 guidelines. J Clin Oncol 2014;32:1855.