OPEN

HER2 Status Determination: Analyzing the Problems to Find the Solutions

Irene Terrenato, PhD, Ilaria Pennacchia, MD, Simonetta Buglioni, PhD, Marcella Mottolese, PhD, and Vincenzo Arena, MD

Abstract: Misdiagnosis in the evaluation of HER2 status in breast cancer may have consequent negative impact on clinical decision-making. Therefore, it has become ever more important to share procedures and interpretation criteria for HER2 testing among laboratories. Herein, we report an interlaboratory survey among 9 hospitals located in the central-south regions of Italy. The centers sent a series of 36 slides, 4 for each HER2 score, to the revising centers. We found a good concordance in HER2 scoring for 0 and 3+ score, but a very low concordance for 1+ and 2+ scores. To focus on factors that may lead to discordant results, we report 4 cases which summarized the most common source of discrepancy in HER2 testing. This methodological approach will help the individual laboratory to minimize technical variables and to reduce the percentage of erroneous interpretations of HER2 status.

(*Medicine* 94(15):e645)

Abbreviations: ASCO/CAP = American Society of Clinical Oncology/College of American Pathologists, BC = breast cancer, CI = confidence interval, DAB = diaminobenzidine, DDISH = Inform HER2 Dual in situ hybridization DNA probe cocktail assay, ER = estrogen receptor, US FDA = US Food and Drug Administration, G = tumor grading, IHC = immunohistochemistry, k_{cs} = kappa category-specific, k_w = weighted linear kappa statistics, MAb = monoclonal antibody, PAb = polyclonal antibody, PC = participant center, pN = lymph node status, PR = progesterone receptor, pT = tumor size, RC = revising center, T-DM1 = trastuzumab emtansine.

INTRODUCTION

It is widely known that HER2-positive breast cancer (BC) significantly differs from HER2-negative tumor both in terms of prognosis and response to therapy. The introduction of novel anti-HER2 treatments,[1] such as lapatinib,[2] pertuzumab,[3] and trastuzumab emtansine (T-DM1),[4] shows how increasingly important it is to correctly identify BC patients who may benefit from these targeted therapies. Hence, the development and clinical availability of these new anti-HER2 drugs have made essential the performance, in a proper and standardized manner, of the diagnostic techniques that are currently available to detect HER2 gene alterations. There are many procedural issues regarding the preanalytical and analytical phases which can interfere with the accuracy of the final result with consequent negative impact on clinical decision-making. Therefore, it has become ever more important to compare and share procedures and interpretation criteria for HER2 testing among laboratories. The aim of this paper is to underline the importance of monitoring and constantly updating the expertise of individual laboratories in HER2 testing, describing and discussing issues that emerge during an interlaboratory survey. For all the 9 laboratories, the survey represented an opportunity to verify the validity of the assays performed in their own laboratory and to acquire novel diagnostic tools that are able to enhance their expertise in HER2 status detection. Furthermore, the survey allowed us to perform proper statistical analyses aimed to highlight the persistent dilemma concerning the interlaboratory variability in preanalytical/analytic issues of HER2 determination.

MATERIALS AND METHODS

An interlaboratory survey among 9 hospitals located in the central-south regions of Italy (Figure 1A) was carried out in June 2013. Revising centers (RCs) of the survey were the Pathology Laboratories of the National Cancer Institute Regina Elena (MM) and of the Catholic University Sacred Heart of Rome (VA). The 9 participant centers (PCs) sent to the RC a series of slides referred to 36 BC cases collected from their archive, 4 for each HER2 score (0, 1+, 2+, and 3+), in which the HER2 status had been already determined using their own procedures. In particular, 3 PCs used the polyclonal antibody (PAb) A0485 (Dako, Milan Italy), 2 used the Hercep Test kit (Dako), 3 the monoclonal antibody (MAb) 4B5 (Ventana, Roche Diagnostics, Milan, Italy), and 1 used the MAb CB11 (Leica, Oracle, Milan, Italy) (Figure 1B). The PCs did not provide data concerning HER2 gene amplification. Ethical approval was not necessary because the samples were anonymized, and the tumor characteristics were obtained from medical records or direct pathological review of tumor tissue. All the collected cases were then retested by the RCs by using immunohistochemistry (IHC) and, regardless of the HER2 score, analyzed by ''InformHER2 dual in situ hybridization DNA probe assay'' (DDISH, Ventana, Benchmark XT) according to the following methods:

(1) IHC. IHC testing was performed using both the PAb A0485 (Dako) and the MAb 4B5 (Ventana Roche). The immunoreactions were revealed by a streptavidin–biotin enhanced immunoperoxidase technique (Super Sensitive MultiLink, Leica) in an automated autostainer (Bond III,

Editor: Sergio Gonzalez Bombardiere.
Received: January 26, 2015; revised: February 18, 2015; accepted: February 19, 2015.
From the Biostatistics, Scientific Direction, Regina Elena National Cancer Institute (IT); Pathology Department Catholic University of Sacred Heart (IP, VA); and Pathology Department Regina Elena National Cancer Institute–Rome, Italy (SB, MM).
Correspondence: Vincenzo Arena, Istituto di Anatomia Patologica, Università Cattolica del Sacro Cuore, L.go F.Vito, 1 00168 Rome, Italy (e-mail: vincenzo.arena@rm.unicatt.it).
The authors have no funding and conflicts of interest to disclose.

ISSN: 0025-7974
DOI: 10.1097/MD.0000000000000645

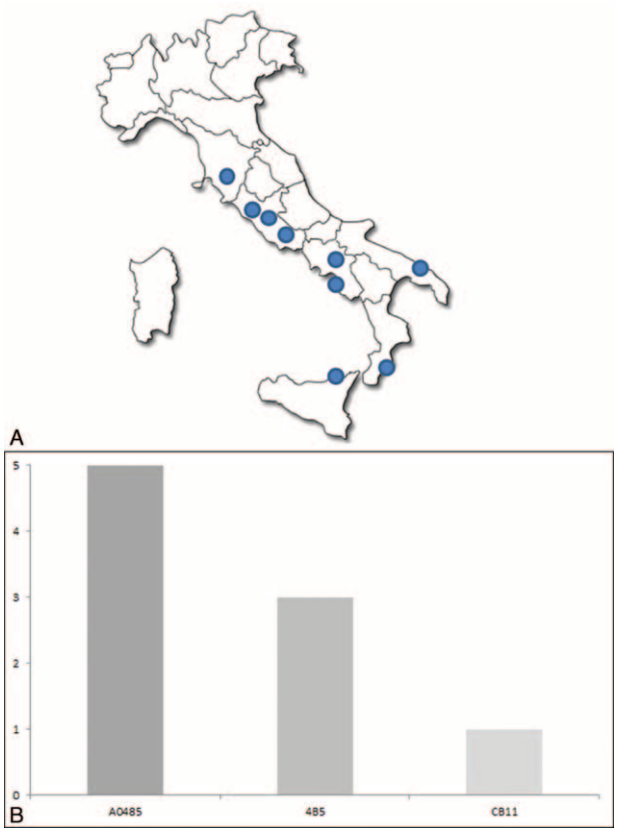


FIGURE 1. Geographical distribution of the 9 hospitals participating in the survey. Histogram reporting the antibodies used by the 9 participating centers.

Leica), and by Ultraview Universal DAB (diaminobenzidine) detection kit in an automated autostainer (Ventana Roche, Benchmark XT), respectively. HER2 positivity was scored as follows: 0 and 1+ negative, 2+ equivocal (to be confirmed by in situ hybridization), and 3+ positive according to the US FDA scoring system.

(2) In situ hybridization. HER2 gene amplification was evaluated using a fully automated DDISH (Ventana Roche). The assay was performed according to the manufacturer's instructions. DDISH results were analyzed by using a light microscope (Nikon, Eclipse 55i, Moncalieri (To) Italy) equipped with a software able to capture images (Eureka Interface System, Menarini, Florence, Italy). HER2 was defined amplified by DDISH when an HER2 gene copy number $\geq$6 or a ratio $\geq$2 was detected in at least 60 nuclei in 6 to 8 randomly selected invasive tumor areas. Lymphocytes and normal breast glandular epithelial cells served as internal controls.

Statistical Analysis

We compared the scores determined by each PC with their own procedures with the scores evaluated by the RCs as described earlier. Furthermore, to validate the performance of the PC, DDISH test was used as the gold standard. The 3+ cases were considered positive and 0/1+ cases were considered negative. Equivocal cases (score 2+) were excluded from this analysis, as they were misleading. To estimate the overall agreement both between PC and RC and between PC and DDISH, weighted linear kappa statistics (k_w) with its relative 95% confidence interval (95% CI) was used. The calculation of the weighted kappa assumes that the categories are ordered and accounts for how far apart the two raters are. In order to evaluate the contribution of each scoring category to the overall agreement (ie the agreement between the score given by the PC and the one given by the RC), the kappa category-specific (k_{cs}) statistic[10] and its 95% CI were calculated. To this end, the slides stained by all the participants were jointly considered. Each k_{cs} value was interpreted in a qualitative manner based on the Landis and Koch classification criteria.[11] Statistical analyses were performed with the SAS software (version 9.2.; SAS Institute Inc., Cary, NC).

RESULTS

Table 1 reports the IHC HER2 score distribution obtained by each PC using their own procedures and reagents in parallel with the results obtained by the RC by using IHC (MAb 4B5 and PAb A0485) DDISH in the 36 BC cases. Data obtained showed a complete overlapping between PC and RC results in the group of 3+ score, whereas we observed a few discrepancies for the other three score groups. The availability of DDISH results allowed a more accurate concordance analysis between PC and RC.

IHC Concordance Analysis Between PCs and RCs

Overall, IHC concordance among the results of PC and RC showed a good level of agreement both with respect to MAb 4B5 ($k_w = 0.692$, 95% CI: [0.527–0.858]) and to PAb A0485 ($k_w = 0.655$, 95% CI: [0.476–0.835]).

Table 2 reports the kappa category-specific statistic values (k_{cs}) and its relative 95% CI to indicate how each score category contributed to the agreement overall. As expected, independent of the testing method, reproducibility between PC and RC resulted to be very high only for 3+ score (with k_{cs} values between 0.81 and 1.0), being the levels of agreement for the other three categories lower. In particular, the agreement between score 1+ obtained by the PC versus the RC-4B5 and the RC-A0485 scores were ''fair'' (k_{cs} values between 0.21 and 0.40), while the agreement resulted ''moderate'' for scores 0 and 2+ (with k_{cs} values between 0.41 and 0.60).

When we stratified the analysis by reagents, we obtained only results for PAb A0485 (5 PCs) due to the small number of PCs that used MAb 4B5 (3 PCs) and MAb CB11 (1 PCs). The observed agreement between PCs and RCs still presented a good level of concordance for 0 and 3+ score ($k_{cs} = 0.69$ and $k_{cs} = 0.87$, respectively), but showed very low concordance between 1+ and 2+ scores ($k_{cs} = 0.37$ and $k_{cs} = 0.20$, respectively; data not shown).

Concordance Analysis Between IHC and DDISH

Table 3 reports the concordance between IHC results of PC and DDISH provided by RCs. Following Landis and Koch classification, we observed a very good agreement ($k_w = 0.84$). In fact, DDISH test identified HER2 gene amplification in 2/18 score 0/1+ and in 9/9 3+ BC cases.

Case Series

All the pathologists analyzed a number of slides related to the most controversial BC cases comparing the results already obtained by the PC with those obtained by the RC using both IHC and DDISH.

TABLE 1. HER2 Score Distribution: Comparison Between PC and RC

PC	Antibody PC	Score PC	Score RC 4B5	Score RC A0485	Score PC	Score RC 4B5	Score RC A0485	Score PC	Score RC 4B5	Score RC A0485	Score PC	Score RC 4B5	Score RC A0485
1	A0485	0	0	1+	1+	2+	2+	2+	2+	2+	3+	3+	3+
2	4B5	0	1+	1+	1+	1+	1+	2+	2+	2+	3+	3+	3+
3	4B5	0	1+	1+	1+	1+	1+	2+	2+	2+	3+	3+	3+
4	CB11	0	1+	2+	1+	3+	3+	2+	2+	2+	3+	3+	3+
5	Herceptest	0	0	2+	1+	1+	1+	2+	1+	2+	3+	3+	3+
6	A0485	0	0	0	1+	1+	1+	2+	1+	1+	3+	3+	3+
7	Herceptest	0	0	0	1+	2+	2+	2+	1+	1+	3+	3+	3+
8	A0485	0	0	0	1+	0	1+	2+	2+	3+	3+	3+	3+
9	4B5	0	0	0	1+	0	1+	2+	0	1+	3+	3+	3+

PC = participating center, RC = revising center.

Through a face-to-face meeting of all the pathologists (simultaneously reading at a multi-head microscope the discordant samples), it was possible to share and discuss the criteria for properly interpreting the HER2 status and assessing the technical issues concerning the procedures applied for determining the HER2 status. Herein, we report a series of 4 controversial cases, which allowed us to discuss the most common criticism in HER2 status interpretation.

Case 1: "*Be Precise*" From the Beginning! The Preanalytical Phase

The first case concerns a 63-year-old female patient with an invasive ductal carcinoma, G2, pT2, pNx, presenting the following immunophenotypic profile: estrogen receptor (ER) 90%, progesterone receptor (PR) 65%, Ki-67 3%, and HER2 (clone CB11) score 0. The HER2 staining performed by the PC proved to be negative showing only a mild-moderate aspecific cytoplasmic positivity. The IHC reassessment by the RC of the HER2 status gave the results plotted in Figure 2 (aspecific cytoplasmic immunostaining). The case tested by DDISH, unexpectedly, presented HER2 gene amplification although this was not foreseeable either on the base of the tumor morphology or on the base of the biological parameters (ER/PR positive, low Ki-67: 3%). The discrepancy between the IHC scores and DDISH was likely due to a delay in the fixation with a potential loss of antigenicity and coexistent cytoplasmic diffusion as emerged during the face-to-face meeting. The PC referred that the tumor specimen came from a hospital located away from the pathology laboratory where the HER2 test was carried out. Therefore, it is likely that the timing and quality of tissue fixation did not meet the standards required by the current guidelines recommendations.[5]

Case 2: A Problem of Interpretation

The second case concerns a 53-year-old female patient with an invasive ductal carcinoma, G2, pT1c, pN0, presenting the following phenotype: ER 90%, PR 80%, Ki-67 8%, and HER2 (clone A0485) score 1+. When the slide was reviewed, all the pathologists participating to the survey agreed on the score of 1+ as reported by the PC. The reassessment of the IHC and the DDISH test performed by the RC gave the results plotted in the Figure 2. The majority of the pathologists who reviewed the slides agreed on a diagnosis of 2+ score especially with the MAb 4B5. Two pathologists (with the MAb 4B5) and 1 pathologist (with the PAb A0485) would diagnosed the case as HER2 positive (score 3+) thus directly eligible for trastuzumab therapy. Conversely, the DDISH did not evidence HER2 amplification. The interpretative difficulties of the IHC are overlapping in the interpretation of HER2 gene amplification. In fact, 5 pathologists diagnosed the tumor as amplified while 2 as nonamplified. Observing the slide prepared by DDISH, we can highlight, as in case 1, some features concerning the preanalytical issues, again likely responsible for both suboptimal immunostaining and interpretative differences of molecular analysis. The DDISH test evidenced, more clearly than IHC, the artifacts due to a delayed fixation. Tissue degradation was well represented by areas with a consistent detachment of section from the slide, areas with empty nuclei, and areas without

TABLE 2. HER2 Evaluation: Comparison Between PCs and RCs

		RC 4B5			RC A0485		
			95% CI			95% CI	
Score PC	N Slides	k_{cs}	Lower Limit	Upper Limit	k_{cs}	Lower Limit	Upper Limit
0	36	0.55	0.24	0.87	0.54	0.21	0.87
1+	36	0.21	−0.13	0.56	0.40	0.08	0.72
2+	36	0.52	0.18	0.85	0.41	0.06	0.74
3+	36	0.92	0.79	1.00	0.86	0.67	1.00

CI = confidence interval, k_{cs} = kappa category-specific statistic, PC = participating center, RC = revising center.

TABLE 3. Concordance Analysis Between HER2 IHC by PCs and HER2 Dual Color Silver In Situ Hybridization by RCs

	RC DDISH		
PC IHC	**NA**	**A**	**Total N**
HER2 0/1+	16	2	18
	88.9%	11.1%	100.0%
HER2 3+	0	9	9
	0.0%	100%	100.0%
Total	16	11	27
	59.3%	40.7%	100.0%

$k_w = 0.84$, $P < 0.001$. A = amplified, DDISH = dual color silver in situ hybridization, IHC = immunohistochemistry, NA = nonamplified, PC = participating center, RC = revising center.

hybridization signals next to areas that can be easily evaluated and are located at the periphery of the section. The variability in the interpretation of the molecular data in DDISH, although less commonly discussed in comparison to that observed in IHC, may significantly influence the planning of anti-HER2 therapy both in terms of ''overtreatment'' and ''undertreatment''.[6]

Case 3: Always Look on the Other Biopathological Parameters!

The third case concerns a 63-year-old woman with an invasive ductal carcinoma, G3, pT2, pN2, with the following immunophenotypic profile: ER and PR negative, Ki-67% 30%, and HER2 (A0485) score 2+. The reassessment of the IHC slides prepared by the RC gave the results plotted in Figure 3. This case, though it showed HER2 amplification by DDISH, did not display a strong and convincing circumferential membrane staining with any of the used antibodies. The case was evaluated in a highly variable manner: 5 pathologists gave a score 0, 10 pathologists gave a score 1+, and 9 pathologists assigned a score 2+. The lack of ER and PR expression, the high proliferative index (Ki-67), the pathological grading (G3), and the lymph nodes status (pN2) induced 9 pathologists to assign a score 2+ to the BC cases examined. A critical evaluation of HER2 status in the light of the clinical/morphological context not only represents a shared approach but also should be the norm as recently suggested by Iorfida et al.[7]

Case 4: It is a True Heterogeneity?

The fourth case concerns a 48-year-old female patient with an invasive ductal carcinoma, G2, pT1c, pN0, presenting the following immunophenotypic profile: ER 98%, PR 95%, Ki-67 16%, and HER2 (clone 4B5) score 2+. The reassessment by RC of the IHC gave the results plotted in Figure 4. This case has raised a wide debate on the ''intratumoral heterogeneity'' of HER2 status. However, the low power view of the slide shows that it is not a heterogeneous case from a biological point of view.[8] In Figure 4, we can appreciate how the IHC HER2 signal fades from the periphery to the center. Conversely, at high magnification a circumferential and strong HER2 signal is observed at the periphery of tumor, while in the center this finding becomes less evident as the nonspecific cytoplasmic staining increases. The variation of staining may be due to

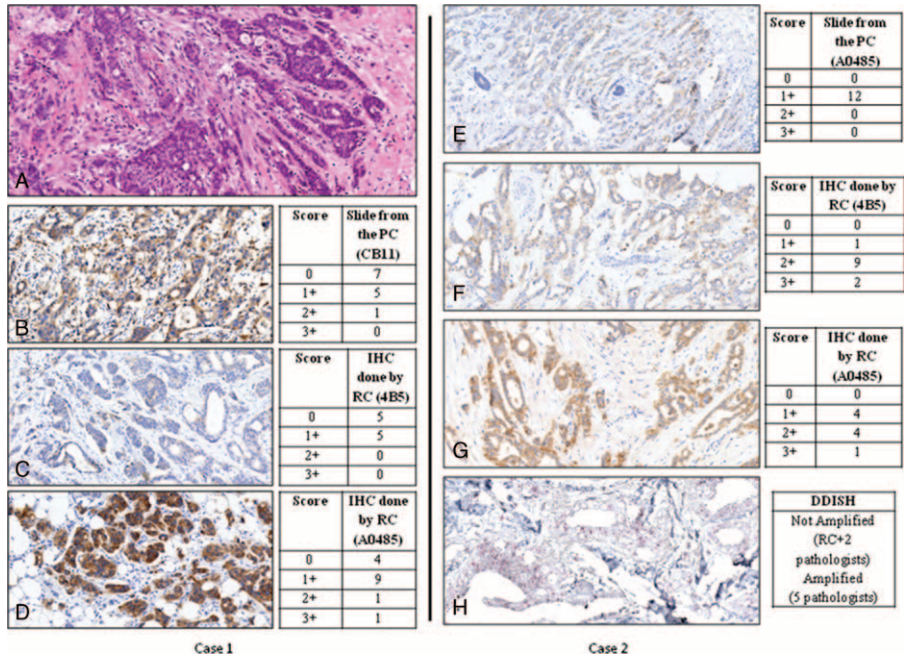
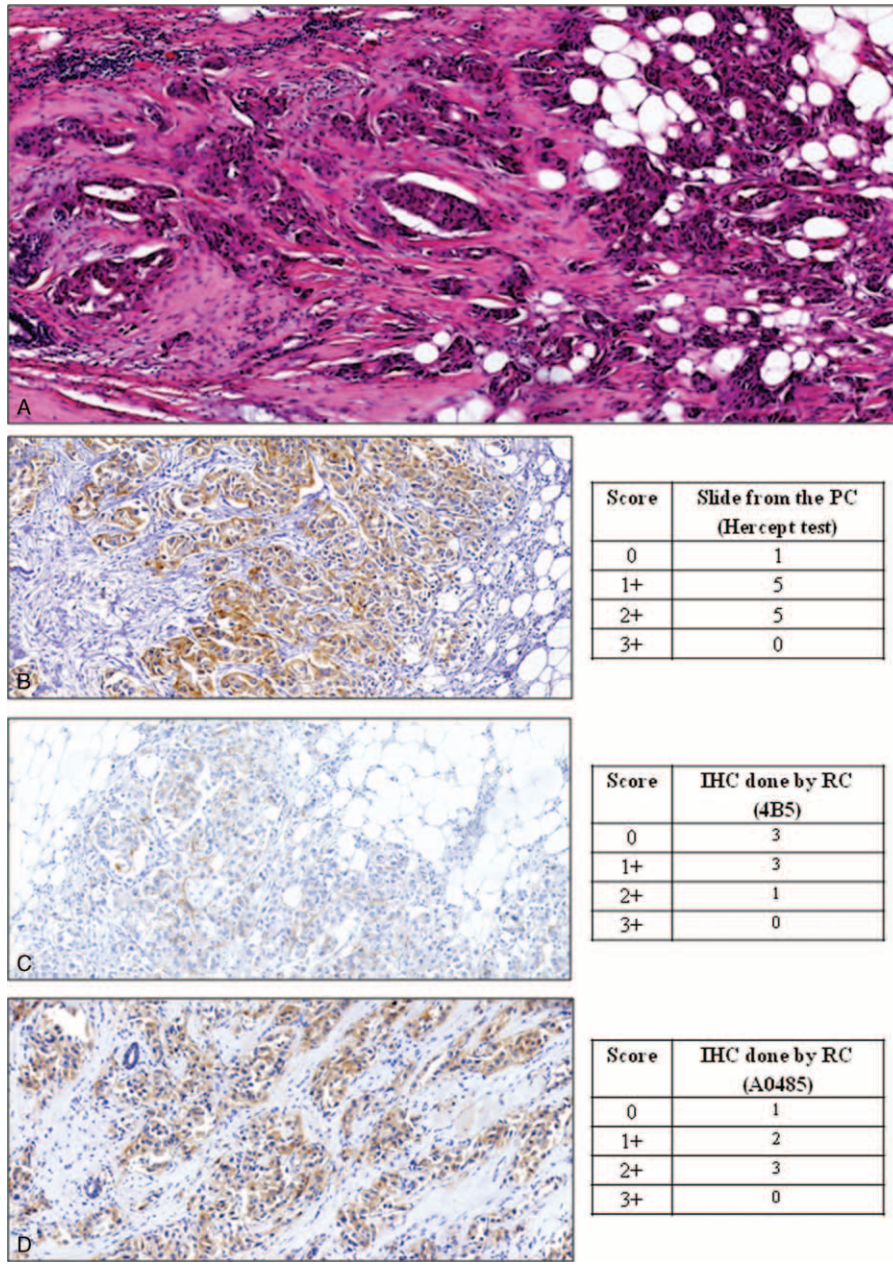


FIGURE 2. Case 1 (A) Hematoxylin/eosin staining. (B) Faint, barely perceptible incomplete membrane staining ≤10% of tumor cells obtained by the PC-4 using the MAb CB11 (score 0). (C) No immunoreactivity obtained by the RC using the MAb 4B5 (score 0). (D) Aspecific cytoplasmic immunostaining obtained by RC using the PAb A0485 (original magnification ×20). Case 2 (E) Faint, barely perceptible incomplete membrane staining in >10% of tumor cells obtained by the PC-1 using the PAb A0485 (score 1+). (F) Faint, barely perceptible incomplete membrane staining >10% of tumor cells obtained by the RC using the MAb 4B5 (score 1+). (G) Circumferential weak, incomplete membrane staining >10% of tumor cells obtained by the RC using the PAb A0485 (score 2+; original magnification ×20). (H) DDISH showing a ratio <2 (not amplified; original magnification ×40). Tables report the pathology consensus for each IHC slide (case 1 and case 2) and for DDISH (case 2).



Score	Slide from the PC (Hercept test)
0	1
1+	5
2+	5
3+	0

Score	IHC done by RC (4B5)
0	3
1+	3
2+	1
3+	0

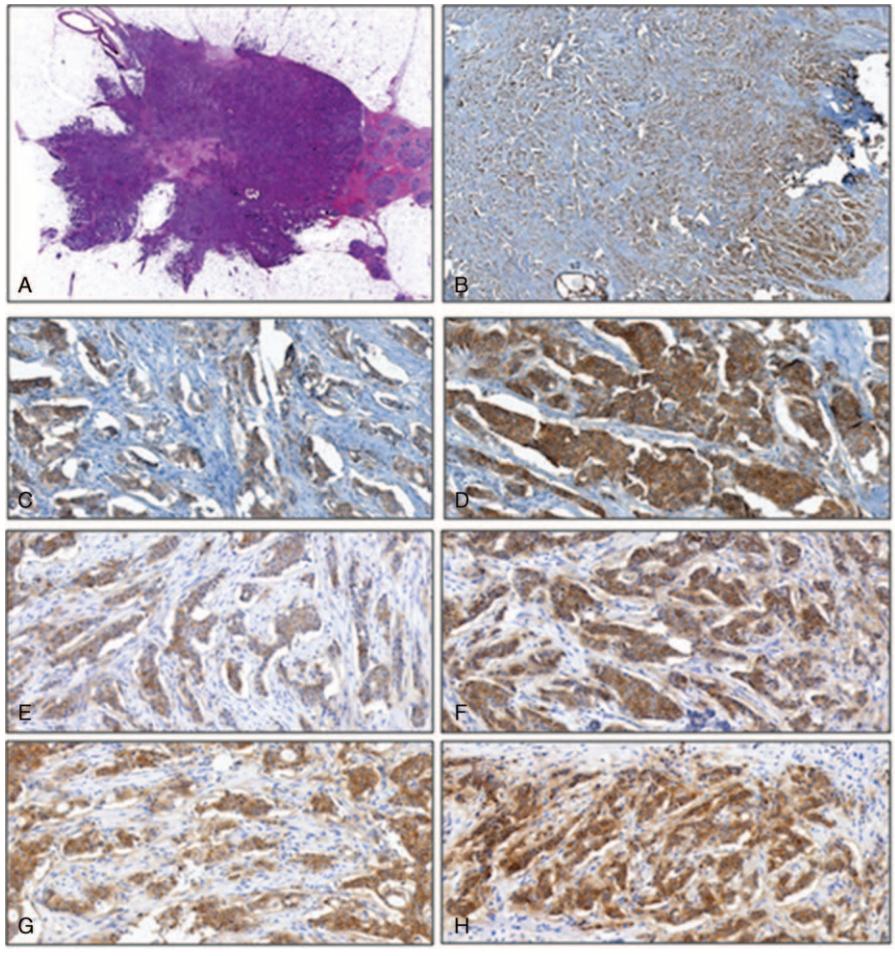
Score	IHC done by RC (A0485)
0	1
1+	2
2+	3
3+	0

FIGURE 3. Case 3 (A) Hematoxylin/eosin staining. (B) Circumferential, weak, incomplete membrane staining >10% of tumor cells obtained by the PC-7 using the Herceptest (score 2+). (C) Faint, barely perceptible incomplete membrane staining in >10% of tumor cells obtained by the RC using the MAb 4B5 (score 1+). (D) Circumferential, weak, incomplete membrane staining >10% of tumor cells obtained by the RC using the PAb A0485 (score 2+; original magnification ×20). Tables report the pathology consensus for each IHC slide.

nonoptimal/suboptimal time of fixation with consequent alcoholic postfixation during the processing of the specimen. Therefore, the fixation occurred by cross-links of the proteins and not by coagulation is the only fact that can guarantee an optimal preservation of cellular antigenicity.[9]

DISCUSSION

The clinical cases that have been presented and discussed raises a single and important question: how is it possible to achieve an adequate level of diagnostic accuracy in the determination of HER2 status that allows to properly plan the treatment of patients affected by breast cancer? In fact, concordance analyses performed between results sent by the PC and results subsequently obtained by the RC showed a high reproducibility of score 3+, but revealed a fair degree of reproducibility of the other classes (0, 1+, and 2+). Regardless of the ''intensity'' or ''completeness'' of the IHC signals and regardless of the number of HER2 gene copy number, the issues related to the preanalytical procedures (and to a lesser extent the variability of the currently available anti-HER2 antibodies) might represent the causes that lead to false-positive or false-negative results in a



Score	Slide from the PC (4B5)
0	0
1+	0
2+	9
3+	3

Score	IHC done by RC (4B5)
0	0
1+	0
2+	10
3+	2

Score	IHC done by RC (A0485)
0	0
1+	0
2+	9
3+	3

FIGURE 4. Case 4 (A) Hematoxylin/eosin staining (PC-2, original magnification ×4). (B) IHC HER2 staining fading from the periphery to the center (original magnification ×10). (C–H) Circumferential moderate, incomplete membrane staining of tumor cells associated with a specific cytoplasmic staining obtained by the PC-9 and by the RC using both the MAb 4B5 and the PAb A0485 (score 2+; original magnification ×20). Tables report the pathology consensus for each IHC slide.

relevant percentage of cases. These issues continue to be addressed also by the most recent guidelines.[5] We believe that the efforts to standardize and to optimize the preanalytical phases still today encounter a number of difficulties on the Italian national territory as it is highlighted by the cases herein reported. It was interesting to note that when the pathologist is aware that technical problems (preanalytical or analytical) had arisen, he frequently asks for a ''reflex test'' (at the time of the survey the last American Society of Clinical Oncology/College of American Pathologists [ASCO/CAP] guidelines had not yet published).[5] Nowadays, the availability of new anti-HER2 therapies, especially in case of drugs directly bound to the chemotherapeutic agent such as T-DM1, opens increasingly difficult challenges for the quality assurance of the test. The discussion made during the consensus meeting around the microscope revealed that a few centers may provide information about the ''time *to* fixation'' or about the ''time *of* fixation''. Due to financial constraints, some hospitals cannot afford ''breast-committed'' technical and medical staff and for this reason, and also minor variations in routine procedures may encounter some difficulties. Similarly, it was not known whether the surgical specimen was placed in 10% buffered formalin following the recommended sampling procedures or whether it was already open by the surgeons, or rather whether it remained intact in buffered formalin until the sampling. It is a concern shared worldwide, already underlined in the 2007 ASCO/CAP guideline[12] and then widely stressed in those of 2013,[5] that pathology laboratories should certificate their HER2 testing performance. This certification may be achieved by participating in quality control programs aimed to detect, reduce, and correct deficiencies in the analytical process involved in HER2 detection. Furthermore, pathology laboratories that handle a limited number of cases a year are likely to benefit from the experience of RCs that should take a leading role in the initiation of such quality control studies. The creation of the Breast Units and the participation in regional/interregional and/or national quality control programs,[13] in our opinion, could be of help to ensure the correct preanalytical standardization[14,15] that, to date, is essential in BC as in other tumor pathology. However, even in the absence of Breast Units, we believe that an effective collaboration between the various professionals involved in BC diagnosis, it will certainly be of help to reduce these kinds of problems. ''*If you want to go fast go alone. If you want to go far go together*'', and it is with regret that we can say that too often this sentence is just an African proverb.

REFERENCES

1. Arteaga CL, Sliwkowski MX, Osborne CK, et al. Treatment of HER2-positive breast cancer: current status and future perspectives. *Nat Rev Clin Oncol.* 2011;9:16–32.
2. Geyer CE, Forster J, Lindquist D, et al. Lapatinib plus capecitabine for HER2-positive advanced breast cancer. *N Engl J Med.* 2006;355:2733–2743.
3. Baselga J, Cortés J, Kim SB, et al. CLEOPATRA Study Group: Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer. *N Engl J Med.* 2012;366:109–119.
4. Verma S, Miles D, Gianni L, et al., EMILIA Study Group. Trastuzumab emtansine for HER2-positive advanced breast cancer. *N Engl J Med.* 2012;367:1783–1791.
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;3131:3997–4013.
6. Petersen BL, Sørensen MC, Pedersen S, et al. Fluorescence in situ hybridization on formalin-fixed and paraffin-embedded tissue: optimizing the method. *Appl Immunohistochem Mol Morphol.* 2004;12:259–265.
7. Iorfida M, Dellapasqua S, Bagnardi V, et al. HER2-negative (1+) breast cancer with unfavorable prognostic features: to FISH or not to FISH? *Ann Oncol.* 2012;23:1371–1372.
8. 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.
9. Burandt E, Sauter G. HER2 ASCO guidelines. The answer to everything? *Pathologe.* 2010;31(Suppl 2):285–291.
10. Fleiss JL. Statistical methods for rates and proportions. 2nd ed. New York: Wiley and Sons; 1981.
11. Landis R, Koch G. The measurement of observer agreement for categorical data. *Biometrics.* 1977;33:117–127.
12. Wolff AC, Hammond ME, Schwartz JN, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *J Clin Oncol.* 2007;25:118–145.
13. Terrenato I, Arena V, Pizzamiglio S, et al. External Quality Assessment (EQA) program for the preanalytical and analytical immunohistochemical determination of HER2 in breast cancer: an experience on a regional scale. *J Exp Clin Cancer Res.* 2013;32:58.
14. Tong LC, Nelson N, Tsourigiannis J, et al. The effect of prolonged fixation on the immunohistochemical evaluation of estrogen receptor, progesterone receptor, and HER2 expression in invasive breast cancer: a prospective study. *Am J Surg Pathol.* 2011;35:545–552.
15. Schiavon BN, Jasani B, de Brot L, et al. Evaluation of reliability of FISH versus brightfield dual-probe in situ hybridization (BDISH) for frontline assessment of HER2 status in breast cancer samples in a community setting: influence of poor tissue preservation. *Am J Surg Pathol.* 2012;36:1489–1496.