CME/SAM

Distinguishing Score 0 From Score 1+ in HER2 Immunohistochemistry-Negative Breast Cancer

Clinical and Pathobiological Relevance

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ABSTRACT

Objectives: *To investigate the clinical and pathobiological significance of distinguishing score 0 and score 1+ within the group of immunohistochemistry (IHC)-negative invasive breast cancers.*

Methods: *We studied HER2 status using both IHC and fluorescence in situ hybridization (FISH) in 150 consecutive breast tumors submitted to our laboratory after a negative IHC result in local testing centers.*

Results: *We were able to discern a group of score 0 tumors that had a lower HER2 copy number than the group consisting of score 1+ tumors. In contrast with the group of score 1+ tumors, HER2 FISH was consistently negative for both copy number–based and ratio-based tumors without equivocal results.*

Conclusions: *In a setting with stringent quality assurance, score 0 and score 1+ tumors emerge as distinct and clinically important subgroups within the HER2 IHC-negative population.*

Upon completion of this activity you will be able to:
- interpret HER2 status of a breast tumor based on given information about immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) results.
- define differences in HER2 FISH results between IHC score 0 and score 1+ breast tumors.
- discuss whether the prevalence of 0 and 1+ scores differs or not between needle biopsies and resected specimens.

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A vast amount of literature exists on HER2 testing in breast cancer, but most articles have focused on HER2-positive cancers.[1] Very few studies have investigated whether differences between the 2 entities can be distinguished by immunohistochemistry (IHC) within the group of HER2-negative tumors (ie, tumors with a score of 0 or 1+). A recent study found that score 1+ tumors had a higher estrogen receptor (ER) expression both at the messenger RNA (mRNA) and protein levels than score 0 tumors.[2] Gilcrease et al[3] reported that HER2-negative cancers with a score of 1+ had a worse prognosis than those with a score of 0, but this finding could not be confirmed by Jensen et al.[4] Differences in treatment regimens could explain this, as mentioned by the authors,[5] but it is also important to realize that these 2 studies used different criteria to define score 0 and 1+. Indeed, the definition of these IHC categories in the American Society

of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines[6] differs from those in the UK recommendations[7] regarding both percentage cutoff and completeness of membranous staining. Other scoring methods have also been applied, especially in studies performed before the ASCO/CAP and UK guidelines were published.[8,9] So, besides (pre)analytical differences, there is already an important postanalytical variability in the literature purely based on differences in scoring methods. Hence, it is very difficult to assess the relative proportion of score 0 and score 1+ cases in the HER2 IHC-negative population and whether there is a difference in concordance with (fluorescence) in situ hybridization ((F)ISH) between the 2 categories. This issue is further complicated by the fact that chromosomal gains and losses in the centromeric region of chromosome 17 can lead to artificial skewing of the *HER2*/CEP17 ratio toward false-positive or false-negative results.[10,11] Finally, the issue of (double) equivocal/ambiguous results and reflex testing has recently gained interest[12,13] but has not been evaluated specifically in IHC score 0 and score 1+ tumors.

To investigate these issues and to assess whether the distinction of HER2 score 0 from score 1+ is biologically and clinically meaningful, we performed IHC and FISH for HER2 on 150 consecutive cases submitted to our laboratory as HER2 IHC negative. We took differences between local and central laboratory results and the role of copy number, ratio, and ambiguity into account when performing our analysis.

Materials and Methods

Tissue Samples

All paraffin blocks with formalin-fixed invasive breast cancer received between June 2011 and September 2012 that were scored as HER2 IHC negative according to the Belgian guidelines[14] by the referring pathologist were considered for inclusion in the study. All cases were sent by pathologists from community hospitals without accreditation for HER2 testing for central testing in our laboratory, which has been ISO15189 accredited for HER2 IHC and FISH testing since July 2009. The study was approved by the ethics committee of the Ghent University Hospital, provided that data that could lead to identification of the referring hospitals would not be disclosed.

Immunohistochemistry

Staining for HER2 was performed on 2-μm-thick sections mounted on SuperFrost slides (Menzel-Gläser, Braunschweig, Germany) using an automatic immunostainer (BenchMark XT, Ventana Medical Systems, Tucson, AZ) according to the manufacturer's instructions. The rabbit monoclonal antibody 4B5 (ready to use; Roche, Basel, Switzerland) was used, and visualization was achieved with the ultraView Universal DAB Detection Kit (Ventana Medical Systems). Heat-induced epitope retrieval was performed using Cell Conditioning 2 (Ventana Medical Systems). 4B5 is an anti-HER2 antibody of which the epitope has been defined by mapping as the amino acid sequence TAENPEYLGL.[15] This antibody does not cross-react with other members of the HER family in formalin-fixed, paraffin-embedded breast carcinoma.[16]

HER2 expression was scored according to the Belgian guidelines,[14] which are identical to ASCO/CAP guidelines,[8] as 0/negative, 1+/negative, 2+/equivocal, or 3+/positive. More specifically, the invasive part of the tumor was scored 0 when there was no membranous staining observed, 1+ when there was incomplete membrane staining in any proportion of tumor cells or weak complete membrane staining in less than 10% of cells, 2+ when there was complete and weak membrane staining in at least 10% of cells or when there was intense complete membrane staining in 30% or less of tumor cells, and 3+ when there was intense complete membrane staining in more than 30% of tumor cells. Scoring was performed by an expert breast pathologist (K.L.).

FISH

After mounting of 2-μm-thick sections on SuperFrost slides, 7 to 10 μL (dependent on the tissue size) of the locus-specific identifier HER2/neu and CEP17 probe (PathVysion HER2 DNA Probe Kit, Abbott Molecular, Abbott Park, IL) was added. After denaturation for 5 minutes at 75°C in a hot water bath, sections were hybridized overnight at 37°C in an incubator. Then, 10 μL DAPI was added, followed by covering with a coverslip.

Red (for *HER2*) and green (for CEP17) nonoverlapping signals were counted in 20 nuclei according to the ASCO/CAP guidelines[8] using a fluorescence microscope equipped with appropriate filters (Olympus BX40, Olympus, Tokyo, Japan). Only nonoverlapping intact nuclei were considered, and nuclei without signals or signals of only 1 color were excluded. The presence of clusters was noted and the number of signals fitting in a cluster was estimated. The mean *HER2* and CEP17 copy number was determined by dividing the total number of signals by the number of counted nuclei. Scoring was performed by an experienced laboratory technician (B.M.) under supervision of a pathologist (K.L.). In agreement with the ASCO/CAP guidelines[8] and the ASCO/CAP clinical notice,[12] ratio-based amplification was considered present when the *HER2*/CEP17 ratio was 2 or more, and copy number–based amplification was considered present when the mean *HER2* copy number was more than 6. The ASCO/CAP clinical notice emphasizes that patients with a ratio of 2 or more, including those with a ratio between 2.0 and 2.2, were eligible for the trastuzumab adjuvant trial. The

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clinical notice also mentions the relevance of reflex testing in case of an equivocal result (ie, a score of 2+ for IHC and a ratio between 1.8 and 2.2 or a mean copy number between 4 and 6 for ISH). Therefore, the issue of (double) equivocal results was also evaluated in the current study.

Statistical Analysis

Data were analyzed using the Mann-Whitney test for continuous data or the χ^2 test for categorical data. Continuous values are reported as mean ± standard deviation. Analyses were performed using StatView 5 (SAS, Cary, NC), and significance was accepted when $P < .05$.

Results

Characteristics of the Samples

In total, 153 cases were submitted for central HER2 testing in the considered period. Insufficient quality of the tissue in the paraffin block and insufficient amount of invasive tumor tissue precluded IHC and FISH analysis in 2 and 1 cases, respectively. The other 150 tumors were included in the study and were received from 5 different community hospitals, of which 4 used the 4B5 antibody from Roche and 1 used the A0485 antibody from DAKO (Glostrup, Denmark). In 116 (77%) cases, the paraffin block contained a needle biopsy specimen, and in 34 (23%) cases, the paraffin block contained tissue taken from a resected specimen. In total, 102 (68%) cases were scored 0 and 48 (32%) cases scored 1+ by the local pathologist. Of 116 needle biopsy specimens, 84 (72%) were scored 0, while of 34 resection specimens, 18 (53%) were scored 0 ($P = .03$).

Central vs Local IHC for HER2

The concordance between local results and the results of HER2 staining at the central laboratory is described in **Table 1**. Concordance was good for local score 1+, whereas most local score 0 cases were score 1+ at the central laboratory. All IHC-positive cases at the central laboratory were locally scored 1+. In contrast with local laboratory results, the prevalence of scores 0 and 1+ at the central laboratory was not different between needle biopsy and resection specimens ($P = .32$).

Ratio-Based and Copy Number–Based FISH vs Central and Local IHC for HER2

The comparison between IHC and FISH data is summarized in **Table 2** and **Table 3**. While 8% of local IHC results were false negative according to the ratio-based FISH data, this decreased to 4% of cases when copy number–based FISH results were used as reference, and all these cases were submitted as score 1+. Similarly, concordance between FISH and central IHC-negative cases increased from 97% to 99% when copy number–based FISH was used as reference instead of ratio-based FISH, with none of the central score 0 tumors showing FISH positivity, irrespective

Table 2
Local IHC vs Central FISH for HER2

	Central FISH Testing, No. (%)			
	Ratio Based		Copy Number Based	
Local IHC Testing	Negative	Positive	Negative	Positive
Score 0	100 (98)	2 (2)	102 (100)	0 (0)
Score 1+	38 (79)	10 (21)	42 (88)	6 (12)
Total	138 (92)	12 (8)	144 (96)	6 (4)

FISH, fluorescence in situ hybridization; IHC, immunohistochemistry.

Table 3
Central IHC vs Central FISH for HER2

	Central FISH Testing No. (%)			
	Ratio Based		Copy Number Based	
Central IHC Testing	Negative	Positive	Negative	Positive
Score 0	16 (100)	0 (0)	16 (100)	0 (0)
Score 1+	110 (97)	3 (3)	112 (99)	1 (1)
Total negative cases	116 (97)	3 (3)	118 (99)	1 (1)
Score 2+	12 (70)	5 (30)	16 (94)	1 (6)
Score 3+	0 (0)	4 (100)	0 (0)	4 (100)

FISH, fluorescence in situ hybridization; IHC, immunohistochemistry.

Table 1
Comparison of Local and Central IHC for HER2

	Central IHC Testing					
Local IHC Testing	Score 0	Score 1+	Score 2+	Score 3+	Total	Concordance, %
Score 0	15	78	9	0	102	15
Score 1+	1	35	8	4	48	73
Total	16	113	17	4	150	86

IHC, immunohistochemistry.

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of the interpretation method of FISH results. Mean *HER2* and CEP17 copy number, *HER2*/CEP17 ratio, and local and central IHC results for the 6 tumors that were only ratio-based FISH positive are described in **Table 4**. All cases that were FISH positive based on copy number were also positive based on ratio.

Equivocal Ratio-Based and Copy Number–Based FISH vs Central and Local IHC for HER2

Of 8 cases that were equivocal on FISH, either ratio based and/or copy number based, 3 were also equivocal on IHC, rendering these tumors double equivocal. One case that was ratio-based equivocal had a ratio higher than 2 and was therefore considered ratio-based FISH positive in the other analyses performed in this study. The data of these tumors are described in **Table 5**.

HER2 Copy Number and Central IHC for HER2

In the group of nonamplified tumors based on copy number, the mean *HER2* copy number was higher in score 1+ tumors than in score 0 tumors ($P = .01$) and was higher in score 2+ tumors than in score 1+ tumors ($P < .001$) **Table 6**. Of the 6 tumors amplified based on copy number, 3 showed *HER2* clusters, and these tumors were all scored 3+. The tumor with score 1+ showed a mean *HER2* copy number of 6.2, the lowest value of all amplified tumors.

Table 5
Data From Tumors That Were Equivocal on FISH[a]

Patient No.	Local IHC Score	Central IHC Score	*HER2* Signals/Cell	*HER2*/CEP17 Ratio
1	1	1	**5.20**	**1.891**
2	1	1	2.50	**2.155**
3	0	**2**	**4.30**	1.509
4	1	**2**	**4.35**	1.192
5	0	1	**4.55**	1.182
6	1	1	**4.60**	1.394
7	1	1	**4.70**	1.593
8	1	**2**	**4.80**	1.171

FISH, fluorescence in situ hybridization; IHC, immunohistochemistry.
[a] Equivocal results are in bold; double equivocal cases are italicized.

Table 6
Mean HER2 Copy Number per IHC Category in Copy Number–Based Negative Tumors

IHC Score	No. of Cases	*HER2* Signals/Cell, Mean ± SD
0	16	2.14 ± 0.25
1+	112	2.57 ± 0.73
2+	12	3.76 ± 1.29

IHC, immunohistochemistry.

Discussion

In the current study, we found that central reassessment of breast tumors scored as IHC negative after local laboratory testing resulted in an important shift of score 0 toward score 1+. Moreover, all tumors found to be equivocal or positive by central laboratory IHC testing were originally scored 1+, never 0. Interestingly, the prevalence of score 0 and score 1+ cases did not differ between needle biopsy and resection specimens based on central laboratory results, while score 0 was less prevalent in the latter than in the former based on local laboratory results. Since local and central testing was performed on the same paraffin block, preanalytical phase differences were negligible. The relative contribution of variability in analytical and postanalytical phases cannot be determined, but it can reasonably be assumed that differences in IHC staining protocols played a substantial role. Since 2 types of anti-HER2 antibodies were used, their known difference in sensitivity[17] may have contributed in the shift of score 0 toward score 1+ after central reassessment. To determine the influence of the scoring process, we searched the literature for articles mentioning the relative proportion of score 0 tumors within an IHC-negative group, only considering publications that specifically reported that the ASCO/CAP scoring guidelines were followed. Remarkably, the proportion of score 0 tumors widely varies, ranging from 18% to 80%.[2,3,18,19] Interestingly, only in our current study

Table 4
Data From Tumors of Which Only the Ratio-Based FISH Was Positive

Patient No.	Local IHC Score	Central IHC Score	*HER2* Signals/Cell	CEP17 Signals/Cell	*HER2*/CEP17 Ratio
1	1+	1+	2.50	1.16	2.155
2	1+	1+	4.45	2.00	2.225
3	0	2+	4.95	1.95	2.538
4	0	2+	5.85	2.05	2.854
5	1+	2+	5.80	1.95	2.974
6	1+	2+	5.10	1.60	3.187

FISH, fluorescence in situ hybridization; IHC, immunohistochemistry.

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and in the study by D'Alfonso et al[18] was scoring consistently done by the same observer. Both studies found a similar low proportion of score 0 tumors (ie, 14% and 18%, respectively), while the other studies and the local scoring results in the current study yielded a much higher proportion of score 0 tumors within the IHC-negative group. It appears important to carefully scan all tumoral cells to exclude any faint and partial membranous staining before assigning the tumor to the score 0 category.

Applying the stringent quality system of our laboratory, we were able to discern a group of breast tumors with score 0 that had a lower *HER2* copy number than the group consisting of score 1+ tumors. In the same line, Pinhel et al[2] found that HER2 mRNA levels were lower in a group of score 0 tumors compared with a group of score 1+ tumors. Overall, this suggests that score 0 tumors differ from score 1+ tumors at the DNA, mRNA, and protein level of HER2.

The very low *HER2* copy number in score 0 tumors also explains why this group was consistently HER2 FISH negative for both copy number–based and ratio-based tumors. Shifting toward a ratio-based positive FISH result due to a low CEP17 copy number occurred only in a small proportion of cases in the group of score 1+ tumors with a slightly higher copy number of *HER2*. For the group of tumors that were IHC negative in the local laboratory, the FISH positivity rate was halved when only the *HER2* copy number was considered rather than the ratio of *HER2*/CEP17. The discrepancy between both types of FISH results vs negative IHC results thus decreased after central IHC testing, but it did not completely disappear, indicating that this discrepancy is an inherent phenomenon that is not solely caused by the quality of HER2 testing. A recent array-based comparative genomic hybridization study has highlighted the possibility that the HER2 ratio can be artificially skewed to positive due to centromeric loss,[10] and Grimm et al[20] found that the IHC-negative and FISH-positive group in their study was enriched for cases with low CEP17 copy numbers. These and our findings clearly suggest that in such cases, the (F)ISH result should be considered false positive rather than the IHC results considered false negative. It has been suggested that a cautionary note should be used when reporting the results of such cases.[10]

We found that 3 (38%) of 8 cases that were equivocal on FISH, either ratio based and/or copy number based, were also equivocal on IHC, with all equivocal cases being scored at least 1+ by central laboratory testing. Clay et al[13] recently found that of 60 cases equivocal on ratio-based FISH, 18 (30%) were also equivocal by IHC testing. To our knowledge, no other reports have involved double equivocal or ambiguous HER2 tumors. The ASCO/CAP guidelines[8] and the clinical notice[12] state that withholding anti-HER2 treatment was not recommended in those patients with an equivocal HER2 test (or tests) whose results fell within ranges that would have allowed them to be treated in the first generation of adjuvant HER2 trials (ASCO/CAP guidelines and clinical notice). Since patients with a ratio of 2 or more were eligible for these trials, this suggests that a patient who has a double equivocal tumor that is IHC 2+ and a *HER2*/CEP17 ratio of 2.1 merits to be treated. However, it is less obvious whether a patient who has a double equivocal tumor that is IHC 2+ and a *HER2* copy number of 4.8 (as was the case for patient 8 in Table 5) should be denied treatment. Clearly, more studies are needed on the subject of double equivocal HER2 tumors.

In the current study, we were able to delineate a group of score 0 tumors that were consistently both ratio-based and copy number–based FISH negative, without equivocal results. Our findings indicate that score 0 tumors are biologically distinct from score 1+ tumors, and one could suggest that expected concordances between (F)ISH and IHC should differ for the latter rather than the former type of tumor. During a CAP conference on HER2 in 2002, it was suggested that laboratories with a FISH-IHC concordance of at least 90% for score 3+ and score 0 tumors should investigate only score 1+ and 2+ by FISH.[21] Thus, one could advocate that within the group of IHC-negative tumors, only cases scored 1+ should be further evaluated with (F)ISH.

In conclusion, our study showed that in the setting of an accredited central laboratory, score 0 and score 1+ tumors emerge as distinct and clinically relevant subgroups within the HER2 IHC-negative population.

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