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Prediction of *HER2* gene status in Her2 2+ invasive breast cancer: a study of 108 cases comparing ASCO/CAP and FDA recommendations

Frédéric Chibon[1], Isabelle de Mascarel[1], Ghislaine Sierankowski[1], Véronique Brouste[2], Hervé Bonnefoi[3], Marc Debled[3], Louis Mauriac[3] and Gaëtan MacGrogan[1]

[1]*Department of Pathology, Institut Bergonié, Bordeaux, France;* [2]*Department of Biostatistics, Institut Bergonié, Bordeaux, France and* [3]*Department of Medical Oncology, Institut Bergonié, Bordeaux, France*

Most Her2 testing guidelines recommend that all cases scoring Her2 2+ by immunohistochemistry should be analyzed by fluorescent *in situ* hybridization (FISH) to determine *HER2* status to confirm eligibility for Trastuzumab therapy in breast cancer. The aim of our study was to determine *HER2* gene and chromosome 17 (CEN17) status in a series of 108 Her2 2+ consecutive cases and study the correlation between pathological characteristics of the tumors and HER2 amplification. Invasive breast cancers were tested by FISH using the Dako *HER2* FISH pharmDx® kit. The Her2 immunohistochemistry protocol was performed using the polyclonal AO485 antibody (Dako®) diluted to 1:1500. *HER2* and CEN17 status were correlated to tumor SBR grade, mitotic count, estrogen receptor, progesterone receptor status and percentage of Her2 immunohistochemistry-positive cells. Following Food and Drug Administration guidelines, ie, *HER2*/CEN17 ratio ≥ 2 and an *HER2* copy number > 4, amplified cases were observed in 36 (33%) and 49 (45%) cases, respectively, and following American Society of Clinical Oncology/College of American Pathologists guidelines, ie, *HER2*/CEN17 ratio > 2.2 and an *HER2* copy number > 6, amplified cases represented 30 and 24% of the study population, respectively. Chromosome 17 polysomy (CEN17 > 2.25) was observed in 39 (36%) tumors. Significant positive correlations were found between FISH *HER2* amplified cases and Her2 immunostaining $> 60\%$ ($P = 1.1.10^{-5}$), SBR grade 3 ($P = 0.0001$), nuclear atypia ($P = 0.03$) and mitotic count ($P = 0.008$). By multivariate analysis, Her2 immunostaining $> 60\%$ ($P < 10^{-3}$) and SBR grade 3 ($P < 10^{-3}$) were independent factors predicting *HER2* amplification status irrespective to cutoff guidelines. All SBR grade 3 cases with more than 60% Her2+ cells had an *HER2*/CEN17 ratio ≥ 2, only one had a ratio ≤ 2.2. In our series of consecutive Her2 2+ cases, one-third demonstrated *HER2* amplification, and one-third had chromosome 17 polysomy. Pathological factors, in particular SBR grade 3 and more than 60% Her2+ cells, were significantly correlated with *HER2* amplification.
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The *HER2* gene encodes an epidermal growth factor receptor tyrosine kinase that is overexpressed in 10–30% of invasive breast cancers. Her2 overexpression occurs primarily through amplification of the wild-type *HER2* gene and is associated with poor disease-free survival and resistance to certain chemotherapeutic agents.[1] Her2 overexpression and amplification are predictive of response to therapy with Trastuzumab (Herceptin®; Hofman La Roche, Bâle),[2] determination of Her2 status in breast cancer is therefore important. The two most widespread used methods for determining Her2 status, in routine practice, include immunohistochemistry and fluorescent *in situ* hybridization (FISH). Immunohistochemistry is used as a screening method to determine the level of Her2 protein expression in breast cancers and Her2 immunohistochemical results are generally expressed in a four scale scoring system ranging from 0 to 3+.[3] Scoring criteria in this system comprise percentage of positive tumor cells and quality of staining including intensity of staining and type of membrane staining. According to clinical trials, expert consensus and US Food and Drug Administration (FDA) and the recent American Society of Clinical Oncology/College of American Pathologists (ASCO/CAPs)

404

recommendations,[4] an invasive breast cancer with an Her2 3 + score is considered a positive Her2 tumor, ie, the patient is eligible for Trastuzumab therapy. Moreover, samples scored as Her2 2 + are considered Her2 equivocal and should be further tested with a validated assay for *HER2* gene amplification, such as FISH, before considering for Trastuzumab therapy. FISH is considered the gold standard for *HER2* status determination in breast cancer. Determination of *HER2* status by FISH can either be done by assessing the mean number of *HER2* copies or by calculating the mean *HER2*/CEN17 ratio in a population of tumor nuclei, where CEN17 represents the number of chromosomes 17. Comparison of Her2 status determined by immunohistochemistry and FISH has been previously performed in numerous studies. Using a cutoff of 60% of positive tumor cells, we had previously found a 95% concordance rate between Her2 immunohistochemistry and FISH in a series of 119 cases of invasive breast carcinomas.[5] However, data on *HER2* FISH or chromosome 17 polysomy statuses are sparse for breast tumors showing equivocal Her2 immunostaining. The ASCO/CAP have recently introduced more stringent criteria defining *HER2* amplification[3] as compared to the FDA criteria formerly used and data on the consequences of such a change are sparse.[6]

HER2 amplification as well as chromosome 17 polysomic statuses are known to be associated with adverse pathological and clinical factors in breast cancer[7] and therefore assessment of such factors in the presence of an equivocal Her2 immunostaining, may help predict *HER2* amplification or chromosome 17 polysomy.

The aim of the present study was therefore to assess the *HER2* gene and chromosome 17 status in Her2 2 + invasive breast cancers, according to the FDA and the ASCO/CAP criteria and to determine if tumor pathological factors and percentage of Her2 2 + cells could predict these different conditions.

Materials and methods

Between 08 January 2005 and 08 January 2006, 275 cases of invasive breast cancer were assessed for their *HER2* gene status by the FISH assay at the Pathology Department of Institut Bergonié. These cases were either sent to the Department by external laboratories or were in-house cases. The FISH assay for determination of *HER2* status was mainly performed for cases with equivocal Her2 2 + immunostaining. However cases with no or high Her2 overexpression were also tested by FISH analysis, for control or confirmation of Her2 status, requested by the patient's physician.

For the purpose of this study, Her2 immunohistochemistry was systematically reperformed on all cases tested by FISH and Her2 expression using the Herceptest score was reassessed by one of the authors (GMG). Of 275 cases, 108 presented with a definite Her2 2 + score and selected for this study. For each case, the original H&E slides and estrogen and progesterone receptor immunostains were requested and the histological type, Elston and Ellis tumor grade, mitotic count, estrogen and progesterone receptor status were assessed.

Her2 Immunohistochemistry

For all the cases tested for Her2 overexpression, antigen retrieval was performed by heating 5 μm tissue sections in 0.01 M citrate buffer pH = 6 in a pressure cooker for 20 min. Tissue sections were then incubated for 60 min with a polyclonal anti-Her2 antibody (Dako®, Trappes France, AO485 dilution 1:1500). A labeled streptavidin–biotin–peroxydase method (LSAB kit K5001, Dako®, France) was used for visualization of the immunoreactions with Diaminobenzidine as a chromogen.

Her2 Immunohistochemical Scoring

The Herceptest scoring system was used to assess Her2 overexpression. Briefly, cases showing no membrane immunostaining or in less than 10% invasive cancer cells were scored 0 +, cases with weak and incomplete membrane staining in more than 10% of invasive cancer cells were scored 1 +, cases with complete membrane staining that was either nonuniform or weak in intensity but with obvious circumferential distribution in at least 10% of cells were scored 2 + and finally cases with strong membrane staining in more than 10% invasive tumor cells were scored 3 + (Dako A/S G, Denmark:HercepTest package insert: http://pri.dako.com/28630_herceptest_interpretation_manual.pdf). In each case, negative benign glands were used as internal controls for specificity of the immunoreaction and an invasive breast cancer case with known Her2 overexpression was used as an external control for sensitivity of the immunoreaction. For the purpose of this study, the percentage of positive Her2 invasive cancer cells and intensity of staining were also assessed.

HER2 FISH

Assessment of the *HER2* gene and chromosome 17 status was performed using the DAKO HER2 PharmDX® kit following the manufacturer's instructions. After deparaffinization and rehydration, specimens were heated in the pretreatment solution provided in the kit for 10 min. Proteolytic digestion was carried out with ready-to-use Pepsin at room temperature for 5–15 min. A ready-to-use probe mix consisting of a mixture of Texas red-labeled DNA probes covering a 218 kb region including *HER2* gene on chromosome 17, and a mixture of

fluorescein-labeled PNA probes targeted at the centromeric region of chromosome 17 (CEN17) was then applied. After 5 min denaturation at 82°C, slides and probe mix were incubated overnight at 45°C in a humidified hybridization chamber. The following morning, after a series of stringent washes, a fluorescence mounting medium containing DAPI was then applied.

HER2 FISH Interpretation

The FISH specimens were analyzed on a Nikon Eclipse 80i fluorescent microscope with appropriate filters and pictures were captured using a Hamamatsu C4742-95 CCD camera and analyzed with the Genikon® software (Alphelys®, France). In each case, a minimum number of 40 invasive tumor cell nuclei were analyzed in at least two distinct areas. The mean number of HER2 copies and the mean number of CEN17 per nucleus were then calculated and the HER2/CEN17 ratio was established. In this study, a case was considered HER2 amplified if the HER2/CEN17 was equal to or more than two following FDA or more than 2.2 following ASCO/CAP guidelines,[3] respectively. In borderline cases, ie, showing $1.8 < HER2/CEN17 < 2.2$, an additional number of 20 invasive tumor cell nuclei were analyzed and the mean number of HER2 copies and CEN17 and the HER2/CEN17 ratio were established in a total of 60 nuclei.

Statistical Analysis

χ^2-Tests were performed to evaluate the relationship between different tumor pathologic features, including estrogen receptor and progesterone receptor status, tumor grade, percentage of Her2 2 + invasive tumor cells and HER2 amplification status or chromosome 17 polysomic status. Reciprocal influence among the different predictive factors was determined by multivariate analysis using a backward logistic regression test. The variables that predicted chromosome 17 polysomy in the first analysis and HER2 amplification in the second analysis were determined. All factors were included in the logistic regression analyses, irrespective of their P-value by univariate analysis; but only those with a P-value $\leq 5\%$ were retained in the final models. Univariate and multivariate analyses (logistic regression model) were performed using SAS software version 8.

Results

HER2 Amplification and Chromosome 17 Polysomy in the Series According to FDA and ASCO/CAP Guidelines

Distribution of mean HER2 copy number per nucleus and mean HER2/CEN17 ratio per nucleus in the study population of 108 Her2 2 + cases are

Table 1 Distribution of mean HER2 copy number per nucleus and mean HER2/CEN17 ratio per nucleus in a series of 108 Her2 2+cases of invasive breast cancer

		HER2		Total
		≤4	>4	
(A) Following the FDA recommendations				
HER2/CEN17	<2	59 (55%)	13 (12%)	72 (67%)
	≥2	0	36 (33%)	36 (33%)
Total		59 (55%)	49 (45 %)	108 (100%)
		≤6	>6	
(B) Following the ASCO/CAP recommendations				
HER2/CEN17	≤2.2	73 (68%)	3 (3%)	76 (71%)
	>2.2	9 (8 %)	23 (21%)	32 (29 %)
Total		82 (76%)	26 (24%)	108 (100%)

FDA, Food and Drug Administration; ASCO/CAP, American Society of Clinical Oncology/College of American Pathologist.

shown in Table 1. Depending on the FDA and ASCO/CAP guidelines, 36 (33%) and 32 (30%), respectively, of the Her2 2 + cases were considered HER2 amplified in the series. All cases with a HER2/CEN17 ratio equal to or more than two had more than four HER2 copies per nucleus. Moreover, 13 cases (12%) with more than four HER2 copies per nucleus showed a HER2/CEN17 ratio less than two. Results were slightly different considering the ASCO/CAP cutoffs, because nine cases with a ratio more than 2.2 had less than six HER2 copies per nucleus and only three cases with more than six HER2 copies per nucleus showed a HER2/CEN17 ratio equal to or less than 2.2.

Using 2.25 CEN17 per nucleus as the cutoff for defining polysomy in the series, 39 (36%) Her2 2 + cases showed chromosome 17 polysomy.

All the 13 cases with more than four HER2 copies but with a HER2/CEN17 ratio of less than two per nucleus demonstrated CEN17 polysomy, with mean CEN17 varying between 2.3 and 4. In the same cases, HER2 copy numbers varied from 4.16 to 6.71 with two cases presenting with more than six HER2 copies per nucleus. Corresponding HER2/CEN17 ratios varied from 1.23 to 1.92, with five cases presenting with an equivocal HER2 status with a ratio of more than 1.8.

Association Between Chromosome 17 Polysomic Status and Tumor Pathologic Features

Using the χ^2-test, in this series of 108 Her2 2 + cases, a significant negative association between chromosome 17 polysomy and more than 60% Her2 2 + invasive tumor cells was found ($P = 0.05$). Chromosome 17 polysomy tended to be more often present in tumors with less than 60% invasive tumor cells showing Her2 2 + intensity of staining.

No significant correlation was found between chromosome 17 polysomic status and other pathologic variables such as tumor grade, mitotic count,

Table 2 Associations between pathologic criteria and *HER2* gene status, using the χ^2-test

Pathologic criteria	HER2 /CENT17	
	FDA ratio ≥ 2	ASCO 2007 ratio > 2.2
Her2 + cells $> 60\%$	1.10^{-5}	1.10^{-5}
Grade	1.10^{-5}	1.10^{-3}
Mitotic count	0.008	0.03
Nuclear atypia	0.03	NS
Differentiation	NS	NS
IHC estrogen receptor	NS	NS
IHC progesterone receptor	NS	NS

NS, not significant; FDA, Food and Drug Administration; ASCO, American Society of Clinical Oncology; IHC, immunohistochemistry.

tumor differentiation, nuclear atypia, estrogen receptor and progesterone receptor status.

Association Between *HER2* Amplification Status and Tumor Pathologic Features

Using the *HER2*/CEN17 ≥ 2 or the *HER2*/CEN17 > 2.2 ratios for defining *HER2* amplified tumors, a strong positive correlation was found between the percentage of Her2 2 + invasive tumor cells and *HER2* gene status. Tumors with more than 60% 2 + invasive tumor cells were more often *HER2* amplified than the others ($P = 1.10^{-5}$) with both cutoffs. Furthermore, a significant positive correlation, again with both cutoffs, was found between tumor grade, mitotic count and *HER2* amplification (Table 2) and a positive correlation was found with nuclear atypia only following FDA guidelines. Finally, no significant association was found between hormonal receptor status and *HER2* status whatever the cutoff value used. By multivariate analysis, using the *HER2*/CEN17 FDA cutoff at two, two factors independently predicted *HER2* status, ie, percentage of Her2 2 + invasive tumor cells $\geq 60\%$ (odd ratio = 12; 95% CI: 3.2–44.5; $P < 10^{-3}$) and tumor grade 3 (odd ratio = 6; 95% CI: 2.3–16.7; $P < 10^{-3}$). All grade 3 invasive breast cancers presenting with more than 60% Her2 2 + invasive tumor cells ($n = 8$) showed a *HER2*/CEN17 ratio of more than two. Using the *HER2*/CEN17 ASCO/CAP cutoff at 2.2, again the same two factors independently predicted *HER2* status, ie, percentage of Her2 2 + invasive tumor cells $\geq 60\%$ (odd ratio = 9.8; 95% CI 2.9–33.6; $P < 10^{-3}$) and tumor grade 3 (odd ratio = 4.5; 95% CI 1.7–12; $P = 0.003$) and all but one case of grade 3 invasive breast cancers presenting with more than 60% Her2 2 + invasive tumor cells had a *HER2*/CEN17 ratio of more than 2.2 (Table 3).

Discussion

HER2 status is relevant for patient care and over-expression of HER2 indicates a poor outcome.[8–10]

The proper algorithm for the assessment of HER2 status has yet to be defined but evidence arising from comparative studies shows that gene amplification correlates better with prognosis and may be more accurate to identify patients who are likely to benefit from Herceptin® treatment.[11–13] Owing to different drawbacks associated with FISH (cost, time consuming, not routinely performed in all laboratories), immunohistochemistry represents the first screening step and FISH should be performed in all cases where immunostaining is doubtful (technical artifact) or equivocal (read as 2 +). FISH being also subject to artifacts, it is therefore of interest to determine histological criteria allowing prediction of the *HER2* gene status established by FISH.

In our study, we analyzed 108 Her2 2 + tumors by FISH with the aim to identify which pathologic tumor factor might predict *HER2* gene status in tumors showing equivocal Her2 staining. The ASCO/CAP having moved to more stringent cutoff values than the FDA, we analyzed our data taking into account the different criteria defining a *HER2* amplified tumor. Considering positive cases when *HER2*/CEN17 ratio is ≥ 2, the two-thirds (67%) of the samples in our series would have been declared as negative and therefore not eligible for Herceptin® treatment. This rate is very close to the one that would have been obtained using the *HER2*/CEN17 ratio > 2.2 (70%). According to the literature, this rate is between 64 and 93% depending on the samples analyzed and on the decision criteria used, ratio or *HER2* copy number.[9,14] Even if very few studies have been carried out considering only Her2 2 + cases, Merola *et al*[7] report a negative rate of 70% in such a group. Generally, the false-positive rate of the immunohistochemistry appears to be around 70%. This very high rate can be explained by genetic mechanisms or technical pitfalls.

First, in our series and others,[15,16] the *HER2* copy number in the majority of Her2 2 + amplified cases is lower than 10 unclustered copies (mean = 6.95) instead of more than 10 copies in cases scored 3 + (data not shown and 15) and in negative cases this copy number is 2.9 as a mean. According to cytogenetic criteria, there is a great difference between gain and amplification. The last is defined as an increase in copy number of a restricted region of a chromosome arm generated by breakage-fusion-bridge cycle. Amplified DNA resides either on extrachromosomal circles of DNA that lack centromeres, called double minutes, or as a cytologically visible chromosomal expansion,[17,18] both presenting in interphase FISH as clustered signals of more than 10 copies (Figure 1a). In our study, most of the Her2 2 + cases with a ratio > 2.2 harbored spread FISH signals and should so be considered as gain instead of true amplification of the *HER2* locus. Even if this gain (from 4 to 10 copies) could explain the weak expression detected in cases scored 2 + in immunohistochemistry and not amplified or borderline, growing evidence is demonstrating that all these

Table 3 Distribution of mean *HER2*/CEN17 ratio per nucleus according to tumor grade and percentage of Her2 2+ cells

Tumor grade and percentage of IHC Her2 2+ cells	HER2/CEN17		Total
	< 2	*≥ 2*	
(A) Following the FDA recommendations			
Nongrade 3 and <60%	51 (48%)	9 (9%)	60 (57%)
Nongrade 3 and ≥60%	4 (4%)	6 (5%)	10 (9%)
Grade 3 and <60%	15 (14%)	13 (12%)	28 (26%)
Grade 3 and ≥60%	0	8 (8%)	8 (8%)
Total	70 (66%)	36 (34%)	106 (100%)
	≤ 2.2	*> 2.2*	
(B) Following the ASCO/CAP recommendations			
Nongrade 3 and <60%	52 (49%)	8 (7 %)	60 (56%)
Nongrade 3 and ≥60%	4 (4%)	6 (6%)	10 (10%)
Grade 3 and <60%	17 (16%)	11 (10%)	28 (26%)
Grade 3 and ≥60%	1 (1%)	7 (7%)	8 (8%)
Total	74 (70%)	32 (30%)	106 (100%)

IHC, immunohistochemistry; FDA, Food and Drug Administration; ASCO/CAP, American Society of Clinical Oncology/College of American Pathologist.
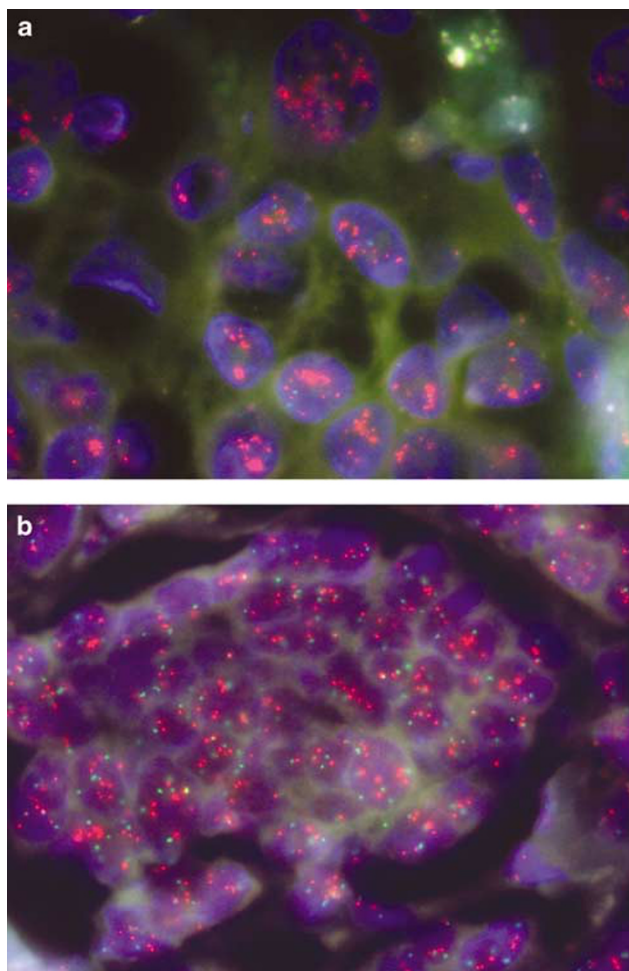
Figure 1 *HER2*/CEN17 FISH on two Her2 2+ invasive breast cancer. Red signal corresponds to *HER2* probe and green signal to CEN17 probe. (**a**) Amplified case harboring more than 20 clustered HER2 copies per nucleus. (**b**) Polysomic case presenting with a mean 3.38 CEN17 copies and 6.71 *HER2* copies per nucleus.

low-grade cases should probably be considered as negative for *HER2* amplification.[8] Arnould et al[13] showed that tumors with a low *HER2* amplification (less than 10 copies per nucleus) had a significant lower rate of complete pathological response to Trastuzumab® neoadjuvant therapy than those with high amplification (more than 10 copies per nucleus). In line with this, the new ASCO/CAP guidelines now consider that at least six *HER2* copies per nucleus are needed to declare a case as positive, and may be a step forward would be to take into account also the *HER2* signal organization, cluster or spread, to declare a case as truly 'amplified' as compared to a case 'eligible' for Herceptin® treatment.

Second, it appears also from few published studies,[14] detecting an abnormal low rate of amplified cases in the Her2 2+ population (7.14%) that the immunohistochemical conditions may be too sensitive and not specific enough. It could so be of interest to establish standard immunohistochemical conditions and interpretation to render it more specific of *HER2* real amplification, meaning Her2 2+ with at least six or eight copies.

Finally, this weak expression, negative for amplification in 70% of the cases, could also be explained, at least in part, by chromosome 17 polysomy.[19] In our series, 40% of the nonamplified cases were polysomic for chromosome 17 (Figure 1b). This mechanism, ie, polysomy, is truly different from amplification in which gene expression can be modulated not only because of the copy number but also by the new genomic environment related to amplification.[20–22] Nevertheless, using 2.25 CEN17 per nucleus as the cutoff for defining polysomy, 36% of Her2 2+ cases showed chromosome 17 polysomy. The χ^2-test demonstrated that polysomy 17 is statistically more frequently observed in cases with less than 60% of Her2 2+ tumor cells and

408

without gene amplification. Impact of polysomy 17 on *HER2* expression and response to Herceptin® is still controversial, but it appears from our results and others that the mechanism for *HER2* amplification is independent of polysomy.[14,23] Following this hypothesis, polysomy could be a surrogate of a general chromosomal instability that is well known to be predictive of poor clinical outcome.[24] In line with this, Salido et al[23] found that nodal involvement was more frequent in polysomic than in nonpolysomic cases.

However, in the polysomic group of our series, 13 invasive breast cancers had more than four *HER2* copies per nucleus, but 10 of these cases had less than six *HER2* copies per nucleus (Table 1) and might be declared as negative for amplification following ASCO/CAP guidelines. The remaining three cases should so be considered with regard to the other pathological and clinical data to determine the eligibility to Herceptin® treatment.

Predictive histologic criteria depend on the *HER2*/CEN17 cutoff value used. Considering the less stringent cutoff (ie; ratio ≥ 2), all the positive cases, in our series, had more than four *HER2* copies per nucleus. Two major pathologic features, tumor grade and the percentage of Her2 2 + invasive tumor cells were independently predictive of *HER2* status and, following our results, an Her2 2 + grade 3 invasive breast cancer had 58% of probability to be eligible for Herceptin® treatment, a tumor with more than 60% Her2 2 + invasive tumor cells had a probability of 77% to be eligible and a tumor harboring both criteria had 100% of probability to be positive for *HER2* amplification. With the more stringent cutoff (ie, ratio > 2.2), not only the pathologic factors still demonstrated an independent predictive value but also became more efficient to predict a nonamplified case. Indeed, cases with less than 60% 2 + cells and histologic grade 1 or 2 harbored less than six *HER2* copies per nucleus or a ratio < 2.2 in 93.3 and 86.7% of the cases, respectively.

Taken together, our data suggest that a proper algorithm for Herceptin® therapy decision in cases scored Her2 2 + should take into account not only FISH (with respect to ASCO/CAP guidelines) or immunohistochemistry but a combination of tumor grading (grades 1–2 vs grade 3), immunohistochemistry (less or more than 60% Her2 2 + invasive tumor cells) and FISH (less or more than six copies, clustered or not).

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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