FISH testing of HER2 immunohistochemistry 1+ invasive breast cancer with unfavorable characteristics

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Abstract. Diagnostic assays for human epidermal growth factor receptor 2 (HER2) expression have a high predictive value because patients with HER2-positive tumors could benefit from HER2-targeted therapy. The aim of the present study is to analyze the incidence of HER2 gene amplification in selected tumors with adverse features that scored 1+ by immunohistochemistry (IHC). For that purpose, 331 consecutive invasive breast cancers (IBCs) were tested by IHC for HER2 expression between January and December 2013, 102 of which (31%) scored 1+. Of these 102 women with IBC who underwent surgery, 75 entered the study (73.5%). A total of 48 out of 75 (64%) IBC samples (patients' median age, 60.75 years) were selected according to ≥1 unfavorable tumor characteristics, and tested by fluorescence *in situ* hybridization (FISH). Of these 48 IBC samples scoring 1+ by IHC, 22 (46%) exhibited high histological grade (G3), 23 (48%) had a high proliferative index (Ki-67, >30%), 27 (56%) showed vascular invasion and 32 out of 41 evaluable cases (78%) were node-positive. Regarding hormone receptor expression, 3 (6%) and 10 (21%) cases were negative for estrogen and progesterone receptors expression, respectively. FISH was performed on 48 IBC cases scoring 1+ by IHC, and 7 infiltrating ductal carcinomas (IDCs) (14.6%) demonstrated HER2 amplification with a high proliferative index. In 42 IDC samples, statistical analysis evidenced a significant association between histological grade and high proliferative index (P=0.0200). In addition, in 48 HER2 scoring 1+ IBCs, Fisher's exact test evidenced a significant association between the presence of gene amplification and high proliferative index (P=0.0033). Based on these biopathological parameters, particularly a high proliferative index, the present results indicate that it is possible to of identify tumors scoring 1+ by IHC with *HER2* amplification by FISH, thus aiding the selection of patients who are suitable for HER2-targeted therapy according to an acceptable cost/benefit ratio.

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Introduction

Clinical studies have revealed that the human epidermal growth factor receptor 2 (*HER2*) gene is amplified in 20-30% of all breast cancers (1), and in ~90-95% of these cases, overexpression is a direct result of gene amplification (2,3). The HER2 protein is a 185-kDa transmembrane growth factor receptor with tyrosine kinase activity involved in cellular signaling, which regulates cell growth and development (4). *HER2* gene amplification or overexpression in breast cancer is a prognostic factor and predictive of a more aggressive clinical course for the patient (5). It is associated with high tumor-grade, hormone receptor-negative tumors, lymph node metastasis (6), increased risk of recurrence after surgery, poor response to conventional chemotherapy and shortened survival (7,8). In addition, diagnostic assays for HER2 expression in breast cancer have also a high predictive value (1) and are important in therapeutic decision-making. Notably, the *HER2* gene product, p185HER2/neu, represents a target for specific therapy with the humanized recombinant monoclonal antibody trastuzumab (Herceptin®; Genentech, Inc., South San Francisco, CA, USA) (9,10).

The efficacy of therapeutic regimens that include trastuzumab, administered in combination with conventional chemotherapy in both the metastatic and the adjuvant setting, requires the accurate determination of HER2 status, since the presence of this alteration is the criteria to determine the patient eligibility for trastuzumab treatment (11,12). Trastuzumab therapy improves survival rate among women with metastatic or localized HER2-positive breast cancer (7,9,11,12). One year of treatment provides a significant disease-free and overall survival benefit, and is the standard of care (13). Analysis requires the application of methods performed on archival formalin-fixed, paraffin-embedded tissue (3). There are two

complementary pathological diagnostic tests in current clinical use to determine HER2 status in breast cancer: Fluorescence *in situ* hybridization (FISH) to evaluate *HER2* gene amplification and immunohistochemistry (IHC) to detect protein overexpression; they examine different aspects of the biology of HER2-driven cancer (14).

Approximately 80% of newly diagnosed invasive breast cancers (IBCs) are tested for HER2 using IHC and 20% are tested using FISH (15,16). The American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) recommend treating patients whose tumor test is IHC 3+ or FISH-positive with trastuzumab, whereas patients whose tumors are IHC 0 or 1+ or FISH-negative are treated with standard chemotherapy. The same guidelines recommend also researching gene amplification by FISH in tumors scoring 2+ (17). Recently, the 2013 ASCO/CAP guidelines recommend either using IHC assays for initial evaluation of HER2 status followed by reflex testing by FISH of certain IHC categories, or the primary use of FISH in initial testing (18). The agreement between IHC 3+ and FISH amplification should be ≥95% (17). The presence of 'incomplete membrane staining that is faint/barely perceptible and within >10% of tumor cells' in those cases scoring 1+, or the absence of HER2 protein immunoexpression, are due to the absence of *HER2* gene amplification in the majority of cases (19); however, a minor but significant number of cases that have faint/perceptible IHC expression (score 1+) (20) or no HER2 protein expression but exhibit *HER2* gene amplification, have been observed worldwide in different cohorts of patients (7). These data were confirmed by extensive internal and external international quality assurance of HER2 testing (21). Discordance between test results may be present in patients with unfavorable tumor characteristics, including high histological grade, high proliferative index and negative or low hormone receptor expression (20), thus leading to false-negative results for HER2 status. In consequence, patients with a false-negative tumor result would be denied the clinical benefits of trastuzumab or other HER2-targeted therapies (22).

In fact, the aim of the ASCO/CAP guidelines update in 2013 was to focus on accurate HER2 testing to ensure access to high-quality cancer biomarker tests that would aid specialists to match the right treatments with the right patients (18). Therefore, even if the absolute number of HER2-positive cases detected could be very low, it would be clinically useful to test *HER2* gene amplification in selected tumors with adverse features scoring 1+ by IHC.

The aim of the present study is to assess the incidence of *HER2* gene amplification in selected tumors with adverse features that scored 1+ by IHC.

Materials and methods

Patients. A total of 331 consecutive IBCs observed between January and December 2013 were tested by IHC for HER2, of which, 42 tumors scored 3+ (13%); 43 tumors scored 2+ (13%), of which 12 cases exhibited HER2 amplification; 102 tumors scored 1+ (31%); and 144 tumors scored 0 (43.5%). In total, 75 out of 102 (73.5%) IBC cases scoring 1+ by IHC, which occurred in women who underwent surgery at the National Cancer Research Institute 'Giovanni Paolo II' (Bari, Italy), were selected for the study. The other 27 out of 102 cases were detected on metastatic sites or core biopsies and were discarded. A total of 48 out of 75 (64%) IBC cases (patients' median age, 60.75 years) were selected according to ≥1 unfavorable tumor characteristics, and subsequently tested by FISH.

Ethics statement. The present study was approved by the Institutional Review Board of the National Cancer Research Institute 'Giovanni Paolo II'. Before undergoing routine surgery, all patients signed an informed consent form authorizing the use of the removed biological tissue for research purposes according to ethical standards.

IHC analysis. Samples were tested by IHC to observe the expression of estrogen receptor (ER) and progesterone receptor (PgR), and to evaluate the Ki-67 cellular proliferation index. Hormone receptors for estrogen and progesterone were tested using monoclonal rabbit anti-human estrogen receptor α (clone SP1; 1:60 dilution; Dako, Glostrup, Denmark) and monoclonal mouse anti-human progesterone receptor (clone PgR 636; 1:100 dilution; Dako) respectively, whereas Ki-67 was detected using monoclonal mouse anti-human Ki-67 antigen [clone mindbomb E3 ubiquitin protein ligase 1 (MIB-1); 1:80 dilution; Dako]. ER, PgR and Ki-67 immunostaining were confined to the nucleus. ER, PgR and Ki-67 index were scored according to the St. Gallen International Breast Cancer Conference guidelines (23,24): ER and PgR receptors were scored as negative/positive when no/any staining was present in the tumor, while Ki-67-labelling index was considered high when staining was present in >30% of tumor cells, intermediate when it was 16-30%, and low when it was ≤15%. In the present study, two subgroups were considered: when Ki-67-labelling index was >30%, it was considered high, whereas when Ki-67 was ≤30%, it was considered low. All samples were also analyzed by IHC using the HercepTest™ kit (Dako) according to the manufacturers' protocol. Cytoplasmic immunoreactivity was ignored. HER2 was scored as 0, 1+, 2+ or 3+ in accordance with the ASCO/CAP guidelines, also adopted by the Italian Society of Pathological Anatomy and Diagnostic Cytology - Italian Division of the International Academy of Pathology (SIAPEC-IAP) (18): 0, no staining observed or membrane staining that is incomplete or faint/barely perceptible in ≤10% of tumor cells; 1+, incomplete membrane staining that is faint/barely perceptible within >10% of tumor cells; 2+, circumferential membrane staining that is incomplete and/or weak/moderate within >10% of tumor cells, or complete and circumferential membrane staining that is intense within ≤10% of tumor cells; and 3+, circumferential membrane staining that is complete and intense within >10% of tumor cells.

FISH for gene amplification. FISH was conducted using a dual HER2/Cep17 probe (Path Vysion HER2 DNA Probe kit; Abbott Molecular, Inc., Des Plaines, IL, USA), combining a *HER2* gene probe (190 kb Spectrum Orange-directly labelled DNA probe) with a centromeric enumeration probe for chromosome 17 (CEP17; 5.4 kb Spectrum Green-directly labelled fluorescent DNA probe specific for the chromosome 17 α satellite DNA sequence). Unstained sections of target tissue (4-μm-thick) were cut from paraffin-embedded blocks. The

Table I. Tumor characteristics in 48 breast cancer cases scoring 1+ by immunohistochemistry for HER2 expression.

Tumor characteristics	IDC histotype	ILC histotype	Total (n)	%
Histological grade (G3)	19	3	22	46
Peritumoral vascular invasion	23	4	27	56
Lymph node-positive	27	5	32	78
Ki-67 >30%	22	1	23	48
ER-negative	3	0	3	6
PgR-negative	10	0	10	21

HER2, human epidermal growth factor receptor 2; ER, estrogen receptor; PgR, progesterone receptor; IDC, infiltrating ductal carcinoma; ILC, infiltrating lobular carcinoma.

sections were baked overnight at 56˚C. Subsequently, the paraffin was removed from the sections with a 15-min wash in warm xylene at 60˚C and 3 x 15-min washes in xylene. The samples were dehydrated twice in 100% ethanol for 5 min and dried in the HYBrite instrument (Abbott Molecular, Inc.) at 45˚C. The sections were fixed in methanol:acetic acid (3:1) for 12 min, dried in the HYBrite instrument at 45˚C, and then immersed in 0.2 M HCl for 10 min, in purified water for 3 min and in a 2X saline sodium citrate (SSC) wash buffer for 3 min. The slides were then placed in a pretreatment sodium thiocyanate solution for 25 min at 84˚C and rinsed in purified water for 3 min, followed by rinsing in 2X SSC wash buffer for 3 min. After incubation in a protease solution at 37˚C for 15 min, the enzymatic reaction was stopped by placing the slides in deionized water for 3 min and air dried. Next, the slides were dehydrated through graded alcohols, and 10 μl PathVysion® HER2/CEP17 probe was applied to the sections. The slides were coverslipped, sealed with rubber cement, and the probe/target tissue was then co-denatured for 5 min at 75˚C using the HYBrite instrument and allowed to hybridize overnight at 37˚C. The coverslip was carefully removed in a 1X SSC/0.1% NP-40 solution, and to remove non-specifically bound probe, the slides were washed in 1X SSC/0.1% NP-40 for 5 min. Stringency wash was performed with 2X SSC/0.3% NP-40 for 5 min at room temperature, and then at 72˚C for 3 min. The sections were washed twice in 1X SSC/0.1% NP-40. Slides were air dried in the dark, counterstained with 10 μl 4',6-diamidino-2-phenylindole (DAPI) and coverslipped.

FISH analysis and interpretation. FISH analysis was performed using an epifluorescence microscope (BX-UCB; Olympus Corporation, Tokyo, Japan) with appropriate filters for Spectrum Orange and Green, a triple bandpass filter set, and an ultraviolet filter for DAPI nuclear counterstain. Normal (x10) and oil fluorescence objectives (x60 and x100) were used for the analysis. Analysis was performed in a dark room, and the DAPI filter set and a low-power objective were used to confirm areas of invasive carcinoma. Using the triple bandpass filter set and a 60X oil objective, the presence of CEP17 signals in ≥75% of cancer cell nuclei was confirmed. Only tumor cells with non-overlapping nuclei were scored. The signals were recorded with a charge-coupled device camera (Olympus Corporation), and analysis of the signal pattern was performed with CytoVision® software (version 4.5.4; Leica Microsystems, Inc., Buffalo Grove, IL, USA).

A total of 60 nuclei from two distinct areas of the invasive carcinoma were scored for green and red signals for each section; red signals represent *HER2* gene copies, green signals represent *CEP17* gene copies. The mean number of CEP17 and HER2 signals was recorded, and the results were expressed as a ratio of red to green signals.

In agreement with the ASCO/CAP guidelines (18), which have also been adopted by SIAPEC-IAP, HER2 ratio-based amplification was considered. Gene amplification was evaluated as present when the HER2/CEP17 ratio was ≥2 or when the mean HER2 copy number was ≥6.

Before and during the study there was a between-laboratory quality assessment exercise involving the circulation of control sections.

Statistical methods. The baseline characteristics of the study population were calculated, and the results were expressed as frequencies and percentages for the categorical variables.

Comparisons of clinical parameters between the groups of interest were performed with the Pearson χ^2 test or the Fisher's exact test, when appropriate, for categorical variables. $P<0.05$ was considered to indicate a statistically significant difference. All the analyses were performed using the Statistical Analysis System software (SAS Institute, Cary, NC, USA).

Results

A total of 48 IBC samples with unfavorable tumor characteristics, including high histological grade (G3) according to the Elston-Ellis classification (25), high proliferative index, lymph node positivity, presence of peritumoral vascular invasion and negative hormone receptor expression, were selected.

Clinicopathological data, including histological grade, peritumoral vascular invasion, lymph node status, Ki-67 index, ER and PgR status are shown in Table I.

In total, 42 out of 48 cases (87.5%) exhibited a histological diagnosis of infiltrating ductal carcinoma (IDC) and 6 cases (12.5%) were diagnosed as infiltrating lobular carcinoma (ILC). A total of 22 out of 48 tumors (46%) displayed high histological grade (G3) (Fig. 1A), and 27 cases (56%) exhibited peritumoral vascular invasion. Regarding lymph node status,

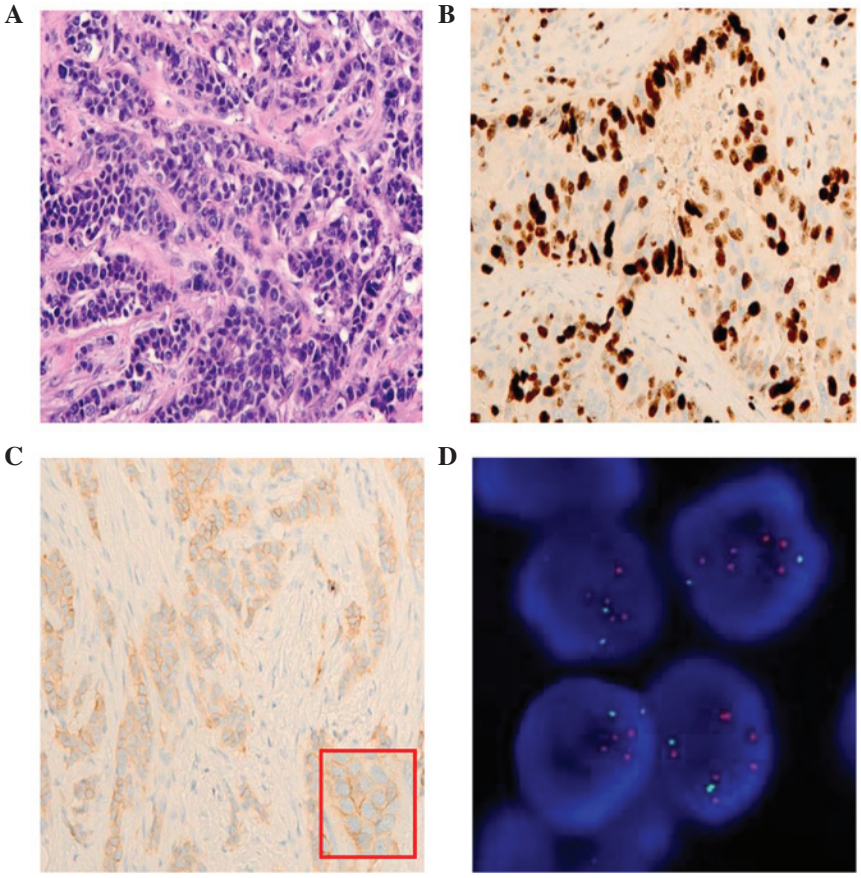

Figure 1. Representative images of H&E and IHC staining and FISH test in an IBC. (A) H&E staining of an IBC with high histological grade (G3) (magnification, x20). (B) High Ki-67 expression in an invasive breast tumor (magnification, x20). (C) IHC HER2 protein expression in an IBC (HER2 score, 1+; magnification, x20). An enlarged image of HER2 protein expression is presented on the bottom right part of the image (red frame; magnification, x40). (D) *HER2* gene amplification tested by FISH. Red signals represent *HER2* gene copies, while green signals represent chromosome enumeration probe 17 copies (oil fluorescence objective; magnification, x60). H&E, hematoxylin and eosin; IHC, immunohistochemistry; FISH, fluorescence *in situ* hybridization; IBC, invasive breast carcinoma; HER2, human epidermal growth factor receptor 2.

Table II. FISH results in 48 breast cancer cases scoring 1+ by immunohistochemistry for HER2 expression.

	IDC histotype (n=42)		ILC histotype (n=6)	
Tumor characteristics	$FISH^+$, n (%)	$FISH^-$, n (%)	$FISH^+$, n (%)	$FISH^-$, n (%)
Histological grade (G3)	5 (12)	14 (33)	0 (0)	3 (50)
Peritumoral vascular invasion	3 (7)	20 (48)	0 (0)	4 (67)
Lymph node-positive	3 (7)	24 (57)	0 (0)	5 (83)
Ki-67 >30%	7 (17)	15 (36)	0 (0)	1 (17)
ER-negative	1 (2)	2 (5)	0 (0)	0 (0)
PgR-negative	3 (7)	7 (17)	0 (0)	0 (0)

HER2, human epidermal growth factor receptor 2; ER, estrogen receptor; PgR, progesterone receptor; IDC, infiltrating ductal carcinoma; ILC, infiltrating lobular carcinoma; FISH, fluorescence *in situ* hybridization.

41 patients out of 48 had axillary lymph node dissection, of which, 32 (78%) were node-positive, 9 were node-negative and 7 had no axillary lymph node dissection. A total of 23 cases (48%) had a high proliferative index (Ki-67, >30%) (Fig. 1B), while 3 (6%) and 10 (21%) cases were negative for ER and PgR expression, respectively. FISH was performed on 48 HER2 samples scoring 1+ by IHC (Fig. 1C) with unfavorable tumor characteristics, and 7 IDCs out of 48 (14.6%) exhibited HER2 amplification (Fig. 1D); all the 7 samples displayed a high proliferative index (Ki-67, >30%) and 5/7 had high histological grade. No amplification was detected in any of the 6 ILCs (Table II). In total, 42 tumors scored 3+ by IHC and 43 cases scored 2+, 12 of which resulted amplified by FISH, indicating that 54 cases (16%) out of 331 were overexpressed and/or

Table III. Association between histological grade and unfavorable tumor characteristics in 42 infiltrating ductal carcinoma cases scoring 1+ by immunohistochemistry for HER2 expression.

		High histological grade (n=19)		Low histological grade (n=23)		
Variable	Category	n	%	n	%	P-value
Peritumoral vascular invasion[c]	0	10	52.63	9	39.13	0.3816[a]
	1	9	47.37	14	60.87	
Ki-67 >30%[d]	0	5	26.32	14	60.87	0.0251[a]
	1	14	73.68	9	39.13	
Lymph node status[e]	0	6	42.86	3	13.64	0.1111[b]
	1	8	57.14	19	86.36	

[a]P-values were calculated with the Pearson χ^2 test. [b]P-value was calculated with the Fisher's exact test. [c]0, absent peritumoral vascular invasion; 1, present peritumoral vascular invasion. [d]0, Ki-67 ≤30%; 1, Ki-67 >30%. [e]0, lymph node-negative; 1, lymph node-positive. HER2, human epidermal growth factor receptor 2.

Table IV. Association between unfavorable tumor characteristics and HER2 amplification in 48 breast cancer cases scoring 1+ by immunohistochemistry for HER2 expression.

		FISH=0[a] (n=41)		FISH=1[a] (n=7)		
Variable	Category	n	%	n	%	P-value[b]
Histological grade[c]	L	24	58.54	2	28.57	0.2226
	H	17	41.46	5	71.43	
Histotype[d]	0	35	85.37	7	100.00	0.5725
	1	6	14.63	0	0.00	
Peritumoral vascular invasion[e]	0	17	41.46	4	57.14	0.6830
	1	24	58.54	3	42.86	
Ki-67 >30%[f]	0	25	60.98	0	0.00	0.0033
	1	16	39.02	7	100.00	
Lymph node status[g]	0	6	17.14	3	50.00	0.1075
	1	29	82.86	3	50.00	

[a]0, FISH-negative cases,; 1, FISH-positive cases. [b]P-values were calculated with the Fisher's exact test. [c]L, low histological grade (G1 and G2); H, high histological grade (G3). [d]0, infiltrating ductal carcinoma; 1, infiltrating lobular carcinoma. [e]0, absent peritumoral vascular invasion; 1, present peritumoral vascular invasion. [f]0, Ki-67 ≤30%; 1, Ki-67 >30%. [g]0, lymph node-negative; 1, lymph node-positive. FISH, fluorescence *in situ* hybridization; HER2, human epidermal growth factor receptor 2.

amplified. A total of 7 cases (2%) cases scoring 1+ by IHC out of 331 exhibited HER2 amplification, indicating that, in total, 61 (18%) tumors out of 331 were overexpressed and/or amplified.

In 42 IDC samples, statistical analysis evidenced a significant association between histological grade and high proliferative index as detected by MIB-1 (P=0.0200), whereas no association was noted regarding peritumoral vascular invasion or presence of metastases (Table III). Additionally, in 48 HER2 breast cancer samples scoring 1+, a significant association between the presence of gene amplification and high proliferative index was also observed (P=0.0033) (Table IV).

Discussion

With the aim of reaching high-quality personalized medicine, one of the major aims of the 2013 ASCO/CAP guidelines update (18) was to aid breast cancer specialists to accurately classify patients for HER2-targeted treatment, thus avoiding false-negative and false-positive HER2 results, as false-negative patients may be denied biological treatment, while false-positive cases may receive potentially toxic, costly and ineffective treatment (26).

Despite the axiom 'the right treatment with the right patient' reported in the ASCO/CAP 2013 updated guideline recommendations (18), Iorfida *et al* (20) demonstrated that

a considerable percentage (13%) of cases that scored 1+ for HER2 protein expression by IHC and tested for gene-copy ratio by FISH exhibited gene amplification. This issue evidenced the possibility to deny an effective therapy in a subset of breast cancer patients.

This observation led to the following open questions: i) Could the percentage of IHC 1+/FISH$^+$ cases be higher than expected?; ii) is the reason for this disagreement technical (quality of the determination) or biological (subset of not-overexpressed/amplified tumors)?; iii) could the selection of certain unfavorable tumor characteristics be helpful in identifying these cases?; and iv) should the selection criteria for the FISH test be reconsidered?

In an attempt to address the above questions, the present study selected 48 IBCs scoring 1+ by IHC for HER2 protein expression according to biopathological parameters of clinical aggressiveness, as previously reported by Iorfida *et al* (20). These cases exhibited a disagreement between absent or very low HER2 protein expression detected by IHC and the presence of gene amplification detected by FISH in 7 IDCs (14.6%). Consistent with previous reports, the present data revealed that there is ~15% of IHC$^-$/FISH$^+$ cases in the selected subset of 48 tumors (20,27). Regarding the causes of IHC/FISH disagreement, Perez *et al* (28) described from a technical point of view the discordance in HER2 results between local and central laboratories participating in clinical trials. Furthermore, the ASCO/CAP guidelines emphasized the requirement for very stringent controls, particularly for new laboratories or when a new assay is adopted (29). Regarding the present study, it should be highlighted that the Molecular Pathology Laboratory (Department of Pathology, National Cancer Research Institute 'Giovanni Paolo II') is well inserted, with good results, in the National Italian program for HER2 testing both for FISH [control quality (CQ) FISH HER2 SIAPEC-IAP] and for IHC (Nordic CQ), promoted by SIAPEC-IAP (18).

Other than technical issues, biological features could also be important, such as the importance of heterogeneity in determining the discrepancy in HER2 status within the tumor or between the tumor and its metastasis (30). Several studies have also demonstrated cell-to-cell heterogeneity of HER2 gene amplification and HER2 protein expression at highly variable rates (1-50%), depending on the methodologies and the sample set used (31-34). However determined, molecular heterogeneity of HER2 gene amplification is recognized in ≥4-5% of breast cancers (32,35) and appears to be more frequent in advanced disease.

In the present study, the detection of areas of heterogeneity in the same tumor was present both for IHC and FISH in the 7 IHC$^-$/FISH$^+$ tumors analyzed. Notably, a number of IHC$^-$/FISH$^+$ areas were present where the sample was less well fixed, as observed by hematoxylin and eosin staining, or a delay in the time of fixation was recorded. These observations led to further emphasize the importance of a correct pre-analytical phase (36) in order to obtain *HER2* gene amplification and protein overexpression level in concert. However, it must be considered that variability in HER2 testing may arise from pre-analytic, analytic and post-analytic factors according to the testing method (28).

The main purpose of the current study was to identify various unfavorable tumor characteristics that could distinguish discordant cases, particularly those that were not overexpressed/amplified. Discordance in HER2 results is often present among tumor cases that are selected according to unfavorable and histological factors. As reported in previous studies, the presence of certain biopathological factors such as peritumoral vascular invasion, high histological grade and high proliferative index, appears to play a fundamental role in the identification of these cases (20,27). In the subset of 48 patients selected in the present study, the incidence of amplified HER2 cases rose to 14.6%, and proved to be significantly associated with an elevated cellular kinetic index. In the present study, when the Ki-67 index was >30%, it was considered high. It must be highlighted that numerous cut-off values have been proposed in the literature (37,38), and that the reproducibility of the test for Ki-67 is still far from being elevated, due to an important inter-observer and inter-laboratory variability (38). Recently, Goldhirsch *et al* (39) classified the Ki-67 index into three classes, considering as cut-off value the presence of ≥20% of neoplastic, invasive Ki-67-positive cells. Furthermore, Iorfida *et al* (20), utilized a cut-off value of 14%, including also cases with moderate proliferative activity, according to the aforementioned Goldhirsch's classification. The present study adopted a cut-off value of 30% (23), which is higher and, in the authors' opinion, more restrictive, in order to identify tumors with a more aggressive phenotype.

The present data suggest that it could be advisable to perform the FISH test in IHC 1+ breast cancers, in order to identify HER2-positive cases that could be misclassified as HER2-negative, thus denying those patients the opportunity of benefitting from HER2-targeted therapy. According to the current ASCO/CAP guidelines (18), FISH is not performed in HER2 cases scoring 1+. It is clear that if the current guidelines were applied, these patients would be denied biological therapy from which they could benefit. However, the magnitude of the problem in terms of cost/benefit ratio must be defined. In the present cases, the global incidence of HER2-positive cases (overexpressed and/or amplified) was 16%, and there was a IHC$^-$/FISH$^+$ disagreement of 2% (7/331), which led to the same increment of HER2-positive cases (18%). Thus, this is the risk, according to our experience.

In terms of cost/benefit ratio, this 2% increment in the study population does not appear to be sufficient to justify extending the FISH test to HER2 cases scoring 1+ by IHC. However, ~7 patients from our Institute, who could have benefited from HER2-targeted therapy, had treatment denied to them or initiated too late.

In conclusion, based on the biopathological parameters discussed in the present study, and particularly a high proliferative index, the results from the current study suggest that there is a higher probability of identifying tumors scoring 1+ by IHC that exhibit *HER2* amplification by FISH, thus aiding the selection of patients who are suitable for HER2-targeted therapy according to an acceptable cost/benefit ratio.

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