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Fluorescent *in situ* hybridization as a screening test for *HER2* amplification in G2 and G3 breast cancers of lobular and ductal histotype and metastases

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Abstract. The aim of the present study was to evaluate the effectiveness of fluorescence *in situ* hybridisation (FISH), as a screening test, in moderately- (G2) or poorly- (G3) differentiated breast cancers of the ductal (IDC) and lobular (ILC) histotypes and distant metastases. *HER2* FISH was performed on 486 G2 and 477 G3 both of IDC and ILC histotypes and in 241 metastases. A significant difference in the *HER2* amplification was observed between G2 (14.8%) and G3 (31.9%), with no difference according to the histotype. However, the rate of amplification increased to 36% in the G2/hormone receptor-negative cases as compared to 10.6% in the G2/receptor-positive cases ($p<0.0001$). *HER2* was amplified in 17% of metastases with some differences depending on the location. These data suggest that the *HER2* FISH analysis may be an effective screening test in breast cancer metastases and G3 tumors, irrespective of the hormone receptor status or presence of lymphovascular invasion.

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Introduction

HER2 is a transmembrane tyrosine kinase of the epidermal growth factor receptor family encoded by the *HER2* oncogene. Since the first publication on HER2 overexpression in breast cancer (1), hundreds of studies have demonstrated the importance of this growth factor in breast cancer prognosis and treatment (2-4). The HER2 status should be incorporated into clinical decision making, by reporting either the results of the

receptor protein overexpression by immunohistochemistry (IHC) or gene amplification by *in situ* hybridization (ISH) procedures. Some authors report a high level of correlation between IHC and ISH assays, the latter considering fluorescent (FISH) and chromogenic (CISH) methods (5). However, FISH is more predictive than IHC in determining the response to trastuzumab (6-10). Consequently, FISH has been used to confirm some or all positive IHC results (11). Recently, an expert panel of the American Society of Clinical Oncology and the College of American Pathologists (ASCO/CAP) recommended that the 'HER2 status should be determined for all invasive breast cancer' and suggested either HER2-validated IHC or FISH assay as the first test for *HER2* assessment (12,13).

The cost involved for the different tests is also important. FISH testing is more costly than IHC. It is likely that the use of *HER2* FISH as a screening test would result in a relevant cost increase. Elkin *et al* (14) reported on an analysis on the cost-effectiveness of alternative HER2 testing and trastuzumab treatment strategies, and concluded that the additional costs associated with FISH should be weighed against the increasing accuracy of FISH testing, as compared to IHC. In other words, because the IHC false positive rate is higher compared to FISH, this would result in cost savings due to the increase in treatment appropriateness. Perhaps it would be worthwhile to use FISH as a screening test when the probability of amplification is higher, and use IHC testing for all other tumors. For example, several studies demonstrated that *HER2* amplification is a rare event in low grade or special type cancers. Hoff *et al* (15) suggest the re-examination of the *HER2* amplified tumor be diagnosed as grade 1 or as a lobular carcinoma to exclude the possibility of histo-pathological misclassification. However, the difference in *HER2* amplification between moderately- (G2) or poorly- (G3) differentiated breast carcinomas has yet to be fully studied in a similar manner to the significance of the grade of differentiation in lobular carcinoma (classical versus pleomorphic) and *HER2* amplification.

Another concern is the study of the *HER2* gene in distant metastases. The assessment of HER2 is performed in the primary tumor, even if metastases appear several years later. However, some authors suggest that a possible discordance of HER2 overexpression between primary tumors and metastases should be considered when making treatment decisions (16). As a result, in the present multi-institutional study, we focused on G2 and G3 invasive breast carcinomas of the ductal (IDC) and lobular (ILC) histotypes and distant metastases to validate the effectiveness of FISH as a screening test in this specific subset of patients.

Materials and methods

Twenty-two Italian pathology laboratories, diagnosing at least 250 breast cancers/year and performing >100 FISH analyses/year, were asked to perform FISH as a first test in invasive primary breast carcinomas, of the ductal and lobular histotypes, graded as G2 and G3 by the Elston and Ellis scoring system (17). A similar analysis was performed on the distant metastases of breast cancer. Cases diagnosed as ILC included tumors that demonstrated a complete lack of duct formation and had typical lobular features. Proven E-cadherin negative pleomorphic lobular carcinomas were also included in the ILC subgroup.

Further collected data were patient age, lymphovascular invasion (LVI) and tumor hormone receptor status (HR).

Fluorescence in situ hybridisation (FISH). A PathVysion *HER2*/neu probe kit (Vysis Inc., Downers Grove, IL, USA) was used for FISH analysis. Sections were baked overnight at 56˚C, dewaxed in xylene, dehydrated in 99% ethanol and air-dried. Slides were then pre-treated with Na-thiocyanate at 80˚C for 30 min and digested with proteases for 15 min at 37˚C and finally hybridized overnight at 37˚C with the probes (HER2/neu/CEP17 SG probe 35-171060, Vysis Inc.) after DNA denaturation at 72˚C. Slides were washed with post-hybridization buffer at 72˚C, counterstained with 4,6-diamidino-2-phenylindole (DAPI) and mounted and stored in the dark prior to signal enumeration. For FISH analysis, slides were examined with the Olympus BX41 fluorescence microscope equipped with a 100x oil immersion objective and a triple band pass filter for the simultaneous detection of Spectrum Orange, Spectrum Green and DAPI signals. Areas of optimal tissue digestion and no overlapping nuclei were then selected in each core for counting. Cells (40-60) were counted for each case. We considered cases with a FISH ratio (*HER2* gene signals to chromosome 17 signals) of ≥2.2 as amplified.

Statistical analysis. To define the correlation between *HER2* amplification and each of the potential predictors of histotype (IDC versus ILC), grade (2 versus 3), LVI (absent versus present), HR status (positive versus negative) and age (as a continuous variable and 10-year age steps), a univariate analysis for categorical data was performed using the Pearson Chi-square test with continuity correction and odds ratios. The significance levels were set at $p<0.05$. All tests were two-sided. A multivariate analysis was performed using binomial logistic regression with stepwise regression (reverse selection). The analyses were performed using SPSS for Windows (v. 13.0).

Results

FISH was successful in 963 primary breast cancers and in 241 distant metastases. HER2 amplification was observed in 23.2% of the primary tumors. HR status and LVI were known in 889 and 874 primary cancers, respectively, because FISH testing was performed in primary tumors as part of the hospital service.

In the primary tumors, the univariate analysis showed statistically significant differences in the frequencies of amplification between G2 (14.8%) versus G3 (31.9%) ($p<0.0001$), absence (16.8%) versus the presence of LVI (28.5%) ($p<0.0001$) and HR positive (17.3%) versus negative (39.6%) ($p<0.0001$). Although IDC showed a higher percentage of the *HER2* amplification (23.9%), no significant difference was observed with ILC (16%) ($p<0.1000$) (Table I). Specifically, 68 out of 440 (15.5%) G2 IDC were amplified versus 4 out of 46 (8.7%) G2 ILC and 143 out of 442 (32.4%) G3 IDC versus 9 out of 35 (25.7%) G3 ILC. A younger age

The correlation of the *HER2* amplification with ological predictors.

	No. of cases	FISH not amplified (%)	FISH amplified (%)	P-value
IDC[a]	882	671 (76.1)	211 (23.9)	0.1000
ILC[b]	81	68 (84.0)	13 (16.0)	
Grade 2	486	414 (85.2)	72 (14.8)	0.0000
Grade 3	477	325 (68.1)	152 (31.9)	
LVI[c]				
Absent	488	406 (83.2)	82 (16.8)	0.0000
Present	386	276 (71.5)	110 (28.5)	
HR status[d]				
Positive	677	560 (82.7)	117 (17.3)	0.0000
Negative	212	128 (60.4)	84 (39.6)	

[a]IDC, invasive ductal carcinoma; [b]ILC, invasive lobular carcinoma; [c]LVI, lymphovascular invasion and [d]HR status, hormonal receptor status.

Table II. The multivariate analysis of *HER2* amplification.[a]

	OR	95% CI	P-value
Histotype (IDC[b] vs. ILC[c])	1.212746	0.6518421-2.256301	0.543
Grade (2 vs. 3)	0.438	0.300-0.639	<0.0001
LVI[d] (Absent vs. present)	0.531	0.371-0.760	<0.0010
HR status[e] (Positive vs. negative)	0.391	0.266-0.575	<0.0001

[a]Model is based on 807 observations. [b]IDC, invasive ductal carcinoma; [c]ILC, invasive lobular carcinoma; [d]LVI, lymphovascular invasion and [e]HR status, hormonal receptor status.

Table III. The significance of hormonal receptor status and lymphovascular invasion in *HER2* amplification of Grades 2 and 3 breast carcinomas.

	FISH not amplified (%)	FISH amplified (%)	P-value
Grade 2			
HR[a] positive	346 (89.4)	41 (10.6)	0.0001
HR negative	39 (63.9)	22 (36.1)	
Grade 3			
HR positive	214 (73.8)	76 (26.2)	0.0010
HR negative	89 (58.9)	62 (41.1)	
Grade 2			
LVI[b] absent	249 (90.5)	26 (9.50)	0.0010
LVI present	129 (79.1)	34 (20.9)	
Grade 3			
LVI absent	157 (73.7)	56 (26.3)	0.0700
LVI present	147 (65.9)	76 (34.1)	

[a]HR, hormonal receptor status and [b]LVI, lymphovascular invasion.

Table IV. Sites of metastases and *HER2* amplification.

Sites	No. of cases	FISH not amplified (%)	FISH amplified (%)
Lymph nodes	56	46 (82)	10 (18)
Bone	4	3 (75)	1 (25)
Central nervous system	6	6 (100)	0 (0)
Liver	37	29 (78.4)	8 (21.6)
Skin	64	55 (86)	9 (14)
Lung/pleura	33	29 (88)	4 (12)
Other	41	31 (76)	10 (24)

was significantly correlated with *HER2* amplification (OR per one-year increase of age: 0.9837791, 95% CI=0.972823-0.9948585, p=0.004 after 904 observations).

Multivariate analysis confirmed that grade, LVI and HR status were the only independent predictors of *HER2* gene amplification (Table II). When we considered the G2/HR positive carcinomas, the amplification rate was 10.6 versus 36.1% of the G2/HR negative cases (p<0.0001). Additionally, G2/LVI absent was amplified in 9.5% of cases versus 20.9% of G2/LVI present cases (p<0.001). In G3/HR positive cases, 26.2% were *HER2* amplified versus 41.1% of G3/HR negative (p<0.001) cases, while the difference in amplification was not significant with or without LVI (34.1 versus 26.3%, p<0.0700) (Table III).

Metastases were amplified in 42 out of 241 (17.4%) cases. In 41 cases, the site of the metastases was unknown. *HER2* amplification was higher (21.6%) in liver metastases relative to skin (14%) and lung lesions (12%). The site of lymph node metastases was unknown, however, 10 out of 56 (18%) cases were amplified. Four cases of bone metastases were tested, only one (25%) was amplified. Notably, none of the 6 central nervous system (CNS) metastasis cases were amplified (Table IV).

Discussion

HER2 amplification in different histological types and grades of female breast cancer has traditionally been a subject of interest. Some studies have shown that *HER2* amplification or overexpression were significantly more likely in IDC than ILC and in higher grade G3 IDC than in lower grade G1/G2 IDC (15,18-20). However, while the percentage of amplified G1 tumors ranged from 1 to 4%, G2 tumors ranged from 9 to 18%

(15-21). Taking these data into account, we focused on G2 and G3 invasive carcinomas of the ductal and lobular histotypes and showed that grade significantly correlates with *HER2* amplification. Few studies have evaluated the frequency of *HER2* amplification in the ILC of grade 2 or 3, the latter corresponding to the majority of cases for the pleomorphic variant (22). Most cases of ILC, in their classic variant, are positive for estrogen and progesterone receptors and negative for *HER2*. On the other hand, 53 to 81% of pleomorphic lobular carcinomas have been described as *HER2* positive (23,24). In our series, 16% of G2/G3 ILC were amplified. This result reinforces the conclusion drawn by Arpino *et al* (25) that 'management decisions should be based on individual patient and tumor biological characteristics, and not on lobular histology'. While it has been suggested that *HER2* gene amplification is more common in younger patients (19), multivariate analysis failed to confirm a significant relationship between *HER2* gene amplification and patient age in our study.

Recent studies have reported *HER2* amplification in 18-20% of samples tested with FISH (26,27). The present study focused on G2/G3 breast carcinomas and the overall percentage of amplification was 23% and 30% when G3 carcinomas were considered. These data indirectly confirm the low impact G1 tumors have on the rate of *HER2* gene amplification. Conversely, ~15% of moderately-differentiated G2 breast carcinomas may be amplified. Other studies have delved deeper into analysing the features related to *HER2* amplification and predicted the *HER2* status of breast cancer from basic histopathology and immunophenotypical data (19,20,28), such as LVI and HR status. Taucher *et al* (18) proposed a scoring system to determine the probability of *HER2* positivity (diagnostic instrument for the validation of *HER2*/neu, DIVER score). This scoring system was determined by prognostic markers that exhibit the strongest correlation with *HER2* status, namely estrogen and progesterone receptors and tumor grade. They concluded that in a subgroup of patients demonstrating hormone-responsive and G1/G2 tumors, the likelihood of HER2 overexpression was very small. We have shown that in the categories of G2/G3 breast cancers, HR status was significantly correlated with *HER2* gene status as well. Only 17% of breast carcinomas expressing HR show *HER2* amplification versus 40% of HR negative carcinomas. Moreover, G2/HR negative breast cancers have an amplification rate similar to those of G3 breast carcinomas. In contrast to the results of Crowe *et al* (19), LVI was determined to be another significant independent variable in our multivariate analysis. However, LVI is not always reproducible even among expert pathologists (29), while the HR status, in particular estrogen receptor expression, is an all or nothing phenomenon that is more easily reproduced on histological slides (30). Finally, Gong *et al* (16) suggested that the *HER2* gene does not appear to be linked directly to tumor dissemination. Our data confirm this hypothesis since only 17% of breast cancer metastases show *HER2* amplification, while we expected amplification rates of at least 23%. Notably, the results of *HER2* gene analysis on CNS metastases in our series indicate the need for larger studies to elucidate whether trastuzumab resistance in CNS metastases is a true phenomenon or a consequence of the absence of *HER2* amplification (31). Eligibility for trastuzumab therapy in advanced breast cancer patients warrants the use of FISH as a screening test whenever possible, particularly considering that samples from FNA of the metastatic lesion are frequently the only specimens available for diagnosis. It is well known that more variability is found in the IHC results compared with the FISH results on FNA samples, and scoring the FISH signals in such small tissue samples is more objective than scoring IHC staining (32,33).

In conclusion, considering the overall logistical difficulties as well as the accuracy, time and cost for the double testing of HER2 (IHC/FISH), gene analysis may be an efficient and useful approach for HER2 screening of breast cancer metastases and G3 tumors, particularly for laboratories running a large number of breast cancer surgical specimens, where the pathological experience would guarantee a correct grading of the tumor. For all the non-G3 tumors, the rational algorithm for HER2 testing would be to perform IHC first, followed by FISH to validate equivocal IHC results (12).

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