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HER2 status in early breast cancer: Relevance of cell staining patterns, gene amplification and polysomy 17

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ABSTRACT

The prevalence of HER2 amplification according to the percentage of positively stained cells, of polysomy 17 and their correlation with clinical and pathologic characteristics were retrospectively evaluated in a population of 415 breast cancers where fluorescence *in situ* hybridisation (FISH) was performed to clarify HER2 status previously determined by immunohistochemistry. Forty-two tumours with intense and complete staining in >50% of cells were selected from the same database as internal controls.

Among the 415 cases, 233 tumours were IHC 1+, 168 tumours were 2+ and 14 tumours showed an intense and complete immunostaining in ⩽50% of neoplastic cells. HER2 was amplified in 3/14 (21.4%) tumours with ⩽50% and in 36/42 (85.7%) tumours with >50% of intense stained cells, ($p < 0.001$).

Polysomy 17 was detected in 77 tumours (16.85%). It was inversely correlated with the percentage of positively stained cells, but not with amplification. Patients with polysomy 17 and no amplification were significantly more likely to have tumours with favourable biological features when compared with patients with HER2 amplification.

Our results suggest that FISH testing should be considered for tumours with ⩽50% positive stained cells and that polysomy 17 without amplification is not associated with poor prognostic features.

1. Introduction

HER-2/neu (HER2) is a proto-oncogene located on chromosome 17, encoding for a transmembrane growth factor receptor with tyrosine kinase activity.[1] Overexpression of the protein and/or amplification of the gene have been reported in approximately 20 to 30% of breast cancers and they are associated with either poor prognosis or with response to the humanised monoclonal antibody trastuzumab.[1]

The most widely used assays to determine HER2 status are immunohistochemical analysis (IHC) and fluorescence *in situ* hybridisation (FISH), which measure protein expression and gene amplification, respectively.[1,2] Although FISH is considered the most accurate and reproducible test with a better

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correlation with prognosis and response to therapy,[2] the IHC determination still remains the most used in many institutions due to the limited related costs and accessibility. Further evaluation with the FISH test is reserved in cases where the results are doubtful (i.e. cases with moderate expression of the protein IHC 2+). In fact, in tumours with weak to moderate IHC expression of the protein, gene amplification has been reported in 16% of cases.[3] Moreover, although limited data are available on the correlation between percentage of positive cells and amplification, some data are consistent in showing a greater accuracy of IHC by increasing the cutoff of positive stained cells above 10%.[4,5]

Aneusomy, mostly polysomy, of chromosome 17 has been reported in 10 to 50% of cases, depending upon the criteria used for the definition of polysomy.[6] The role of polysomy on clinical behaviour and response to targeted therapies is still unclear.[7] It has been hypothesised that both an increased number of the gene due to multiple chromosome 17 copies and gene amplification might identify HER2 positive tumours,[8] but the biological and clinical relevance of polysomy 17 remains under investigation.

The objectives of the present study were:

1) to retrospectively evaluate the prevalence of HER2 amplification in tumours where HER2 status was previously defined equivocal at IHC,
2) to evaluate the prevalence of polysomy of the chromosome 17, defined as $\geqslant 3$ copies of the chromosome and its correlation with HER2 status either in terms of protein expression and gene amplification,
3) to investigate the correlation of polysomy 17 with clinical and pathological characteristics as compared with tumours with HER2 amplification.

2. Patients and methods

We collected, in a dedicated database, information on all consecutive breast cancer patients operated on at the European Institute of Oncology (EIO) in Milan between April 1999 and December 2005. Pathological assessment included evaluation of the primary tumour size, grade and histological type, and of lymph node status following axillary lymph node dissection or a sentinel node biopsy. All patients signed an informed consent for further assessment of their pathological sample for research purposes.

Among a total number of 13,900 patients, we retrospectively selected patients presenting with tumours with c-erbB-2 expression defined at IHC as negative (faint and partial staining in >10% of cells = 1+) and equivocal (faint and complete staining in >10% of cells = 2+ and intense and complete staining in $\leqslant$50% of cells), where a FISH test was performed in order to clarify the HER2 status. In particular, FISH was requested by the clinician because it might be helpful in making a clinical decision. A total of 415 patients with these characteristics were identified. We also selected from the same database 42 patients presenting with tumours with intense and complete staining in >50% of cells where FISH was performed to determine the eligibility to a clinical trial.

Immunostaining experiments for the localisation of oestrogen and progesterone receptors (ER and PgR), Her2/neu protein and Ki-67 antigen were performed on consecutive tissue sections. Four micrometer thick sections of formalin – fixed, paraffin – embedded tissue were pretreated with heat-mediated antigen retrieval in 1 mM EDTA buffer at pH 8 in a water bath at 99 °C for 30 min and subsequently incubated with the following primary antibodies: the monoclonal antibody (MAb) to ER (clone 1D5, Dako, at 1/100 diluition), the MAb to PgR (clone PgR 636, Dako, at 1/400 diluition), the MIB-1 MAb to the Ki-67 antigen (Dako, at 1/200 diluition) and the polyclonal rabbit antiserum (Dako, at 1/800 diluition) to the Her2/neu protein for 30 min at room temperature, and then with a commercially available detection kit (Dako EnVision Plus HRP) according to the manufacturer's instructions. Peroxidase activity was developed with a liquid DAB chromogen system (DAKO, liquid DAB + substrate chromogen system) according to the manufacturer's instructions. The immunostained slides were evaluated independently by two of the authors. Only nuclear reactivity was taken into account for ER, PgR, and Ki-67 antigen, whereas only an intense and complete membrane staining $\geqslant$10% of the tumour cells was taken as evidence of Her2/neu overexpression (3+). The results were recorded as the percentage of immunoreactive cells over at least 2000 neoplastic cells. The value of Ki-67 labelling index was used as a cut-off in distinguishing tumours with low (<20%) and high ($\geqslant$20%) proliferative fraction. The value of 20% was selected based on previous data from our group indicating that this threshold significantly correlated with higher response rate to preoperative chemotherapy. Steroid hormone receptor status was classified as absent (oestrogen receptor and progesterone receptor 0% of the cells positive), low (oestrogen receptor and/or progesterone receptor $\geqslant$1% <10% of the cells), or positive (oestrogen receptor and progesterone receptor $\geqslant$10% of the cells), as previously reported.[9]

Tumour grade was evaluated according to Elston and Ellis and peritumoral vascular invasion (VI) was assessed according to Rosen et al., as previously reported.[9]

A commercially available dual colour FISH assay (PathVysion, Vysis, Downers Glove, IL) was used to simultaneously evaluate HER-2/neu gene and chromosome 17 copy number according to the manufacturer's instructions. The locus specific identifier HER-2/neu probe is a 190 kb SpectrumOrange (Abbott-Vysis, Inc.) directly labelled fluorescent DNA probe for the HER-2/neu gene locus (17q11.2-q12). The chromosome enumeration probe is a 5.4 kb SpectrumGreen (Abbott- Vysis, Inc.) directly labelled DNA probe specific for the alpha satellite DNA at the centromeric region of chromosome 17 (17p11.1-q11.1). At least 100 cells in the tumour component were evaluated for gene amplification. Specimens with ratio of HER-2/neu gene copies to chromosome 17 centromere copies $\geqslant$2.0 were considered amplified. Polysomy was defined as the occurrence of three or more copy numbers of centromeres for chromosome 17 per cell, according to Salido et al.[10] Digital images were obtained using a Leica DMRB epifluorescence microscope equipped with a Leica digital camera DC250 (Leica Imaging Systems, Ltd., Cambridge, UK). FITC, Cy3, and DAPI fluorescent signals were detected using specific filters. The images were recorded, pseudocoloured and merged using the QFluoro software (Leica, Inc., Deerfield, IL). The Fisher's

exact test was used to compare the distributions of polysomy 17 and HER-2 amplification in tumours characterised by different IHC and FISH status.

Univariate logistic regression and Fisher's exact test were used to evaluate the association between clinical and pathological variables and the FISH status, categorised as 'Not amplified with polysomy17' and 'Amplified with and without polysomy'. The odds ratio (OR), with 95% confidence intervals (CI), were computed for each factor considered.

Multivariate logistic regression models were also fitted to the data to assess the association between FISH status and variables that were identified as important in univariate analysis.

All statistical analyses were performed with SAS software (SAS Institute, Cary, N.C.). All reported *p*-values are two-sided.

3. Results

Both IHC and FISH results were compared for negative and equivocal cases ($n = 415$) and for the control cases ($n = 42$) issued from the selection process for FISH assay previously described (Table 1). Our results showed that HER2 was amplified in 18/415 cases (4.33%); fifteen cases of amplification were observed among tumours with weak to moderate IHC staining (3.74%). In particular, tumours with weak (1+) and moderate (2+) expression, had amplification in 3/233 tumours (1.29%) and 12/168 (7.14%), respectively. Only three out of 14 (21.4%) tumours with positive immunostaining in $\leqslant$50% of cells also showed amplification. On the contrary, 36 out of 42 (85.7%) tumours with staining in >50% of cells were amplified (*p*-value Fisher's exact test $p < 0.001$).

We then analysed the prevalence of polysomy 17 in the whole population of 457 tumours. Polysomy 17 was observed in 77 tumours (16.9%) and it was equally distributed among tumours without (68/403, 16.9%) and with (9/54, 16.7%) HER2 amplification, respectively (*p*-value Fisher's exact test = 1.00). On the other hand, polysomy 17 was inversely associated with the percentage of positively stained cells in that 6/14 (42.9%) of tumours with intense and positive immunostaining in $\leqslant$50% of cells also showed polysomy while only 6/42 (14.3%) of tumours with positive staining in >50% were polysomic (*p*-value Fisher's exact test = 0.05).

Results of univariate analysis comparing non-amplified tumours with polysomy and tumours with HER2 amplification, selected from the whole population of 457 tumours, are shown in Table 2. Non-amplified tumours with polysomy 17 were more likely to be smaller than or equal to 2 cm (57.4% versus 35.2%, respectively $p < 0.05$), to be node negative (50% versus 24.1%, respectively $p < 0.01$), to have expression of steroid hormone receptors (95.6 % versus 50%, respectively; $p < 0.01$), grade 1–2 disease (47% versus 13.5%, respectively; $p < 0.01$) and low Ki-67 (26.5 % versus 1.9%, respectively; $p < 0.01$) and not to show vascular invasion (32.8% versus 51.9%, respectively, $p < 0.01$) when compared with tumours with HER2 amplification. When the analysis was restricted to tumours with negative or equivocal HER2 status, the results were similar (data not shown).

Finally, in multivariate analysis, HER2 amplification was significantly associated with hormone receptor status, nodal involvement and tumour grading, but not with vascular invasion and tumour size (Table 3). The effect of ki67 was not estimated because only one patient with ki67 < 20% was included in the HER2 amplified group.

4. Discussion

HER2 status is relevant for patient care as recently stated in the 9th St.Gallen Consensus Conference.[11] Despite issues related to reproducibility of IHC staining or FISH testing, overexpression of HER2 indicate a poor outcome.[1,2,10,11] Overexpression and/or amplification of HER2 also have a predictive value.[12–16]

The proper algorithm for the assessement of HER2 status has yet to be defined. A series of assays are available and some of them, specifically HercepTest (DAKO) and CB11 (Ventana) for IHC and PathVysion (Abbott) and INFORM (Ventana) for FISH have been approved by the FDA.[1,2,17] Evidence arising from comparative studies shows that gene amplification correlates better with prognosis and is more accurate in identifying patients who are likely to benefit from trastuzumab treatment, while very limited data are available on the activity of trastuzumab in patients with IHC positive/FISH negative tumours.[1,18–20] However, a number of drawbacks are associated with FISH testing: it is expensive, time consuming, not easily performed in all laboratories and discrepancies have been reported between reference and peripheral laboratories.[2,21] Therefore, while IHC represents the first screening test, a FISH test should be performed in all cases where IHC results are doubtful or equivocal.[1,17]Based on these considerations it may thus be useful to define subsets of tumours

Table 1 – Incidence of HER2 amplification and polysomy 17 according to immunohistochemical scores in 457 breast cancers

FISH	Polisomy 17	IHC				Total
		Weak	Moderate	Intense / complete $\leqslant$ 50%	Intense / complete > 50%	
Not Amplified	Absent	196	127	6	6	335
	Present	34	29	5	0	68
Amplified	Absent	3	10	2	30	45
	Present	0	2	1	6	9
Total		401		14	42	457
			415			

IHC: Immunohistochemistry; FISH: fluorescence *in situ* hybridisation.

Table 2 – Association between HER2 amplification and polysomy 17 status with clinical and pathological variable - results of univariate analysis

Variable	FISH Not Amplified / Polysomy 17 Present (N = 68)	FISH Amplified (N = 54)	OR (95% CI)	p-value
HR status				
ER – / PgR –	3 (4.4%)	27 (50.0%)	1.00	–
ER + / PgR –	8 (11.7%)	8 (14.8%)	0.11 (0.24–0.52)	<0.001
ER + / PgR +	57 (83.8%)	19 (35.2%)	0.04 (0.01–0.14)	<0.001
Grading				
3	35 (53.0%)	45 (86.5%)	1.00	–
2	29 (44.0%)	7 (13.5%)	0.19 (0.07–0.48)	<0.001
1	2 (3.0%)	0 (0.0%)	–[a]	0.205
Ki67				
<20 %	18 (26.5%)	1(1.9%)	1.00	–
⩾20 %	50 (73.5%)	53 (98.1%)	19.1 (2.45–148.3)	0.005
Age				
⩽35 years	3 (4.4%)	6 (11.1%)	1.00	–
>35 years	65 (95.6%)	48 (88.9%)	0.37 (0.09–1.55)	0.174
Histotype				
Ductal	65 (95.6%)	50 (92.6%)	1.00	–
Lobular	1 (1.5%)	0 (0.0%)	[a]	1.000
Other	2 (2.9%)	4 (7.4%)	2.60 (0.46–14.77)	0.281
Tumour Size				
pT ⩽ 2 cm	39 (57.4%)	19 (35.2%)	1.00	–
pT > 2 cm	29 (42.6%)	35 (64.8%)	2.48 (1.19–5.17)	0.016
Nodal status				
N0	33 (50.0%)	13 (24.1%)	1.00	–
N1–3	22 (33.3%)	19 (35.2%)	2.19 (0.90–5.33)	0.083
N ⩾ 4	11 (18.9%)	22 (40.7%)	5.07 (1.93–13.36)	0.001
Vascular Invasion				
No	45 (67.2%)	26 (48.1%)	1.00	–
Yes	22 (32.8%)	28 (51.9%)	2.20 (1.05–4.61)	0.036

FISH: fluorescence *in situ* hybridisation.
OR: odds ratio (and 95% confidence interval) comparing variable distributions in 'FISH Amplified' *versus* 'FISH Not Amplified / Polysomy 17 Present'.
ER –: oestrogen receptor negative; PgR –: progesterone receptor negative; ER +: oestrogen receptor positive; PgR +: progesterone receptor positive.
a Odds ratio not calculated because of zero cell value. *p*-value based on Fisher's exact test.

when FISH test is required or, on the contrary, it might be avoided. In fact, the criteria for determining the need for FISH testing are debated. While a general agreement on testing 2+ IHC tumours exists, largely inconsistent data are available for 3+ IHC tumours for which a discordance, generally ranging from 3 to 15%, has been reported.[22] Recently, the American Society of Clinical Oncology and the College of American Pathologists have renewed guideline recommendations for HER2 testing and also included, as equivocal cases, protein positive staining in ⩽30% of cells. This cut-off was established according to the cumulative experience of panel members and previous published reports using cut-off values higher than 10% in order to reduce the rate of false positive 3+ tumours.[5,6,17] Our data, arising from a large volume laboratory using standard immunohistochemical methods, indicated that using a cut-off of 50% of positively stained cells, only three out of 14 (21%) tumours had gene amplification, with a discordance rate of 79%.

On the other hand, 36 out of 42 (85.7%) tumours with positive staining in >50% of cells were amplified, indicating that the large discordance in the former group is not due to methodological biases. Moreover, in the present study we observed a low rate of amplification in tumours considered IHC negative (weak staining). In fact, only 1.3% of tumours with weak (1+) staining were amplified. In 2+ IHC as well, the proportion of gene amplification (7.14%) was lower than expected according to literature data where amplification was reported in 12–36%.[2]

The results of our study confirm that, as previously reported, HER2 overexpression is not so infrequent in the absence of gene amplification.[23] Since gene amplification is functionally more significant either in terms of poorer prognosis or of predicting response to trastuzumab,[1,19,22] overexpressing tumours with positive staining in ⩽ 50% of cells deserve the FISH test in order to proper define HER2 status. Our data, consistent with other reports,[24] showed that amplification was significantly associated with node positive, hormone receptor negative and poorly differentiated tumours. Interestingly, in our series, no well differentiated tumours, and 2% of tumors with low Ki-67 expression, showed gene

Table 3 – Association between HER2 amplification and clinical and pathological variables - results of multivariate analysis

Variable	OR (95% CI)	*p*-value
HR status		
ER – / PgR –	1.00	–
ER + / PgR –	0.09 (0.01–0.52)	0.017
ER + / PgR +	0.03 (0.01–0.15)	<0.001
Grading		
3	1.00	–
1/2[a]	0.29 (0.09–0.96)	0.040
Tumour Size		
pT ⩽ 2 cm	1.00	–
pT > 2 cm	0.72 (0.23–2.28)	0.574
Nodal status		
N0	1.00	–
N1–3	5.81 (1.45–23.18)	0.013
N ⩾ 4	11.76 (2.50–55.29)	0.002
Ki67		
<20 %	1.00	–
⩾20 %	[b]	
Vascular Invasion		
No	1.00	–
Yes	0.98 (0.31–3.04)	0.972

OR: odds ratio (and 95% confidence interval) comparing variable distributions in 'FISH Amplified' versus 'FISH Not Amplified / Polysomy 17 Present'.
ER –: oestrogen receptor negative; PgR –: progesterone receptor negative; ER +: oestrogen receptor positive; PgR +: progesterone receptor positive.
a G2 and G1 were grouped together because no patient in the FISH A Poly –/+ had G1 tumour.
b The effect of Ki67 ⩾ 20% in multivariate analysis was not estimable because only one patient with Ki67 < 20% was included in the FISH Amplified group.

amplification, suggesting that in these subgroups of patients further FISH testing should not be performed.

An increased copy number of chromosome 17 has been claimed as one contributing factor to the presence of protein staining despite the lack of gene amplification.[8] Conflicting results have been reported in literature about the prevalence and role of polysomy 17, depending on the criteria used for defining a polysomic status.[6,7,25,26] Different cut-off points have been used to define polysomy tumour's genomic heterogeneity as well as different proliferative activity but also artifacts arising from tissue sectioning may account for the differences reported. In addition to the discrepancy on the prevalence, inconsistent data are available on the association with HER2 IHC expression, gene amplification, and with different clinical and biological characteristics of the tumour.[6,10,25,27]

In our series, polysomy 17, defined as three or more chromosome copies, was reported in about 17% of tumours, a figure which is almost comparable with that reported by Salido et al..[10] Differently from other authors[28] no significant correlation with gene amplification was observed, while polysomy appeared to be inversely related to the percentage of positively stained cells.

An issue of clinical relevance is whether polysomy is associated with a clinical behaviour similar to that of HER2 amplified tumours. Inconsistent findings have been reported.[10,29] Our results indicated that tumours with polysomy 17, but no HER2 amplification, showed different clinical and biological features than amplified tumours, strengthening the hypothesis that polysomy 17 does not represent a poor prognostic feature itself.

Currently, no studies correlating polysomy 17 and either long term prognosis or clinical response to trastuzumab are available. Given the association with hormone receptor positive, low grade and low proliferating tumours, it is likely that long term studies are required to point out an independent prognostic role of polysomy 17.

According to the prevalence observed in our series (16.7% in amplified and 29.4% in IHC 3+ tumours, respectively), the independent role, if any, of polysomy 17 in contributing to response to targeted agents may be investigated in 3+ IHC and/or FISH positive tumours only within large phase III trials.

The results of our study confirm that HER2 amplification can be detected in a small proportion of tumours with moderate protein expression or intense expression in a low percentage of cells, although a different cut-off of positive stained cells from that previously published has been considered. According to the present results, we suggest that tumours with HER2 positive staining in ⩽50% of cells should be candidated for further FISH testing. In addition, in these tumours HER2 status should also be determined in axillary lymph node metastasis, if present. On the other hand, in low proliferating and well differentiated tumours, amplification was a rare finding, therefore indicating that a FISH test might not be performed in these patients. Finally, our findings suggest that tumours with polysomy 17 significantly differed in terms of biological features when compared with tumours with HER-2 amplification, supporting a possible different clinical behaviour to be confirmed in large phase III trials.

Conflict of interest statement

None declared.

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