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Analysis of aneusomy level and HER-2 gene copy number and their effect on amplification rate in breast cancer specimens read as 2+ in immunohistochemical analysis

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ABSTRACT

Our aim was to determine the aneusomy level and the *HER-2* gene copy numbers, by fluorescence *in situ* hybridization (FISH) and to analyze their impact on the amplification rate in breast carcinomas considered HER-2 weakly positive cases by immunohistochemistry. We evaluated 343 breast carcinomas using double colour FISH (LSI *Her-2/neu* gene and *CEP* 17). Monosomy and polysomy were demonstrated in 24.2% and 46.1% respectively and 101/343 (29.6%) of the specimens were amplified by FISH. A statistically significant difference was observed, when we compared the amplification percentage in polysomic and monosomic specimens ($P < 0.0001$) and, among polysomic specimens, when tumours were compared with *HER-2* gene signals number per cell between 3 and 10 and >10 respectively ($P < 0.0001$). Logistic regression analysis showed that *HER-2* signals >10 and polysomy absence were independently associated with amplification. Our results confirm that the majority of 2+ IHC cases express the HER-2 protein without gene amplification and highlight the effect of chromosome 17 aneusomy and the HER-2 gene copy number on amplification.

1. Introduction

HER-2 is a protoncogene located on chromosome 17. It encodes a transmembrane growth factor receptor with tyrosine kinase activity. Overexpression and/or amplification of HER-2 are detected in approximately 20% to 30% of invasive ductal carcinomas of the breast[1,2] and have been associated with a poor prognosis.[3] Amplification of the *HER-2* oncogene and concomitant overexpression of protein are currently implicated in breast carcinoma as important biomarkers for predicting response to trastuzumab (Herceptin, Roche, Basel, Switzerland), which has significant anti-tumour activity both as a single agent and in combination with chemotherapy in these patients.[4,5]

For this reason, laboratory assessment of HER-2 status is becoming a key step in the optimal management of patients with advanced breast cancer. Immunohistochemical (IHC) and fluorescence *in situ* hybridization (FISH) have emerged as the two most widely used assays to evaluate HER-2 status in breast cancer. Both methods are recommended by national and international guidelines.[6–8] Numerous studies have shown that the overexpression of HER-2 protein is closely

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correlated with amplification of the *HER-2* gene in IHC 3+ cases, but not IHC 2+ cases. Benefit from trastuzumab treatment in patients with breast cancer, IHC 2+ for HER-2, appears to be limited to those tumours that are FISH-positive for *HER-2* amplification.[9,10] Furthermore, patients with these apparent IHC positive, FISH-negative tumours appear to have clinical outcomes similar to patients with no alteration.[11] Aberrant numerical changes in chromosome 17 copy number are frequently encountered in invasive breast cancer[12] and may complicate the scoring of *HER-2* amplification.[13] It has been suggested that some of these IHC low positive may in part be due to increased copy numbers of chromosome 17 resulting in increased HER-2 protein expression.[14] For that, some authors consider the chromosome 17 copy correction to be essential to establish the true amplification. On this basis, in the present study we performed a FISH assay using the dual colour system on 343 cases of invasive primary or metastasis breast cancer, read as 2+ in immunohistochemical analysis. The aim was to determine the aneusomy level (monosomy and polysomy), HER-2 gene signals number per cell and their impact on the amplification rate in a selected representative group of carcinomas considered weakly positive cases by IHC.

2. Materials and methods

2.1. Tissue Specimens

A total of 343 formalin-fixed paraffin-embedded breast carcinomas, which were sent to the cytogenetic laboratory of the Regina Elena Cancer Institute Rome for FISH testing, were included in this study. All cases had an immunohistochemical score of 2+ for HER-2 and would not have been eligible for trastuzumab therapy unless gene amplification had been detected by FISH. In this series, 128 cases were referred from other centers after immunohistochemical screening.

2.2. Fluorescence in situ hybridization

FISH was performed using the PathVysion assay kit (Vysis, Inc., Downers Grove, IL), which includes two directly labelled DNA probes: a locus specific probe for the *HER-2* gene labelled with SpectrumOrange (LSI Her-2/neu) and an alpha satellite probe targeting the centromere region of chromosome 17 labelled with SpectrumGreen (*CEP* 17). The assay was performed according to the manufacturer's instructions. In brief, after deparaffinization, specimens were incubated in pre-treatment solution (80 °C, 10 min) and then digested with protease (37 °C, 15 min). The LSI *Her-2/CEP 17* probe was applied, a coverslip sealed to the slide, and the specimens hybridized overnight at 37 °C. Slides were then washed, counterstaining with DAPI and analyzed under a fluorescence microscope. In each breast cancer specimen an average of 200 nuclei were enumerated in the invasive part of the tumour. *HER-2* gene copy number, chromosome 17 copy number and the average *HER-2* gene to chromosome 17 signal ratio were reported as FISH genetic variables. Samples with a ratio value $\geqslant$2.0 were considered to be amplified. A ratio ranging between 2 and 4 was qualified as low amplification, between 4 and 10 as moderate and >10 as high. Chromosome 17 numerical status was defined as monosomic or polysomic when the cancer cell population showed single or multiple (>3) 17 centromere signals, respectively. The cut-off values for chromosome 17 aneusomy were estimated according to our previous published results.[12] On the basis of the cut-off values, we classified one group of monosomic tumours and one group of polysomic tumours, considering at the same time, the observed mean percentage values of monosomy (48.9 ± 10.7) and polysomy (46.5 ± 25.8).

Specimens with a chromosome 17 copy number in the range of 2 to 4 signals per cell were considered to have low 17 polysomy, while a copy number >4 signals per cell as having high polysomy.

In order to avoid the probable low polysomy underestimation due to tissue cutting, we used as controls for chromosome 17 number determination, slides performed by touch imprinting from 10 frozen biopsies which were already evaluated as paraffin-embedded section.

2.3. Statistical analysis

The χ^2 test was used to evaluate the association of amplification rate with monosomy, polysomy and *HER-2* gene copy number. The accepted statistically significant difference was $P < 0.05$.

Logistic regression analysis was used to assess the impact of genetic variables on the ratio. The cut-off p-values to enter in or to be removed from the model were set to 0.10 and 0.15. Results are reported as odds ratio (OR) with 95% CI.

3. Results

3.1. Fluorescence in situ hybridization assay results in a cohort of 343 cases considered 2+ by immunohistochemistry (IHC)

As illustrated in Table 1, among the 343 patients with a low level of HER-2 expression and not eligible for trastuzumab therapy if not amplified by FISH, 29.7% of patients had a disomic FISH pattern, 24.2% monosomy, whereas 46.1% showed polysomy. In the group of 158 patients evaluated as polysomic, 87.3% had a low polysomy level, whereas 12.7% had a high

Table 1 – FISH patterns in 343 specimens scored as 2+ by IHC

FISH genetic variables		Patients	
		N	%
Disomy		102	29.7
Monosomy		83	24.2
Polysomy		158	46.1
Low polysomy		138	87.3
High polysomy		20	12.7
Ratio	*Mean ratio*		
$\leqslant$ 0.9 (deletion)	0.82	28	8.2
> 0.9–< 1.30 (No apparent HER2 anomaly)	1.04	170	49.6
1.30–< 2 (duplication)	1.57	44	12.8
$\geqslant$ 2 (amplification)	5.46	101	29.4

polysomy level. Using a spreadsheet, the *HER-2/CEP 17* signal ratio was calculated for each tumour considered weak positive by IHC. The HER-2 anomalies were categorized with use of the criteria described in Table 1. Of the 343 specimens, 8.2% demonstrated deletion of *HER-2*, 49.6% no apparent *HER-2* anomaly, 12.8% duplication of *HER-2* and 29.4% *HER-2* amplified. Moreover, among the 101 amplified tumours, we classified 50.5% with low amplification (23 monosomic and 19 polysomic), 32.7% with moderate amplification (10 monosomic and 16 polysomic), whereas 16.8% had a high amplification level (8 monosomic and 3 polysomic) (Table 2). In Fig. 1 we show the representative pictures of two tumours with a duplicated *HER-2* gene (Fig. 1A: low polysomy and ratio = 1.21, Fig. 1B: high polysomy and ratio = 1.36) and two tumours with an amplified *HER-2* gene (Fig. 1C: low polysomy and ratio = 3.01, Fig. 1D: high polysomy and ratio > 10).

Table 2 – Distribution of ratio in specimens scored as 2+ by IHC and amplified by FISH

		Patients	
		N	%
	Ratio		
Low amplification	2 – < 4	51	50.5
Moderate amplification	4 – < 10	33	32.7
High amplification	>10	17	16.8

3.2. *Association of amplification rate with monosomy, polysomy and HER-2 gene copy number*

In the second part of our study, contingency Table and the χ^2 test were used to evaluate the association between rate amplification and genetic alterations observed in the 343 samples examined. As observed in Table 3, a statistically significant difference ($P < 0.0001$) was present, when we compared the amplification percentage in polysomic (24.1%) and monosomic (49.4%) specimens. Moreover, we noticed a positive trend when we examined the rate amplification in samples with low (21.7%) and high polysomy (40.0%), even if this was not statistically significant ($P = 0.07$). Taking into consideration the *HER-2* gene copy number as variable, in polysomic specimens, tumours with signals >10 had an amplification percentage higher (95.0%) than tumours with signals between 3 and 10 (23.5%) with a statistical difference ($P < 0.0001$). Whereas, when we compared the amplification rate in monosomic tumours with signals >10 (100%) and tumours with signals between 3 and 10 (87.5%) there was no statistical difference ($P = 0.22$).

3.3. *Logistic regression analysis*

The results of the logistic regression analysis of factors associated with amplification (ratio value) are summarized in Table 4. The following variables were studied: monosomy, polysomy, low and high polysomy and HER-2 signals. Only HER-2 signals and polysomy were significantly associated

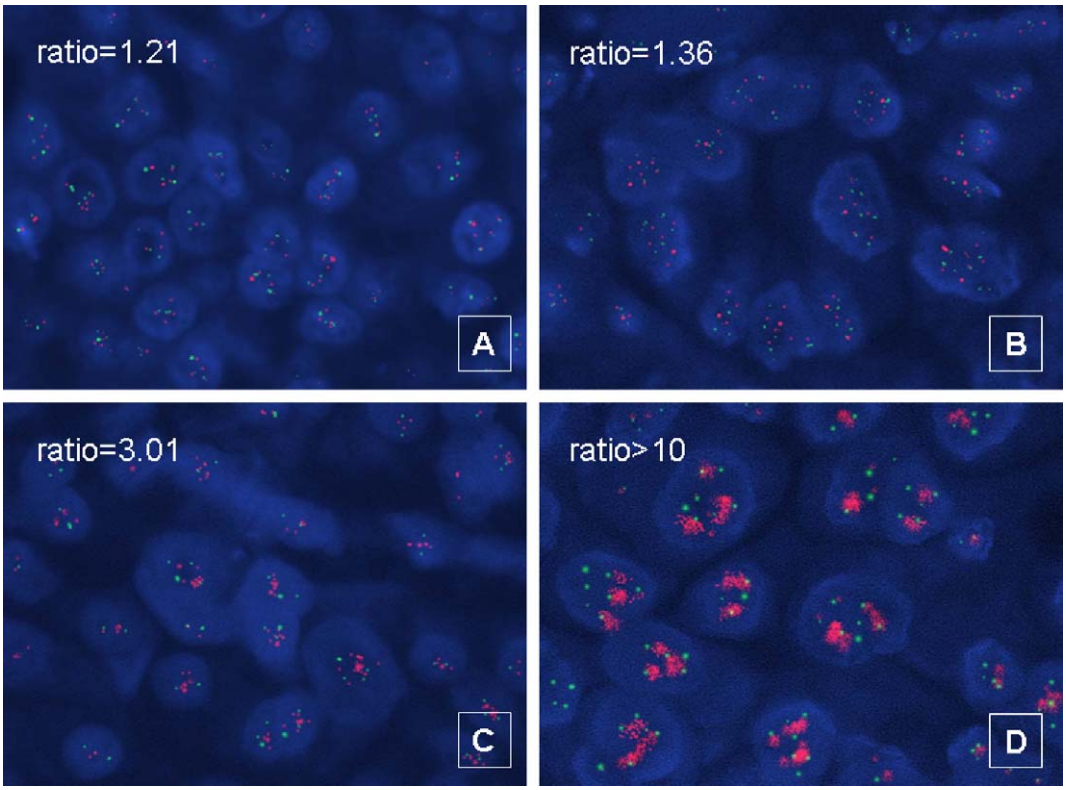

Fig. 1 – FISH image: *CEP 17* (green) and *HER-2* gene (red) in two tumours with duplicated gene (A: low polysomy and ratio = 1.2, B: high polysomy and ratio = 1.3) and two tumours with amplified *HER-2* gene (C: low polysomy and ratio = 3.01, D: high polysomy and ratio > 10).

Table 3 – Amplification rate (HER-2 gene/chromosome 17 ratio ⩾2) in breast cancer scored as 2+ by IHC according to aneusomy level and HER-2 signals number/cell

FISH genetic variables	N	% Amplification	P	Mean ratio
Polysomy	158	24.1	<0.0001	1.96
Monosomy	83	49.4		3.27
Low Polysomy	138	21.7	0.07	1.85
High Polysomy	20	40.0		2.74
HER-2 gene signals number/cell 3–< 10[a]	81	23.5	<0.0001	1.75
HER-2 gene signals number/cell >10	20	95.0		5.47
HER-2 gene signals number/cell 3–< 10[b]	24	87.5	0.22	3.48
HER-2 gene signals number/cell >10	11	100		8.73

P value:χ^2 test.
a Evaluation performed in polysomic specimens.
b Evaluation performed in monosomic specimens.

Table 4 – Logistic regression analysis of amplification (ratio value) in IHC 2+ specimens

Variable	OR	95% C.I.	P-value
HER-2 signals (3–< 10 vs >10 signals)	0.016	0.002–0.125	<0.0001
Polysomy (presence vs absence)	0.095	0.031–0.293	<0.0001

OR, odds ratio; CI, confidence interval.

with OR. HER-2 signals >10 and polysomy absence (monosomy) were independently associated with amplification (OR 0.016, 95% CI 0.002–0.125 and OR 0.095, 95% CI 0.031–0.293, respectively).

4. Discussion

The determination of HER-2 status in breast cancer is of the utmost importance, not only in metastatic disease to determine eligibility for trastuzumab therapy but also in the adjuvant setting for selection of appropriate chemotherapy.[15–17] For clinical HER-2 status determination, the US Food and Drug Administration has approved the HercepTest (Dako, Carpinteria, CA) and Ventana Pathway (Ventana, Tucson, AZ) as immunohistochemical assays for detection of HER-2 protein expression in breast cancer and PathVysion (Vysis, Downers Grove, IL) as a FISH method to detect *HER-2* amplification. Early studies have reported a high level of concordance between the two methods. Researchers at the Memorial Sloan-Kettering Cancer Center, NY, found a very high concordance rate between immunohistochemical scores of 0, 1+, and 3+ and FISH data in the 2279 cases studied, whereas only 25% of cases, scored as 2+ by IHC, demonstrated gene amplification in FISH studies.[18] In consideration of that, in this work we set out to determine the aneusomy level (monosomy and polysomy), *HER-2* gene copy numbers and to examine their impact on the amplification rate in a representative group of carcinomas considered weakly positive cases by IHC.

We used the FDA-approved PathVysion FISH assay, which includes a dual probe set for the *HER-2* gene and the chromosome 17 centromere to adjust for the effects of aneuploidy and to establish the true amplification. Numerical aberrations in specific chromosomes have been shown to occur in a high proportion of breast carcinomas. We have already reported that aneusomy 17 is present at different rates depending on the series considered.[12] and it has been shown that chromosome 17 alterations correlate to lymph node metastasis.[19] as well as other poor prognostic factors.[20] Chromosome 17 aneusomy, deviation from the normal state of disomy, can be polysomy (>3 copies per cell) or monosomy (<2 copies per cell). On the basis of established criteria, all tumours from our patients were defined monosomic or polysomic. As concerns polysomy, the comparison between paraffin-embedded sections and the corresponding tissue touches imprinting demonstrated the marginal influence of tumour section on low polysomy (trisomy) counting and thus the insignificant risk of triploidy underestimation (data not shown) . It is conceivable that protein overexpression can result not only from an increased number of genes secondary to gene amplification but also as a result of a concomitant increase in the numbers of chromosome 17. Varshney[21] demonstrated that a weak overexpression of HER-2 protein in a majority of cases seems to represent an artificial staining pattern and that chromosome 17 polysomy is a major factor in strong HER-2 protein overexpression in 3+ non amplified cases. Other recent studies have dealt with the role of polysomy in terms of HER-2 expression and its significance in the clinical assessment of HER-2 status.[9,22] Ma[23] showed that a net increase in *HER-2* gene copy number consecutive to polysomy, in the absence of specific gene amplification, might lead to a strong protein overexpression in a small subset of breast carcinomas.

In our specimens, referred to our laboratory as 2+ IHC, we found the chromosome 17 polysomy in 158/343 (46.1%) specimens: most of the polysomic cases, 87.3%, showed low polysomy. Wang in breast cancer specimens, including most IHC-positive and some IHC-negative cases, found aneusomy 17 present in more than 50% of breast carcinomas, mainly in the form of low polysomy 17.[24] Moreover, we observed *HER-2* amplification (ratio: ⩾ 2) in 29.6% compared with the 10% to 25% reported in the literature.[25,26] Recently, for cases with immunohistochemical scores of 2+, Lan C reported an amplification of 44.4%. This startling high amplification rate, in 2+ cases, was probably affected by various factors (standardization of all preanalytic variables including tissue handling, fixation) and by great variability (specimens from 24 pathology departments) in performing immunohistochemical evaluations as different antibodies use.[27] 242/343 (70.5%) of

our IHC weakly positive cases showed no evidence of gene amplification by FISH. Among the amplified cases, 50.5% showed low amplification (ratio between 2 and 4), as illustrated in Table 2. In addition, on the basis of ratio values between 0.9 and 1.30, we observed 49.6% of examined cases with no apparent HER-2 anomaly. These results are similar to those of Perez[26] who reported a 57% of cases without apparent HER-2 anomaly, among specimens with 2+ IHC.

An interesting point is the incidence of extra copies of HER-2 relative to *CEP 17* in 12.6% of specimens in this cohort. Some authors interpreted these levels of *HER-2* copy as low-level amplification,[28] while we interpreted these specimens as having *HER-2* duplication (ratio between 1.30 and 2). The presence of polysomy of 17q arm in the tumour nucleus will result in an apparent duplication of *HER-2* and consequently with immunohistochemical false positive and/or in false positive amplification when these specimens are evaluated with other assays, such as chromogenic *in situ* hybridization (CISH), that lacks chromosome 17 information, as reported by Isola.[29]

In the second part of our study, we evaluated the association between chromosome 17 monosomy, polysomy and *HER-2* gene copy number with the rate amplification, as shown in Table 3. Comparing the percentage of amplification between monosomic and polysomic specimens, we saw higher values in monosomic tumours ($P < 0.0001$). The FISH method, which defines amplification on the basis of the ratio of the average number of *HER-2* gene copies to that of *CEP 17* copies, might underestimate the presence of amplification in a proportion of cases with increased numbers of *HER-2* gene copies secondary to chromosome 17 polysomy. In addition our data showed an association between the copy number of chromosome 17 and the amplification rate, even if statistically not significant ($P = 0.07$), indicating a general association of increased chromosome 17 copy numbers with *HER-2* gene amplification. When we examined the amplification rate among polysomic cases, considering *HER-2* gene copy number as variable, a statistically significant difference ($P < 0.0001$) between cases with gene copy number between 3 and 10 and >10 was present. On the contrary, the same variable did not induce statistical difference ($P = 0.22$) among the monosomic cases. Moreover the results obtained by multivariate logistic regression analysis suggest that a *HER-2* signal >10 and polysomy absence may be important predictors for identifying tumours with *HER-2* gene amplification (ratio $\geqslant 2$), among specimens considered 2+ by IHC. Our results show that the effect of these two variables could be important and should be incorporated in the assessment of *HER-2/neu* status and only quantitative analysis performed by FISH should be recommended for an accurate determination in equivocal IHC.

Our investigations confirm and expand previously described findings, supporting the concept: a) that the majority of 2+ IHC cases express the HER-2 protein without gene amplification; b) that chromosome 17 polysomy may be responsible for the low levels of expression in absence of true gene amplification; and c) that *HER-2* gene copy number and polysomy absence could be considered as predictors of amplification in weakly positive cases by IHC. The clinically relevant question is whether or not patients with polysomy, IHC 2+ and FISH negative might also benefit from trastuzumab therapy. Hoffmann[30] showed that all responders to trastuzumab had a high level of protein expression, but did not have amplification as defined by the ratio and showed polysomy 17. Considering that response to target therapy has been associated with true amplification and/or high gene copy number in lung and colon cancer,[31,32] and that HER-2 gene-amplified breast cancer with monosomy of chromosome 17 has a poor response to trastuzumab-based treatment,[33] we considered additional clinical studies to be necessary in order to determine if this subset of tumours IHC 2+ and FISH negative, but related to polysomy and gene copy number, should nevertheless be considered for trastuzumab therapy and to correlate trastuzumab response and prognosis in these patients.

Conflict of interest statement

None declared.

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