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Clinical Cancer Research

Predictive Biomarkers and Personalized Medicine

HER2 Protein and Gene Variation between Primary and Metastatic Breast Cancer: Significance and Impact on Patient Care

Alessandra Fabi[1], Anna Di Benedetto[2], Giulio Metro[1], Letizia Perracchio[2], Cecilia Nisticò[1], Franco Di Filippo[3], Cristiana Ercolani[2], Gianluigi Ferretti[1], Elisa Melucci[2], Simonetta Buglioni[2], Isabella Sperduti[4], Paola Papaldo[1], Francesco Cognetti[1], and Marcella Mottolese[2]

Abstract

Purpose: To analyze HER2 status in primary breast cancer (PBC) compared with correspondent metachronous metastases and to investigate whether BC phenotype may be predictive of change in HER2 expression.

Experimental Design: HER2 was investigated by immunohistochemistry, silver *in situ* hybridization (SISH), and FISH, in a series of 137 tumors, building up a tissue microarray to concurrently analyze each single PBC and metastatic (MBC) on the same slide.

Results: HER2 status was discordant in 14 cases (10%): 12 negative in PBC and positive in metastases and two positive in PBC and negative in metastases ($P = 0.04$). These findings were confirmed by a PCR based test termed Multiplex Ligation-dependent Probe Amplification (MLPA). HER2 status changed in hormone receptor-positive BC more frequently than in negative ones ($P = 0.002$). In addition, we evaluated *HER2* gene and chromosome 17 copy number by SISH in the 123 cases with unchanged HER2 status during progression. We found consistent *HER2* gene copy number stability in the 100 nonamplified cases. Conversely, of the 23 amplified PBC, 13 (57%) demonstrated a significant increase in the *HER2* gene and chromosome 17 copy number in their paired metastases ($P = 0.01$), as defined by SISH ($k = 0.54$, $P < 0.0001$) and MLPA. Patients who changed HER2 status from negative to positive, presented significant longer time to progression when treated with trastuzumab compared to those who were untreated ($P = 0.04$).

Conclusions: When feasible, HER2 reassessment in metastatic lesions should be carefully taken into account, especially for metastases coming from primary hormone receptor-positive BC. *Clin Cancer Res; 17(7); 2055–64. ©2011 AACR.*

Introduction

HER2 is one of the most important therapeutic targets in breast cancer (BC) and its overexpression, in the majority of cases, is due to the amplification of the *HER2* oncogene. The introduction of trastuzumab (Herceptin, Genentech), the humanized monoclonal antibody (mAb) against HER2 into the metastatic setting and, more recently, also in the (neo)adjuvant setting (1, 2) has completely changed the natural history of HER2 positive BC patients both in terms of time to recurrence and survival. Despite the benefits shown by trastuzumab, a percentage of these patients have demonstrated clinical resistance. In metastatic BC (MBC), 44% to 64% of patients show upfront resistance to trastuzumab as a single-agent therapy (2, 3), whereas 12% to 22% of patients are primarily resistant to trastuzumab when given in combination with various cytotoxic drugs (2). Even though the efficacy of trastuzumab is first dependent on the accuracy in assessing HER2 status, various mechanisms are also involved in the resistance to the mAb. Nagata and colleagues (4) identified PTEN as a key modulator of trastuzumab sensitivity and Berns and colleagues demonstrated that the concomitant loss of PTEN and oncogenic mutation in *PIK3CA* can significantly contribute to resistance mechanisms (5). Although HER2 is usually evaluated in primary BC (PBC), knowledge of the HER2 status in metachronous metastatic dissemination could be of potential value for therapeutic decision making. It has recently been reported that HER2 status is mostly unchanged between primary tumors and their synchronous lymph node metastases (6), but may be discordant in 6% to 48% of metachronous metastases (7–14). This discordance may be due to the increasing

Authors' Affiliation: [1]Medical Oncology; [2]Department of Pathology; [3]Department of Surgery; and [4]Biostatistics, Regina Elena Cancer Institute, Rome, Italy

Note: A. Fabi and A. Di Benedetto contributed equally to this work.

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Corresponding Author: Marcella Mottolese, Department of Pathology, Regina Elena Cancer Institute, Via Elio Chianesi 53, 00144 Rome, Italy. Phone: 39-06-52666139; Fax: 39-06-52662920; E-mail: mottolese@ifo.it

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Translational Relevance

The introduction of trastuzumab, a monoclonal antibody against HER2, into metastatic and (neo)adjuvant settings has completely changed the natural history of HER2-positive breast cancer (BC) patients. Although HER2 is usually evaluated in primary tumor, knowledge of the HER2 status in metastases may be of potential value for therapeutic decision making. In this study, the extent of HER2 changes between primary and metastatic BC was investigated by immunohistochemistry and silver *in situ* hybridization. We show that HER2 status changes in 10% of metastases and that the increase of *HER2* gene copy number, together with chromosome 17 centromere gain, is a frequent event during progression. Our results were confirmed by Multiplex Ligation-dependent Probe Amplification, a quantitative PCR-based test. Patients who changed HER2 status from negative to positive presented longer time to progression when treated with trastuzumab. According to our findings, HER2 measurement in metastatic lesions seems advisable, especially in primary tumors with positive hormonal receptors.

level of genetic instability occurring throughout disease progression that can significantly influence the alterations of the *HER2* gene and chromosome 17 (Chr17) (7). In particular, chromosomal rearrangements occurring during the metastatization process may substantially determined the clinical management of MBC patients. In fact, some recent studies (15–18) demonstrated that true Chr17 polysomy is a rare event in BC and that an increase of centromere17 copy number is mostly related to gain or amplification of the centromeric region (15–19). These findings provided evidence that correcting the *HER2* gene copy number with centromere17 enumeration probe (CEP17) might induce misleading results in *HER2* amplification.

The primary aim of our study was to assess the extent of HER2 changes in a series of 137 PBC and their correspondent metachronous metastases paired on the same tissue microarray (TMA). In our series, we studied HER2 expression by immunohistochemistry (IHC) and gene amplification together with CEP17 polisomy by silver *in situ* hybridization (SISH) in each single case. To verify *HER2* gene variation and Chr17 alterations during progression, we tested selected paired cases by the Multiplex Ligation-dependent Probe Amplification (MLPA), a novel molecular assay which allows the concomitant analysis of a set of genes along Chr17. Furthermore, we investigated whether the PBC phenotype could be predictive of change in HER2 during neoplastic progression, evaluating the impact of trastuzumab treatment on the outcome of HER2 positive MBC patients who were previously diagnosed as a HER2 negative PBC.

Materials and Methods

Case selection and tissue microarray construction

One hundred and thirty-seven patients diagnosed with invasive BC between 1999 and 2007, underwent biopsies to pathologically confirm the presence of a metastasis during follow up, were selected from the surgical pathology files of the Regina Elena National Cancer Institute, Rome, Italy. In all the 137 PBC, which were all trastuzumab untreated, the HER2 status had already been assessed at the time of surgery. To concomitantly evaluate HER2 protein overexpression and/or gene amplification in PBC and metachronous MBC, a TMA was constructed from the original formalin fixed paraffin embedded (FFPE) blocks. To this end, 2 representative tumor areas were carefully selected on routine haematoxylin and eosin-stained sections. Two core cylinders (1 mm diameter) were taken from each PBC and MBC and deposited into 2 separate recipient paraffin blocks using a specific arraying device (Alphelys, Euroclone). In cases where informative results on TMA were absent due to missing tissue, no tumor tissue, or unsuccessful staining or hybridization, we reanalyzed the correspondent routine tissue section. In addition to tumor tissues, the recipient block also received normal breast tissue and cell line pellets as negative and positive controls. Three-micrometer sections of the resulting microarray block were made and used for IHC or gene amplification analysis after transferring them to SuperFrost Plus slides (Menzel-Gläser).

Immunohistochemistry

HER2 immunostaining on TMA was performed by using the polyclonal antibody A0485 (Dako) whereas estrogen (ER) and progesterone (PgR) receptors were analyzed by using the mAbs 6F11 and 1A6, respectively (Novocastra). Immunoreactions were revealed by a streptavidin-biotin enhanced immunoperoxidase technique (Super Sensitive MultiLink) in an automated autostainer. Diaminobenzidine was used as chromogenic substrate.

TMA immunostaining was evaluated by 2 expert pathologists (LP, MM). Discordant cases were independently reviewed by another pathologist who was blinded to the previous results.

Silver *in situ* hybridization and fluorescent *in situ* hybridization

To assess *HER2* gene and Chr17 polisomy on TMA we used a fully automated single color *in situ* hybridization assay based on the use of a validated silver deposition technology (SISH, Inform HER2 DNA Probe; Inform Chr17 probe, Ventana, Roche Diagnostic) to detect *HER2* gene and Chr17 status (20). The silver precipitation was visualized as a black dot in cell nuclei.

FISH (pharmDX, Dako) was performed using a *HER2* DNA probe directly labeled with Texas Red fluorochrome targeting the *HER2* amplicon (red signals) and a CEN-17 PNA probe directly labeled with fluorescein (FITC)

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targeting the centromeric region of the chromosome (green signals). The assay was performed according to the manufacturer's instructions.

The 100× oil immersion objective was used to score signals in all the neoplastic cells present in each duplicate TMA cores both for SISH and FISH.

SISH results were analyzed by using a light microscope (Nikon, Eclipse 55i) equipped with a software able to capture images (Eureka Interface System) and the FISH results were assessed with an epi-fluorescence microscope (Zeiss, Axioscope 40) equipped with Image Processing analysis software (Media Cybernetics) able to DAPI/specific Texas Red and FITC single filters.

Multiplex ligation-dependent probe amplification

A 50–100 μL of the genomic DNA solution, extracted from 2 whole 4 μm paraffin BC sections using the QIAamp Mini kit (Qiagen, Medicalproducts), was used in the MLPA analysis following the manufacturer's instructions. The kit (P004-B1 kit, MRC Holland) contains 3 probes for the *HER2* gene, and 21 probes for other genes on Chr17 and 6 control probes located on other chromosomes. All tests were performed in duplicate in an ABI 9700 PCR machine. PCR products were analyzed on an ABI3130 capillary sequencer (Applied Biosystems). Gene copy numbers were analyzed using Genemapper 4.0 and Coffalyser (version 7.0) software. For genes with more than 1 probe present in the kit, the mean of all the probe peaks of the gene was calculated in duplicate. A mean value below 1.5 was defined as normal, between 1.5 and 2.0 as low level amplification (LA) and a value >2.0 as high level amplification (HA), according to the definitions in the Coffalyser software (21).

Scoring criteria

Immunohistochemistry. HER2 IHC positivity was determined according to ASCO-CAP guidelines (22) and was scored as follows: 0 and 1+ negative, 2+ equivocal, and 3+ positive. ER and PgR were considered positive when >10% of the neoplastic cells showed distinct nuclear immunoreactivity.

SISH and FISH. Following the manufacturer's guidelines, scoring of SISH results was carried out assuming that a single signal was counted as 1 gene copy, a small cluster as 8 gene copies, a large cluster as 16 gene copies. According to the ASCO-CAP guidelines (22), PBC and MBC were defined as "nonamplified" (NA) by SISH when a *HER2* gene copy number <4 was observed and by FISH when a *HER2*/CEP17 ratio <1.8 was detected. Cases were defined as "amplified" (A) when SISH displayed a gene copy number >6 or when the FISH ratio was >2.2. Polysomy 17–intended as an increased CEP17 copy number (CEP17CN)—is considered to be present in BC when a mean number of ≥3 signals is shown.

For the purpose of our study, we defined "low amplification" by SISH when BC presented >6 signals/nucleus, "moderate amplification" when BC presented >10 signals/nucleus and "high amplification" when BC presented >20 signals/nucleus. Furthermore, "low polysomy" by SISH was a CEP17CN ≥3 and "high polysomy" a CEP17CN >4. Lymphocytes and normal breast glandular epithelial cells served as an internal control.

Statistical analysis

Descriptive statistics were used to describe the patient's characteristics. The proportions are presented as numbers and percentages. For the statistical analysis, HER2 negative cases are defined those with an IHC score of 0, 1+, and 2+ lacking gene amplification and positive cases those with IHC score 3+ and 2+ displaying gene amplification. The Mc Nemar paired test was performed to evaluate statistical significant differences in HER2 status between PBC and MBC. The rate of concordance between *HER2* and CEP17 copy gains in PBC and MBC was analyzed with the k test. Significance was assessed at a level of 5%. The statistical software package used for this analysis was SPSS for Windows version 17.0 (SPSS Inc).

Results

Patient characteristics

One hundred and fourteen of the 137 PBC included in the analysis were infiltrating ductal carcinomas, 14 invasive lobular carcinomas and 9 other histotypes. As summarized in Table 1, 11 (8%) tumors were graded, using the Bloom and Richardson scoring system, as well differentiated (G1), 66 (48.2%) and 60 (43.8%) as moderately (G2) and poorly differentiated (G3) carcinomas, respectively. Furthermore, 60 (43.8%) patients were node negative and 77 (56.2%) node positive. ER and PgR were positive in 82 (59.8%) BC and HER2 was positive in 25 (18.3%) cases.

Staging was performed by following the Unione Internationale Contre le Cancer tumor-node-metastasis (TNM) system criteria (23).

In our series, 80 (58%) patients were administered (neo) adjuvant chemotherapy. In particular, 46 patients were given an anthracycline-based therapy, 8 and 5 taxane or anthracycline plus taxane regimens respectively, 30 other chemotherapies and 40 hormone therapy alone. Only 8 women did not undergo any treatment. None of the HER2 positive patients received anti HER2 therapy alone or in combination as (neo)adjuvant treatment. Twenty-six (19%) patients developed visceral metastases (11 in the liver, 6 in the pleura, 8 in the lung and 1 in the ovary) and 111 (81%) nonvisceral metastases (25 in the lymph nodes, 26 in the soft tissue and 60 experienced a local recurrence). The median interval between the PBC and the first recurrence of the disease (disease free progression) was 45.4 months (range 1–94 months).

The study was reviewed and approved by the Local Ethical Committee at the Regina Elena National Cancer Institute, and a written informed consent was obtained from all patients.

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Table 1. Clinicopathological characteristics of 137 primary breast carcinomas

Total number of patients	137
Age, median years (range)	56 (26–92)
Grading	
1	11 (8%)
2	66 (48.2%)
3	60 (43.8%)
Node	
Negative	60 (43.8%)
Positive	77 (56.2%)
HR status	
ER and/or PgR positive	82 (59.8%)
ER negative PgR negative	55 (40.2%)
HER2 status	
0/1+/2+NA	112 (81.7%)
2+A/3+	25 (18.3%)
Neoadjuvant/adjuvant therapy	
Anthracycline-based	46 (33.6%)
Taxane-based	8 (5.8%)
Anthracycline plus taxane-based	5 (3.7%)
Other	30 (21.9%)
Hormone	40 (29.2%)
None	8 (5.8%)
First metastatic site	
Visceral disease[a]	26 (19%)
Nonvisceral disease[b]	111 (81%)
Median DFS (months/range)	45.4 (1–94)

Abbreviations: HR, hormonal receptor; ER, estrogen receptor; PgR, progesterone receptor; NA, nonamplified; A, amplified; DFS, disease free survival.
[a]Visceral: liver, pleura, lung, and ovary.
[b]Nonvisceral: lymph node, soft tissue, and local recurrence.

HER2 status in primary and paired metastatic breast carcinomas by SISH and MLPA

Table A in Figure 1 summarizes IHC and SISH results obtained in the 137 PBC and MBC paired on the same TMA. HER2 immunoreactivity was scored as follows: 97 cases (71%) as 0/1+, 17 (12%) as 2+, and 23 (17%) as 3+. The 97 0/1+ score PBC were also nonamplified by SISH. Of the 17 scoring 2+ PBC, only 2 (12%) were amplified. All the 23 cases with a score of 3+ resulted amplified.

When we concurrently analyze each single primary tumor in comparison with the metachronous metastases, we found that of the 97 HER2 negative PBC (score 0/1+ by IHC, NA by SISH), 74 (76%) maintained a concordant score in the matched metastases, whereas 23 (16+7, 24%) displayed an increased IHC score (Fig. 1, Table B: panels a and b). Among the 17 PBC scoring 2+ by IHC, in 4 cases (23%) there was a decrease of the IHC score (0/1+) during progression, in 10 cases (59%) the same score was maintained and in 3 cases (18%) an increase of the IHC score (3+), associated with gene amplification, was registered (Fig. 1, Table B: panels c and d). Two (8.6%) of the 23 PBC scoring 3+ by IHC and amplified by SISH, showed a decrease in the score (0/1+) and a change in the *HER2* gene CN in the correspondent metastasis.

Overall, a significant change of HER2 immunoreactivity and gene amplification in metachronous metastases was observed as compared with the primary tumors (Mc Nemar test, IHC: $P < 0.0001$, SISH: $P = 0.01$). Among the 14 patients who changed HER2 status, 7 (50%) and 4 (28.5%) had received anthracycline or no anthracycline-based therapy in (neo)adjuvant setting, respectively.

We summarized our findings in a flow chart by taking into account both the IHC and SISH findings. Of the 137 PBC, 123 (90%) maintained a concordant HER2 status during disease progression whereas 14 cases (10%) changed HER2 status (12 from negative to positive and 2 from positive to negative, Fig. 2A, gray boxes; Fig. 2B, panels a and b). These findings were further confirmed by MLPA assay (Supplementary Table S1, panel a) in 7 of 8 cases (88%; cases 1–6, 8, 13 illustrated in Table 2) that had previously been evaluated by SISH. In Figure 2C a descriptive case clearly highlighted that *HER2* gene, as determined by MLPA, was nonamplified in the PBC (ratio 1.36, panel a) and highly amplified in the paired metastasis (ratio 8.02, panel b).

Comparison between IHC, SISH, and FISH in the 14 primary breast cancer changing HER2 status during breast cancer progression

We correlated HER2 protein overexpression with gene amplification detected by SISH and FISH in 14 cases all presenting a variation in HER2 status between primary and MBC. As summarized in Table 2, the agreement between IHC and *HER2* gene amplification, evaluated either by SISH or FISH, was 100% in all the 12 cases displaying an increase and in the 2 cases displaying a decrease of HER2 overexpression during disease progression. In regards to CEP17CN, we found that 2 nonamplified PBC (cases no. 6 and no. 12) were disomic by SISH and low polysomic by FISH. The latter 2 cases were amplified and polysomic in the autologous MBC with both SISH and FISH. Furthermore, only 1 case (case no. 8) was found disomic by SISH and polysomic by FISH in MBC.

HER2 change according to biological and clinical features

As summarized in Table 3, in the group of 82 HR positive PBC, 74 (66%) were HER2 negative, and 8 (7%) HER2 positive. Among the 74 HR positive/HER2 negative PBC, 10 (13.5%) changed HER2 status from negative to positive, whereas none of the 8 (100%) HR positive/HER2 positive patients showed HER2 change ($P = 0.002$). No statistically significant differences were seen in the group of patients with negative HR.

HER2 variation was not significantly related to tumor size ($P = 0.11$), node status ($P = 0.48$), grading ($P = 0.41$), site of metastasis ($P = 0.41$), previous anthracycline- and/or taxane-based adjuvant therapy (0.12) and disease free progression (0.14, data not shown).

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A

No of total cases (%)	Primary Breast Cancer		Metastatic Breast Cancer	
	IHC score*	SISH**	IHC score*	SISH**
97 (71%)	0/1+	97: NA	74: 0/1+	74: NA
			16: 2+	12: NA
				4: A
			7: 3+	1: NA
				6: A
17 (12%)	2+	15: NA	4: 0/1+	4: NA
			9: 2+	9: NA
			2: 3+	2: A
		2: A	1: 2+	1: A
			1: 3+	1: A
23 (17%)	3+	23: A	1: 0/1+	1: NA
			1: 2+	1: NA
			21: 3+	21: A

B

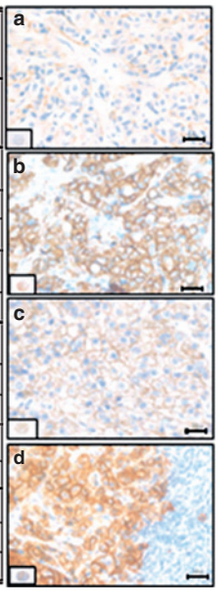

Figure 1. HER2 status in 137 primary and paired metastatic breast cancer Table A: comparison of HER2 status in 137 primary and paired metastatic breast cancer as determined by immunohistochemistry and SISH. Table B: Two representative examples of HER2 immunohistochemical variation between primary and metastatic breast cancer: (a) a primary breast cancer with HER2 score 0 which becomes (b) score 3+ in the metachronous liver metastasis; (c) a primary breast cancer with HER2 score 2+ which becomes (d) score 3+ in the metachronous supraclavear metastatic lymphnode. Scale bar = 30 μm. IHC, immunohistochemistry; NA, nonamplified; A, amplified; *, $P < 0.0001$; **, $P = 0.01$.

HER2 gene and chromosome 17 copy gains in primary and metastatic breast cancer with unchanged HER2 status

We analyzed *HER2* gene CN and CEP17CN by SISH in the 123 PBC that maintained a concordant HER2 status in the paired metastases.

The 100 nonamplified PBC (≤6 signals/nucleus) maintained the same *HER2* gene CN in their paired metastases (Table A in Fig. 3), whereas 13 of 23 (57%) cases, amplified both in PBC and in MBC, showed an increased *HER2* gene CN in the paired metastases (Fig. 3C, panels a and b). Concomitantly (Table B in Fig. 3), an increased CEP17CN was also detected in MBC. In detail, of 108 disomic PBC, 8 became polysomic in the autologous metastases (5 low polysomic and 3 high polysomic; Fig. 3C, panels c and d). Moreover, of 7 low polysomic PBC, 6 (86%) displayed high polysomy in their paired metastases. The *k* test indicated a significant concordance between *HER2* gene CN and CEP17CN gain during BC progression ($k = 0.54$, $P < 0.0001$).

These results were subsequently supported by MLPA assay (Supplementary Table S1, panel b) in representative 4 cases that displayed concordant HER2 status in PBC and MBC. Figure 3D, panels a and b shows an representative case: *HER2* ratio by MLPA in PBC was 3.72 and in MBC 10.02. Moreover, *WSB1* and *NOS2A* ratio in PBC was 1.45 and 1.25 and 3.13 and 2.39 in MBC, respectively.

Clinical outcome of HER2 positive metastatic breast cancer patients

In our series, of 18 patients who underwent trastuzumab-based therapy at the appearance of the first progression, 12 were HER2 positive both in PBC and in MBC (HER2+/+) and 6 changed HER2 status (HER2−/+) in the metastasis. These 18 patients had a median time to progression (TTP) of 10.3 months whereas the TTP of the remaining 17 HER2 positive BC patients (11 HER2+/+, 6 HER2−/+) not administered the mAb therapy, was 5.2 months ($P = 0.04$, Supplementary Fig. S1). In detail, in the 12 trastuzumab treated patients with HER2+/+, the TTP was 11 months and in the 6 patients with HER2−/+ 8 months. Furthermore, in the 11 trastuzumab untreated patients with HER2+/+ the TTP was 2 months and in the 6 with HER2−/+ was 5 months.

Discussion

HER2 overexpression/amplification in BC is of particular clinical relevance when selecting patients eligible for anti HER2 based therapy. In a metastatic setting, the evaluation of HER2 status is mostly performed on the primary tumor based on the notion that the HER2 status does not undergo significant change during disease progression (24, 25).

In the last few years, several studies delved deeper into the matter by reporting a significant discordance between PBC and paired asynchronous metastases ranging between 6% and 48% (7–14). Due to this wide variability of results, this study analyzed HER2 status in 137 PBC and autologous metachronous metastases from trastuzumab-näive patients using IHC and SISH on paired TMA. In addition, we evaluated the amplification of *HER2* gene and of genes (*WSB1*/*NOS2A*) located very close to the centromeric region by the means of MLPA in a selected group of PBC and MBC. To our knowledge, this is the first study

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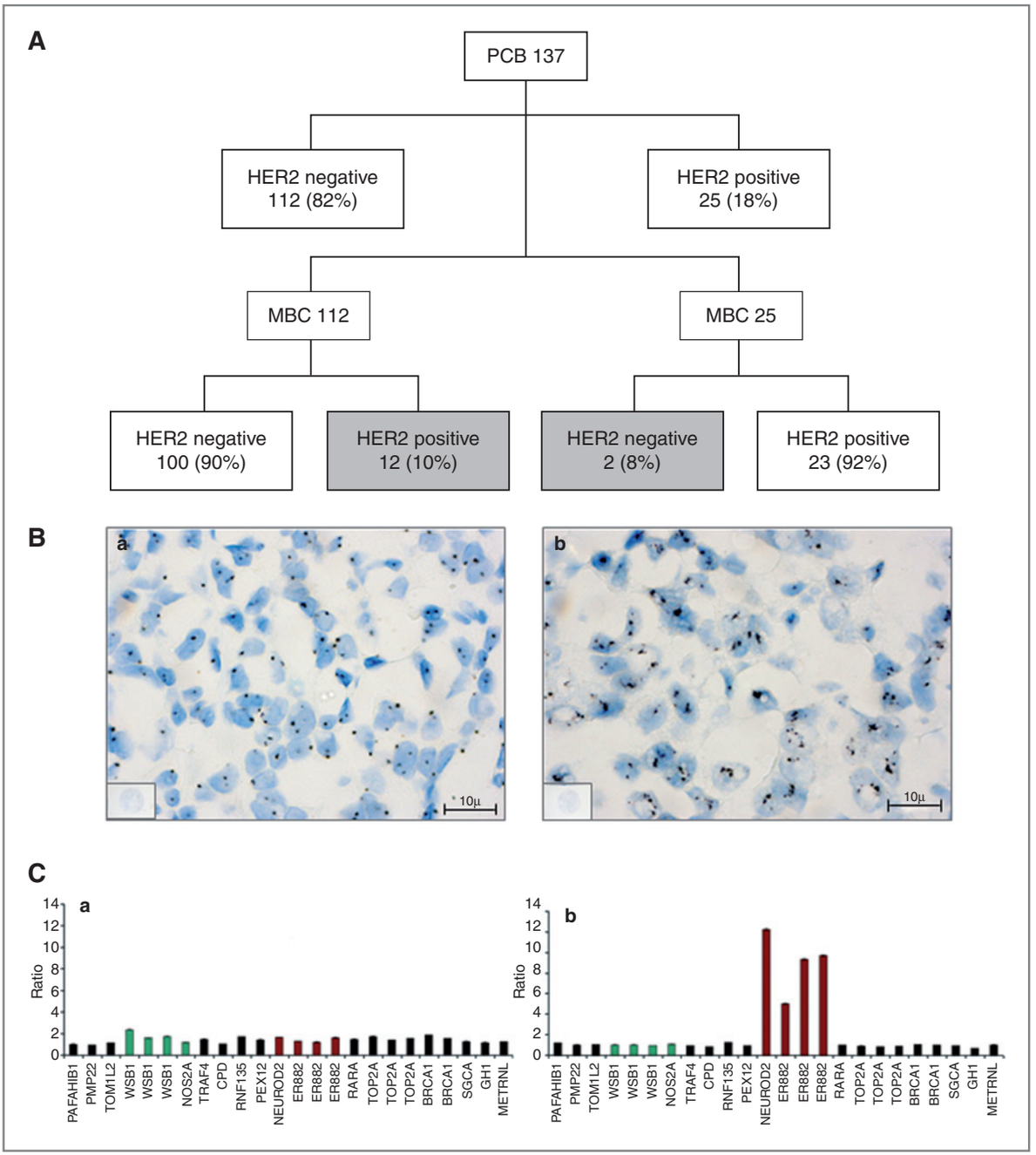

Figure 2. HER2 variation in primary and metastatic breast cancer: A, The flow chart summarizes HER2 change during disease progression in the entire series of 137 primary and metastatic breast cancers. One hundred and twenty-three cases (100 HER2 negative and 23 HER2 positive) maintained the same HER2 status (90%; white boxes) whereas 14 cases (10%) changed HER2 status (gray boxes), $P = 0.04$. B, SISH images demonstrate: (a) no *HER2* amplification in a primary breast cancer (gene copy numbers ≤6) and (b) *HER2* moderate gene amplification in the metachronous liver metastasis (gene copy numbers >10); Scale bar = 10 μm. C, the BC illustrated in panel b has been also analyzed by MLPA showing: (a) a normal HER2 status in PBC and (b) an amplified HER2 status in the paired metastasis (*HER2* ratio 1.36 vs. 8.02). PBC, primary breast cancer; MBC, metastatic breast cancer; HER2 negative, score 0, 1+, 2+ nonamplified; HER2 positive, score 2+ amplified, 3+.

where HER2 variation was investigated, case-by-case, in a large series of trastuzumab untreated 137 patients. We aimed to concurrently analyze PBC and metastatic lesions, paired on the same TMA, by IHC and SISH (26). Because one the major limit of TMA is the reduced amount of tissue analyzed which may be not representative of the phenotypic and genotypic patterns of the tumor, we supported the morphological-based assays using MLPA, a molecular

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Table 2. Comparison between IHC, SISH, and FISH in the 14 primary breast carcinomas modulated in the metastatic lesions

Primary breast cancer						Metastatic site	Metastatic breast cancer				
	IHC	*HER2* SISH		*HER2* FISH			IHC	*HER2* SISH		*HER2* FISH	
Histotype	Score	Average gene CN	Average CEP17CN	FISH ratio	Average CEP17CN		Score	Average gene CN	Average CEP17CN	FISH ratio	Average CEP17CN
1 IDC	0	4.20	3.70	1.30	3.30	NV	3+	>20	3.40	6.70	3.20
2 IDC	2+	2.30	2.10	1.00	2.00	V	3+	>20	2.00	5.00	2.00
3 IDC	1+	3.40	3.40	1.00	3.40	NV	3+	>20	5.20	5.20	5.40
4 IDC	1+	2.10	2.00	1.00	2.00	NV	2+	6.5	3.20	2,7	3.20
5 IDC	0	4.20	3.40	1.00	3.20	NV	2+	6.3	3.40	2,3	3.30
6 IDC	0	2.10	2.00	0.70	3.30	NV	2+	>10	5.00	2.50	5.00
7 IDC	0	3.30	2.00	1.50	2.00	NV	3+	6.7	3.40	2.70	3.10
8 IDC	0	2.00	2.00	1.00	2.00	NV	3+	>20	2.00	3.30	3.40
9 IDC	1+	3.50	2.00	1.30	2.00	NV	3+	>10	5.20	2.50	5.20
10 IDC	1+	3.30	2.00	1.50	2.00	NV	3+	6.2	2.00	3.30	2.00
11 IDC	2+	2.00	2.00	1.00	2.00	NV	3+	6.1	3.40	2.30	3.30
12 IDC	0	2.40	2.00	1.00	3.00	NV	2+	6.4	3.30	2.70	3.40
13 IDC	3+	>10	2.00	5.20	2.00	V	2+	3.4	2.00	1.30	2.00
14 IDC	3+	6.40	2.00	3.20	2.00	V	1+	2.00	2.00	1.00	2.00

Abbreviations: IHC, immunohistochemistry; SISH, silver *in situ* hybridization; CN, copy number; CEP17CN, centromere enumeration probe 17 copy number; V, visceral; NV, not visceral; IDC, infiltrating ductal carcinoma.

technique (16) able to determine relative gene copy numbers in a quantitative way. Our results showed that HER2 status significantly changed in 10% of cases. In particular, 11% of the HER2 negative PBC expressed HER2 in their metastatic sites whereas 8% of HER2 positive PBC became negative in their paired asynchronous metastases. In addition, in the group of the 14 cases undergoing HER2 variation, the SISH results were further confirmed by FISH and, in a subset of available cases, by MLPA. Interestingly, we found that HER2 status changed more frequently in HR positive PBC patients than in the negative counterpart. These findings might reflect acquired resistance to tamoxifen treatment in the adjuvant setting of HR positive BC patients. It has been recently reported that acquired endocrine resistance in positive ER/negative HER2 BC may be associated with an adaptive increase in HER2, although exactly how aberrant HER2 signaling affects the ERα pathway is poorly understood (27).

The discordance between PBC and MBC reported in our series resembles other retrospective and prospective studies

Table 3. Changes in HER2 status between primary breast and paired metastatic breast carcinomas according to hormonal receptor status

HR status		HER2 status				
PBC	No. of patients (%)	PBC	No. of patients (%)	MBC		*P**
				HER2– (%)	HER2+ (%)	
ER and/or PgR+	82 (60)	HER2–	74 (66)	64 (86)	10 (14)	0.002
		HER2+	8 (7)	0	8 (100)	
ER/PgR–	55 (40)	HER2–	38 (69)	36 (95)	2 (5)	0.68
		HER2+	17 (31)	2 (12)	15 (88)	

Abbreviations: HR, hormonal receptor; PBC, primary breast cancer; MBC, metastatic breast cancer; ER, estrogen receptor; PgR, progesterone receptor; HER2−, score 0, 1+, 2+nonamplified; HER2+, score 2+amplified, 3+.
*Mc Nemar paired test.

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A

Primary Breast cancer		Metastatic Breast Cancer			
		HER2 Gene CN			
HER2 Gene CN	No. of cases	≤6	>6	>10	>20
≤6	100	100	0	0	0
>6	11	0	3	4	4
>10	9	0	0	4	5
>20	3	0	0	0	3
Total of cases	123	100	3	8	12

B

Primary Breast cancer		Metastatic Breast Cancer		
		CEP17CN		
CEP17CN	No. of cases	2	≥3	>4
2	108	100	5	3
≥3	7	0	1	6
>4	8	0	0	8
Total of cases	123	100	6	17

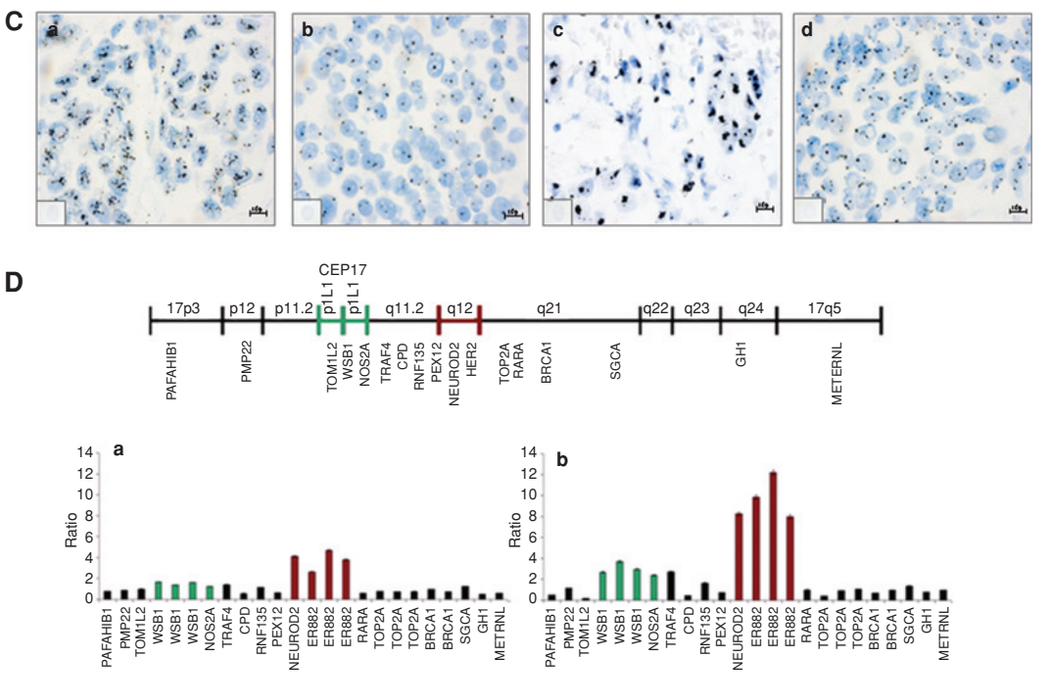

Figure 3. *HER2* gene and chromosome 17 gain in the 123 primary and metastatic breast carcinomas with unchanged HER2 status. Table A: The 100 nonamplified PBC maintained the same *HER2* gene CN in their paired metastases whereas 13 of 23 (57%) cases, amplified both in primary and in metastatic BC, showed an increased *HER2* gene CN in MBC as compared to PBC. Table B: An increased CEP17CN was observed in MBC as compared to their paired PBC. The *k*-test indicated good concordance between *HER2* gene CN and CEP17CN gain during BC progression ($k = 0.54$, $P < 0.0001$). C, An illustrative SISH case showing: (a) a *HER2* low amplification (gene CN >6) associated (b) to Chr17 disomy (CEP17 = 2) in the primary breast cancer and (c) a *HER2* high amplification (gene CN >20) associated (d) to high polisomy (>4 CEP17 copy number) in the autologous supraclavear metastatic lymphnode. Scale bar = 10 μm. D, Schematic diagram of chromosome 17. The positions of *HER2*/*NEUROD2* (17q12), *WSB1* and *NOS2A* (17q11) are shown as red and green lines, respectively. The illustrative SISH case (C) was also analyzed by MLPA showing**:** (a) a low level of *HER2* and *WSB1* amplification in PBC as compared (b) to paired metastasis (*HER2* ratio 3.72 vs. 10.2; *WSB1* ratio 1.45 vs. 3.13).

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(9, 12, 14, 28). Several authors demonstrated primary intratumoral heterogeneity for both HER2 overexpression and gene amplification (10, 11, 29). This heterogeneity may arise via random genetic alterations with clonal progression, likely resulting in genetic subclones of cells within the PBC. Consequently, one may hypothesize that metastatic cells enhanced HER2 alterations in MBC as compared with the PBC.

In the study by Lower (10), which included 382 BC and is the largest report up to now, 23.6% of cases, evaluated by IHC, changed from HER2 positive to HER2 negative and only 9.6% from negative to positive. The authors suggested that the decrease in immunoreactivity may be a possible misclassification of the IHC 2+ score patients not confirmed by FISH. In addition, Lorincz and colleagues (30) found that half of PBC with *HER2* amplification lost this genotype in the correspondent bone metastases. Furthermore, even though *HER2* amplification was retained in the MBC, the copy number decreased compared to the primary tumor. These findings may be explained by technical limitations of the FISH analysis known not to be consistently successful on decalcified bone metastasis due to DNA breakdown (11). For this reason, we excluded bone metastases from the IHC and SISH analyses in our study. In addition, some of the causes of the discordant results in the above cited studies may be due to heterogeneity in case selection, tissue sampling and processing procedures. Furthermore, unlike our study, the other reports rarely provided case-by-case results. Some authors reported that the specimens were handled as part of routine clinical care and were not restained, but rather reread by at least 2 pathologists according to the initial procedures (either IHC or FISH). Retesting was done in cases considered unsuitable for re-evaluation (9, 10, 30). In other studies HER2 status was assayed on histological samples in PBC and on cytological samples in MBC (13, 28, 29). Zidan and colleagues and Gancberg and colleagues (8, 14) retested their series of paired PBC and MBC through the use of IHC whereas FISH was performed exclusively on cases presenting a 2+/3+ score by IHC.

Only Regitning and colleagues (11) have analyzed, through the use of IHC and FISH, a case-by-case TMA, which included PBC and paired MBC similar to our study. The authors demonstrated that, even in a very small group of 31 cases, HER2 IHC expression changed at a high percentage rate (48.4%) in distant metastases. Nevertheless, HER2 immunoreactivity is attributable to gene amplification in only 14.3% of the MBC tested. In our series HER2 protein expression increased in 26 (23%) MBC as compared with 114 HER2 negative PBC whereas gene amplification occurred only in 11% of metastases. All the authors agreed on the concept that the HER2 status may be different in the metastasis in comparison with the primary tumor and stressed the need to verify these results with a larger number of patients in order to apply these findings to clinical practice.

Unlike other authors, we took our analyses one step further by evaluating *HER2* gene and Chr17 status by SISH in the 123 cases showing a concordant HER2 status (100 NA and 23 A). We demonstrated that MBC derived from the 100 PBC with a gene CN ranging between 2 and 6 is consistent with *HER2* gene stability whereas MBC derived from the 23 amplified PBC (gene CN >6) had a significant increase in *HER2* gene CN as well in CEP17CN during metastatization. These findings were further supported and quantitatively confirmed by MLPA analysis in a small group of unchanged 4 primary and metastatic paired cases. An increase in CEP17CN, detected by fluorescent or chromogenic *in situ* hybridization, raises the question whether it could reflect true "polysomy" 17 or rather is related to unbalanced chromosomal rearrangements. Recent studies analyzed HER2 status by comparative genomic hybridization (CGH) or MLPA methods, both in BC diagnosed as polysomic by routine FISH (15, 17) and in randomly selected BC (16, 18). These authors reported that true Chr17 polysomy is a very rare event in BC and that CEP17CN >3, detected by FISH or CISH assay, is most often related to gain or amplification of the centromeric region. So far, nonamplified polysomic BC, presenting a *HER2*:CEP17 ratio <2 by *in situ* hybridization, are not eligible for trastuzumab therapy. Conversely, based on CGH or MLPA data, we may have misinterpreted *HER2* amplification. As previously discussed, in the group of our series of BC patients with unchanged HER2 status we found a 11.4% increase in CEP17CN during metastatization. Some of these cases were considered amplified, but about 5% displayed a high CEP17 polisomy and were thus considered nonamplified. Because abnormal CEP17CN might arise from high level gains or amplification of CEP17, correcting CEP17 probes may provide misleading HER2 status assessment lowering the number of cases in which a change in HER2 status may occur during metastatization.

Focusing on the outcome of HER2 positive MBC patients both in changing (negative/positive) and nonchanging tumors (positive/positive), we observed significant longer TTP (10 months vs. 4 months) in patients treated with trastuzumab compared with those who were not treated with the mAb. Despite the limited number of cases, these data not only underline the importance of testing HER2 status in metastases, possibly using alternative molecular techniques, but also open up the possibility of significantly improving the prognosis of these subsets of patients. In the era of targeted therapy, an accurate definition of the metastatic disease in patients who can experience great benefit by trastuzumab or any novel anti HER2 molecule, represents a pivotal commitment in the clinical management of BC patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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