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Whether to determine HER2 status for breast cancer in the primary tumour or in the metastasis

M.P. Santiago[1], Á. Vázquez-Boquete[2], B. Fernández[3], C. Masa[3], J.R. Antúnez[2], M. Fraga[2], J. Forteza[2] and T. García-Caballero[3]

[1]Department of Pathology, University Hospital of A Coruña, Spain, [2]Department of Pathology and Forensic Sciences, School of Medicine-University Clinical Hospital, University of Santiago de Compostela, Santiago de Compostela, Spain and [3]Department of Morphological Sciences, School of Medicine-University Clinical Hospital, University of Santiago de Compostela, Santiago de Compostela, Spain

Summary. Trastuzumab has substantially changed the prognosis of breast carcinomas. As HER2 over-expression/amplification is a prerequisite for treatment with trastuzumab, an accurate assessment of HER-2 status is the first step for successful treatment. In metastatic breast cancer, we routinely assess HER2 expression in the primary tumour, assuming that HER2 status remains stable through cancer progression. However, it is frequent to find reports that describe discordance between HER2 expression in primary and metastatic tumours. The aim of this paper was to verify whether HER2 status of breast carcinomas is maintained in the corresponding axillary metastasis. Immuno-histochemistry was performed on 52 breast carcinomas and their matched axillary metastasis. HercepTest results were concordant in 46 out of 52 cases (88.5%). FISH proved that the differences observed were clinically relevant in only one of the 52 cases studied (98% concordance). We concluded that HER2 status was stable during axillary metastatic progression. Evaluation of gene HER2 status in axillary metastasis rather than in the primary can be useful in certain situations, e.g., small invasive component intimately mixed with *in situ* component and difficult to recognize in dark field, no tumor after biopsy, or axillary relapse (in this case we can find occasional *de novo* amplifications susceptible to trastuzumab treatment).

Key words: HER-2, Immunohistochemistry, fluorescence *in situ* hybridization, Primary breast carcinoma, Axillary metastasis

Offprint requests to: Prof. Tomás García-Caballero, Department of Morphological Sciences, School of Medicine, c/San Francisco s/n, 15782 Santiago de Compostela, Spain. e-mail: tomas.garcia-caballero@usc.es

Introduction

One of the most important advances in cancer research in recent years has been the development of targeted tumour-specific drugs aimed at blocking key signalling pathways in tumour growth and dissemination. Two types of drugs have been developed: monoclonal antibodies targeted at the extracellular domain of growth factor receptors and small-molecule tyrosine kinase inhibitors acting at the intracellular level. The first recombinant humanized monoclonal antibody developed for cancer therapy was trastuzumab (Herceptin®, Genentech, South San Francisco, CA) approved for clinical use in 1998 by FDA and targeted against the extracellular domain of the HER2 receptor. HER2 is overexpressed in approximately 20% of breast invasive carcinomas (Pauletti et al., 2000; Ridolfi et al., 2000; García-Caballero et al., 2006), although a lower proportion of HER2 positive breast cancers was recently reported (15% on a total of 17,270 cases) (Walker et al., 2008). As HER2 overexpression/amplification is a prerequisite for trastuzumab treatment, an accurate assessment of the HER2 status is the first step for successful treatment. HER2 determination is routinely assessed in the primary tumour, even in cases with metastatic dissemination, but it is frequent to find reports that describe discordances between HER2 expression in breast primary and metastatic tumours (Lorincz et al., 2006; Rossi et al., 2006). Conflicting results in this respect were very recently reported (Tapia et al., 2007; Lower et al., 2008). It is worth questioning whether the HER2 status is maintained in the metastatic lesions, which ultimately determine the course of the disease. Moreover, less than half of the patients with high HER2 expression respond to trastuzumab therapy (Slamon et al., 2001) suggesting that discrepancies may exist between primary and metastatic tumours with respect to HER2 expression.

The purpose of the current paper was to verify, by immunohistochemistry and FISH, whether HER2 status of breast primary carcinomas is maintained in the corresponding lymph node metastasis.

Materials and methods

Tumour specimens

We selected 52 consecutive cases of breast invasive carcinoma with axillary lymph node metastasis from the files of the Pathology Department of the Xeral-Cíes Hospital in Vigo (Spain). Samples had been fixed in 10% buffered formalin for up to 24 h and embedded in paraffin routinely. Sections 4 μm thick were mounted on ChemMate capillary gap microscope slides (Dako, Glostrup, Denmark) and heated in an oven at 60°C for 1 h.

Immunohistochemistry

The immunohistochemical technique was automatically performed using an Autostainer Plus (Dako). Whole tissue sections of 52 breast carcinomas and matched lymph node metastasis were immunostained in the same staining run. The HercepTest kit (Dako) was employed following the recommendations provided by the manufacturer. Briefly, after epitope retrieval in 10 mM sodium citrate buffer (pH 6.0) using water bath for 40 min at 95-99°C, the slides were allowed to cool for 20 min at room temperature. The HER-2 immunostaining protocol includes incubation in: (1) primary A485 polyclonal antibody to HER-2 for 25 min; (2) peroxidase-blocking reagent 3x2.5 min; (3) visualization reagent (dextran polymer conjugated with horseradish peroxidase and affinity-isolated goat anti-rabbit immunoglobulins) for 30 min; (4) 3,3'-diaminobenzidine tetrahydrochloride chromogen solution 3x5 min; and (5) hematoxylin counterstain for 1 min. As positive control we used a slide provided with the kit that contains three pelleted, formalin-fixed, paraffin embedded human breast cancer cell lines with scores of 0 (MDA-231 cell line), 1+ (MDA-175) and 3+ (SK-BR-3). The control slide was included in each staining run. Evaluation was performed following the algorithms of the manufacturers and the American Society of Clinical Oncology College of American Pathologists (ASCO/CAP) guidelines (9). Scores of 0 and 1+ were considered as negative, a score of 2+ was regarded as equivocal, and a score of 3+ was considered as positive (overexpression). The HercepTest slides were independently scored by two of the authors (TG-C and AV-B). In cases of discrepancy (3.8%), a consensus was reached by joint evaluation. Sections were observed and photographed using an Eclipse E400 microscope (Nikon, Tokyo, Japan).

Fluorescent in situ hybridization (FISH)

FISH was performed in cases that showed HercepTest discordances. The HER2 FISH pharmDX kit (Dako) was employed. This test allows a simultaneous determination of HER-2 gene copies and chromosome 17 copies. The kit contains pre-mixed probes: (1) HER2 DNA probes labelled with FITC (with a total coverage of 218 kb including the HER2 gene) and (2) CEN-17 PNA probes (specific for the centromeric region of chromosome 17).

The technique was performed according to the manufacturer's recommendations. Briefly, the sections were pretreated in MES (2-[N-morpholino]ethane-sulphonic acid) buffer for 10 min at 95-99°C; immersed in wash buffer (2x2 min); and incubated in pepsin for 10 minutes at RT. Sections were dehydrated through a graded ethanol series. Ten μL of HER2/CEN17 probe mix were applied and a coverslip was placed over the probe mix. Denaturation of specimen DNA and probes (5 min at 84°C) and hybridization (overnight at 45°C) were performed in a Hybridizer (Dako). Post-hybridization washes were done in stringent wash for 10 min at 65°C. DAPI nuclear stain and a glass coverslip were applied after dehydration. Sections were observed and photographed using an Eclipse E400 Nikon fluorescence microscope equipped with DAPI (nuclei) and Texas red (HER-2) / FITC (CEP 17) dual bandpass filter sets. Following the criteria established by the American Society of Clinical Oncology / College of American Pathologists (ASCO/CAP) we considered polysomy 17 when cells had three or more copies of CEP17 and the criterion for gene amplification was a FISH ratio (HER2 gene signals to chromosome 17 signals) of more than 2.2 (Wolff et al., 2007).

Statistical analysis

A contingency table was used to evaluate the concordance of HercepTest results obtained in the primary and in the corresponding metastatic lesion. Agreement was expressed by concordance of the HER2 status and by the Cohen's Kappa coefficient calculated by SPSS 11.5 (SPSS Inc, Chicago, Ill).

Results

The mean age of patients was 57.4 (range: 29-84 years). All tumours were invasive ductal carcinomas except cases no. 32 and 44, which were lobular carcinomas.

The HercepTest result was identical in the primary tumour and in the lymph node metastasis in 46/52 cases (88.5%) (Fig. 1a-h and Table 1). The average score obtained for the metastatic lesions (1.38) was slightly higher than the corresponding primary lesions (1.27). Concordance of data was analyzed by contingency tables (Table 2) and the Kappa index was 0.798±0.076.

In the six cases with HercepTest result discrepancy we performed fluorescence *in situ* hybridization (FISH) to determine whether the discrepancies in HercepTest results were due to a different gene amplification status or were merely due to technical reasons (primarily

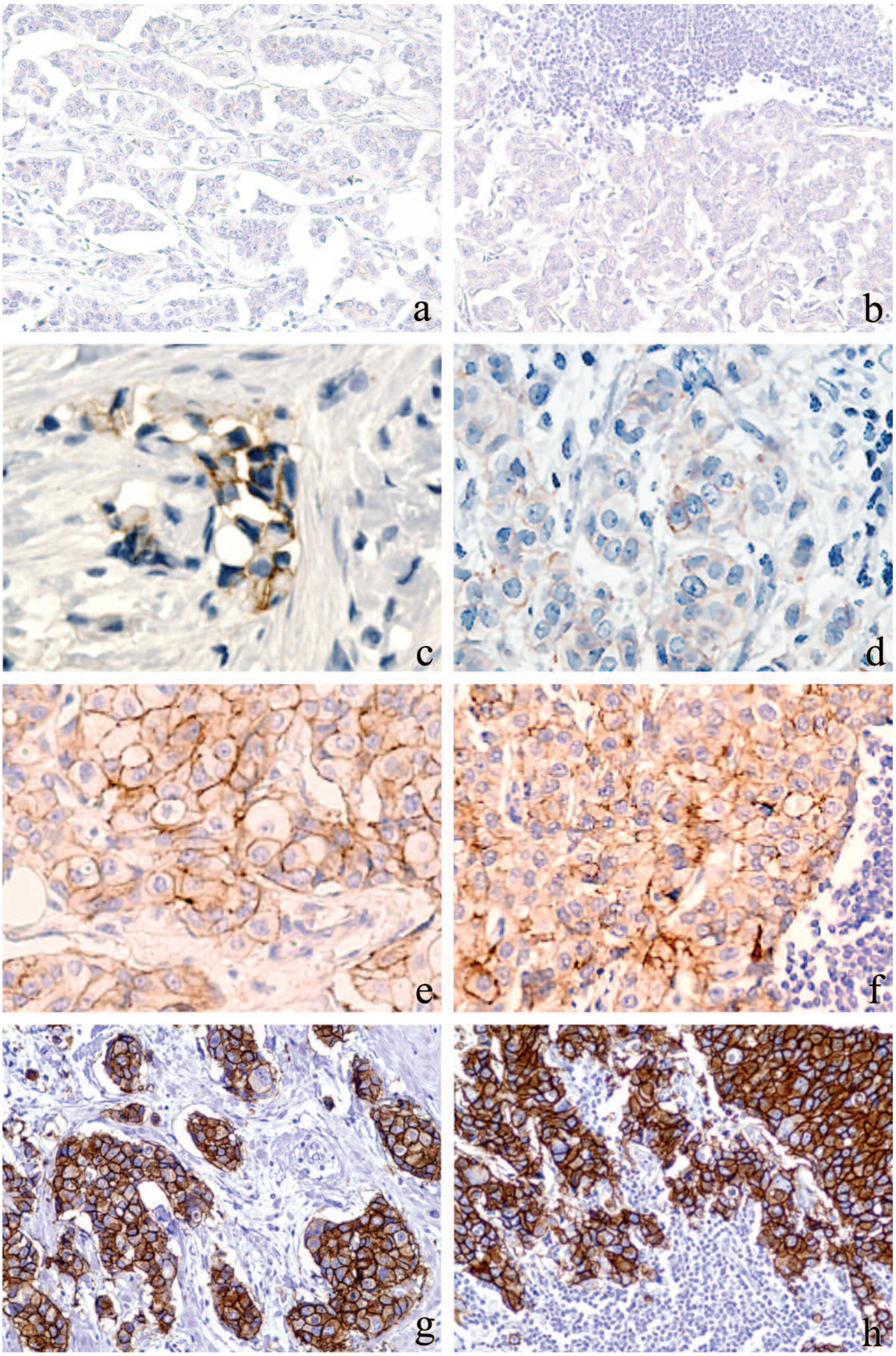

Fig. 1. HercepTest concordance in primary tumours (left column) and matched metastasis (right column) in cases scored as 0 (**a and b**, case 46, x 20), 1+ (**c and d**, case 15, x 40), 2+ (**e and f**, case 45, x 40) and 3+ **(g and h**, case 10, x 20).

fixation differences between the primary tumour and the metastasis). In addition, this technique allowed us to determine the clinical impact of the HercepTest discrepancies.

The two cases with 2+/3+ discrepancies (no. 22 and 26) showed the same gene status in the primary tumour and in the metastasis, i.e., amplification in case 22 and polysomy 17 in case 26. Case 23, 0 score in the primary tumour and 3+ in the metastasis (Fig. 2a,b), showed no amplification in the primary tumour but amplification in the metastasis (Fig. 2c,d). Cases 25 and 34, with 1+/2+ discrepancies, showed no amplification. Finally, case 44 showed 1+ score in the primary tumour and 3+ in the metastasis (Fig. 2e,f) (with staining heterogeneity but with more than 30% positive cells, Fig. 2f inset) and had no amplification in either primary tumour and metastasis

Table 1. HercepTest and FISH results in primary breast carcinoma and their corresponding axillary metastasis (N: Negative; E: Equivocal; P: Positive) and clinical significance of discordances (Clin. Signif.).

CASE	HercepTest Primary	HercepTest Metastasis	Coincidence	FISH Primary	FISH Metastasis	Clin. Signif.
1	N (0)	N (0)	YES			
2	N (0)	N (1+)	YES			
3	N (0)	N (0)	YES			
4	N (0)	N (0)	YES			
5	N (1+)	N (1+)	YES			
6	N (1+)	N (0)	YES			
7	N (1+)	N (1+)	YES			
8	N (0)	N (0)	YES			
9	N (0)	N (0)	YES			
10	P (3+)	P (3+)	YES			
11	N (1+)	N (1+)	YES			
12	N (1+)	N (1+)	YES			
13	N (0)	N (0)	YES			
14	P (3+)	P (3+)	YES			
15	N (1+)	N (1+)	YES			
16	P (3+)	P (3+)	YES			
17	N (0)	N (0)	YES			
18	N (0)	N (1+)	YES			
19	N (0)	N (0)	YES			
20	N (0)	N (0)	YES			
21	P (3+)	P (3+)	YES			
22	E (2+)	P (3+)	NO	Amplified	Amplified	NO
23	N (0)	P (3+)	NO	No amplified	Amplified	YES
24	N (0)	N (0)	YES			
25	E (2+)	N (1+)	NO	No Amplified	No Amplified	NO
26	P (3+)	E (2+)	NO	Polysomy	Polysomy	NO
27	P (3+)	P (3+)	YES			
28	N (0)	N (1+)	YES			
29	N (0)	N (1+)	YES			
30	E (2+)	E (2+)	YES			
31	N (0)	N (0)	YES			
32	N (0)	N (0)	YES			
33	P (3+)	P (3+)	YES			
34	E (2+)	N (1+)	NO	No amplified	No amplified	NO
35	P (3+)	P (3+)	YES			
36	E (2+)	E (2+)	YES			
37	P (3+)	P (3+)	YES			
38	N (1+)	N (0)	YES			
39	P (3+)	P (3+)	YES			
40	P (3+)	P (3+)	YES			
41	N (0)	N (0)	YES			
42	N (0)	N (0)	YES			
43	E (2+)	E (2+)	YES			
44	N (1+)	P (3+)	NO	No amplified	No amplified	NO
45	E (2+)	E (2+)	YES			
46	N (0)	N (0)	YES			
47	N (0)	N (1+)	YES			
48	P (3+)	P (3+)	YES			
49	N (1+)	N (1+)	YES			
50	E (2+)	E (2+)	YES			
51	P (3+)	P (3+)	YES			
52	E (2+)	E (2+)	YES			

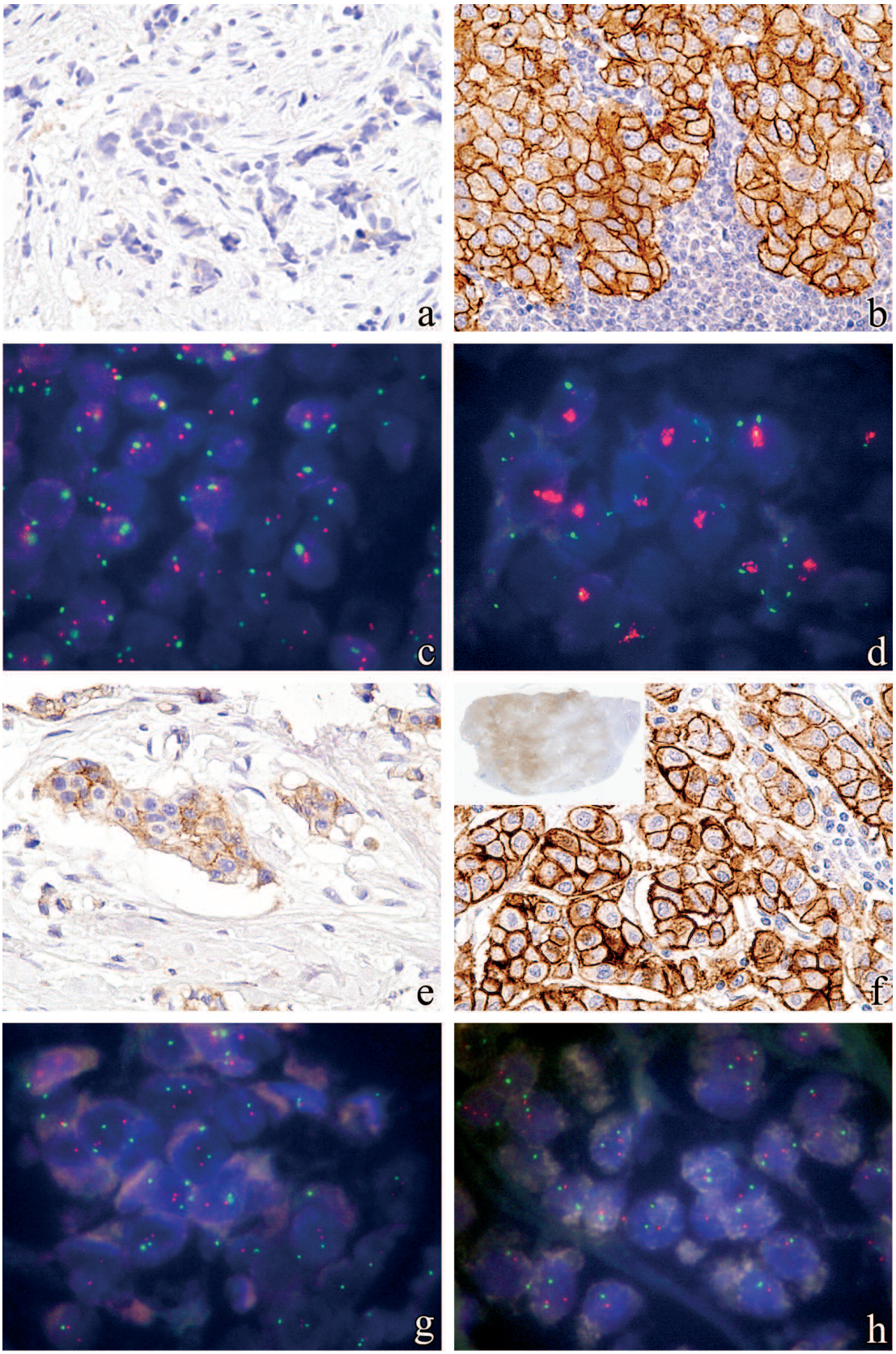

Fig. 2. a-d. Case 23. The primary tumour was negative (0) for HercepTest (**a**) whereas the metastasis was positive (3+) (**b**) (x 40). The HER2 gene by FISH was not amplified in the primary tumour (**c**) and showed high amplification in the metastasis (**d**) (x 100). **e-h** Case 44. In this case, the primary tumour was also negative for HercepTest (1+) (**e**) but the metastasis was positive (3+) **(f)**, with intense immunostaining in more than 30% of cells, but with a heterogeneous pattern (inset). The HER2 gene was not amplified either in the primary (**g)** or in the metastasis (**h**) (x 100).

Table 2. Correlation between HercepTest results of primary tumours and paired lymph node metastasis (n=52).

HercepTest Primary	HercepTest Metastasis			Total
	Negative	Equivocal	Positive	
Negative	28	0	2	30
Equivocal	2	6	1	9
Positive	0	1	12	13
Total	30	7	15	52

(Fig. 2g,h).

These results showed that only one of the 52 cases studied (case no. 23) presented clinically significant differences in HER2 status (2% discrepancy). This case presented a *de novo* amplification in the metastasis that would be susceptible to trastuzumab treatment.

Discussion

The role played by HER2 in breast cancer has changed from a prognostic foe to a predictive friend (Ferretti et al., 2007) since the development and approval of trastuzumab. The administration of trastuzumab after adjuvant chemotherapy reduces the risk of recurrence of HER2-positive breast cancer by roughly 50% (Piccart-Gebhart et al., 2005; Piccart-Gebhart, 2006) and the risk of death by 33% (Romond et al., 2005).

Trastuzumab treatment decisions are based on positivity for HER2 in the primary tumour, but cancer progression can influence HER2 status and it has been reported that HER2-negative tumours can be associated with HER2-positive circulating tumour cells (Hayes et al., 2002). However, it has also been shown that the molecular program of a primary tumour is generally retained during progression to metastasis (Perou et al., 2000; Lacroix et al., 2004; Weigelt et al., 2005) and the expression of different markers, including HER2, is generally concordant between primary tumours and metastasis (Lacroix et al., 2004; Lacroix, 2006).

The results obtained in the present paper show that there is concordance in HER2 status between primary breast tumours and their corresponding lymph node metastasis. The differences observed were clinically relevant in only one of the 52 cases studied (98% concordance). This case (no. 23), with a HercepTest 0 without gene amplification in the primary tumour and HercepTest 3+ with gene amplification in the metastasis, demonstrated that HER2 gene amplification may develop as breast cancer progresses (Meng et al., 2004). This phenomenon could be explained by mutation (Meng et al., 2004), or by a specific selection of HER2-positive cells during tumour progression (Lacroix et al., 2006) (i.e., the metastasis may derive from a subclone of cells presenting amplification). Neoangiogenesis promoted by HER2 overexpressing cells (Bagheri-Yarmand et al., 2000; Sopel et al., 2005) might favor metastatic dissemination of these amplified cells (Lacroix et al., 2006).

In contrast, no amplification was found in the metastasis of case 44, which showed a similar immunohistochemical result to the above mentioned case (negative 1+ in the primary tumour and positive 3+ in the metastasis). In case 44, the metastasis showed heterogeneous HER2 intense immunostaining and this pattern was found to predict gene amplification with only 68% accuracy, while uniform intense staining has an accuracy of 98%. Overexpression (3+) in the absence of amplification was reported in 2.8% of cases (Varshney et al., 2004) and may be due to upregulation of translation, decreased degradation of the protein, or polysomy (case 26) (Varshney et al., 2004), which in fact is the major cause of response to trastuzumab in FISH-negative cases (Hofmann et al., 2008).

Simon et al. (2001) analyzed HER2 (by immunohistochemistry and FISH) in 125 breast primary tumours and their corresponding lymph node metastasis. More than ninety-five percent of their patients had a homogeneous HER2 status determined in their primary tumour and identical findings in all their metastasis. They concluded that a high rate of nonresponse to trastuzumab therapy cannot be explained by heterogeneity in HER2 status between primary tumours and their lymph node metastasis. Our findings concur with this conclusion.

Xu et al. (2002) found concordance of amplification between the primary tumour and the synchronous axillary lymph node metastasis and this concordance was closer in patients with HER2 overexpression in the primary tumour than in patients without overexpression. Carlsson et al. (2004) conducted a study on 47 cases and found only small changes in seven cases when the HER2 scores of the lymph node metastasis were compared to their corresponding primary tumours. Park et al. (2006) analyzed 116 cases and showed that HER2 status is maintained in 93% of metastatic nodes.

Although multiple reports are based on the study of synchronous lymph node metastasis, other papers analyze asynchronous distant metastasis, for which less concordance would be expected. However, Tanner et al. (2001) studied 46 cases and showed that HER2 amplification status always remained the same between primary tumour and its regional or distant metastasis, despite the fact that in some cases the metastasis appeared more than 10 years after the primary tumour. Masood and Bui (2000) studied 56 primary breast cancers and their corresponding metastatic lesions by immunohistochemistry (HercepTest) and found the same immunostaining pattern in all but one case. Gancberg et al. (2002) showed that HER2 status in primary breast cancer and corresponding distant metastatic lesions had a high level of concordance: 94% and 93% when analyzed by IHC and FISH, respectively. Gong et al. (2005) compared HER2 status by FISH in primary and metastatic tumours (locoregional and distant disease). The results obtained agreed in 58 out of 60 patients (97% concordance) and locoregional and distant metastasis presented similar concordance rates with the

primary tumours (98% and 94%, respectively). However, Zidan et al. (2005) found discordance in HER2 overexpression between primary and metastatic sites in eight out of 58 patients (14%) (in one patient, HER2 was positive in primary and negative in metastasis while HER2 was negative in primary and positive in metastasis for seven patients). They suggested that a possible discordance should be considered when making treatment decisions in patients with primary HER2 negative tumours. Moreover, Pectasides et al. (2006) studied by IHC and/or CISH the HER2 status of primary and corresponding metastatic sites in 16 patients treated with trastuzumab. They showed that six out 16 patients (37%) lost HER2 overexpression and the authors considered that it is not clear whether this finding implies resistance or sensitivity to trastuzumab. Very recent reports showed conflicting results, namely, while Tapia et al. (2007) showed discordant HER2 status between primary tumour and matched distant metastasis in only 2.9% of 105 patients, Lower et al. (2008) found discordances in 33.2% of 382 cases. The use of different methods to determine HER2 status (cytological specimens and FISH in the first report and paraffin-embedded specimens and immunohistochemistry with CB11 in the second one) could explain the large discrepancies observed.

Numerous studies have analyzed the HER2 concordance between primary tumours and bone metastasis. Lorincz et al. (2006) showed that about 10% of initial HER2 genotype change in bone metastasis and conversion occurred more frequently in cases with HER2 amplification than in cases without amplification. They suggested that the modification of the original genotype might be due to a selection process during tumour progression or to the effect of trastuzumab treatment. Solomayer et al. (2006) showed a concordance rate of only 62% between primary breast tumour and disseminated tumour cells in bone marrow. These authors detected HER2 positive disseminated tumour cells in 12 out of 20 patients with negative primary tumours and they proposed that the antigenic profile of disseminated tumour cells may be considered for treatment decision, since these patients might actually benefit from trastuzumab. Discrepancies in the immunohistochemical HER2 results between primary breast tumours and bone marrow metastasis could at least in part be explained by the special technical requirements of bone marrow specimens. However, Vincent-Salomon et al. (2007) reported that negative and positive HER2 status remained stable between the bone marrow micrometastasis and the primary tumour in the majority of the cases.

In summary, HER2 status of breast carcinoma was generally stable during axillary metastatic progression and, hence, HER2 discordances between primary tumour and metastasis cannot explain the high rate of nonresponse to trastuzumab in HER2 positive patients. However, in some instances (2% in our study) a de novo amplification can occur in the metastasis. Evaluation of gene HER2 status in axillary metastasis rather than in the primary can be useful in certain situations (small invasive component intimately mixed with in situ component, no tumour after biopsy, axillary relapse, etc.). Moreover, in this way we can find occasional de novo amplifications in the metastatic node.

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