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HER-2/*neu* Amplification by Fluorescence In Situ Hybridization in Cytologic Samples from Distant Metastatic Sites of Breast Carcinoma

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BACKGROUND. Amplification of the HER-2/*neu* oncogene has been proposed as a target for antibody-based therapies and as a predictor of chemoresponsiveness in advanced breast carcinoma. Few studies have concentrated on HER-2/*neu* gene evaluation by fluorescence in situ hybridization (FISH) on distant metastatic sites and none have been performed on cytologic samples. The current study evaluated HER-2/*neu* amplification by FISH on cytologic samples obtained from distant metastatic lesions of breast carcinoma to update HER-2/*neu* characterization through a safe and easier procedure than biopsy.

METHODS. Twenty-two cytologic samples from distant metastases (12 hepatic samples, 4 skin samples, 3 pleural samples, and 3 peritoneal samples) were submitted to HER-2/*neu* evaluation by FISH. Seventeen corresponding primary breast tumors also were evaluated by FISH on paraffin histologic sections or on destained archival cytologic smears.

RESULTS. Seven of the 22 metastases (32%) were amplified. Amplification was observed in 4 of the 12 liver metastases, in 1of the 3 ascitic fluid specimens, and in 2 of the 4 skin metastases. In all the three pleural fluid specimens, HER-2/*neu* was unamplified. Matched results from primary and metastatic lesions were obtained in 14 cases (5 were amplified and 9 were unamplified on both primary and metastatic tumors).

CONCLUSIONS. The results of the current study emphasized the feasibility and advantages of two rapid and very informative techniques, such as fine-needle aspiration biopsy and FISH. Both procedures were performed to ascertain the malignant nature of a suspicious lesion and to obtain predictive markers for response. Since the advent of trastuzumab, the characterization of the molecular profile in metastatic breast disease has become increasingly important for targeted therapy selection. ***Cancer (Cancer Cytopathol)* 2003;99:310-5.**

KEYWORDS: fine-needle aspiration (FNA), breast carcinoma, fluorescence in situ hybridization (FISH), HER-2/*neu*.

Amplification and overexpression of the HER-2/*neu* protooncogene represent a requisite for successful therapy with trastuzumab. Trastuzumab is a high-affinity, humanized anti-HER-2/*neu* antibody developed by Genentech (San Francisco, CA) for the treatment of advanced breast carcinoma.[1,2] Although metastases are the target for trastuzumab-based therapy, HER-2/*neu* status is usually evaluated only in the primary tumor because metastatic lesions are rarely removed or biopsies are rarely performed for a biologic characterization. It has been demonstrated that, when compared with the primary lesion, disseminated breast carcinoma displays a genetic

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heterogeneity[3] that may eventually correlate with the expression of different patterns of predictive biomarkers.[4] Theoretically, this could explain the high proportion of patients that ultimately do not respond to trastuzumab-based therapies. A number of studies have shown a high level of concordance, although not complete, between HER-2/*neu* status evaluated on primary tumors and lymph node metastases by means of both immunohistochemistry (IHC)[5–9] and fluorescence in situ hybridization (FISH).[9,10] However, to our knowledge few studies to date have compared HER-2/*neu* status between primary tumors and paired distant metastases.[8,11–15] These studies showed a heterogeneous pattern of results.

Fine-needle aspiration biopsy (FNAB) and FISH are two rapid and reliable techniques for cytologic diagnosis and biologic characterization of primary breast carcinoma.[16,17] In our experience, FNAB, possibly coupled with ultrasound for the sampling of deep lesions, is a relatively safe and less invasive alternative to surgical biopsy for cytologic sampling of metastases. The aim of the current study was to evaluate the feasibility of HER-2/*neu* status assessment by FISH on cytologic samples from distant metastases, allowing an updated definition of this predictive factor and circumventing many drawbacks correlated with immunocytochemistry.[18] Moreover, the results of FISH cytology on metastatic lesions have been compared with those obtained by FISH on paired primary tumors.

MATERIALS AND METHODS

The current series included cytologic preparations obtained from 22 patients with primary breast carcinoma who presented during follow-up with secondary lesions localized to the liver ($n = 12$), the pleura ($n = 3$), the peritoneum ($n = 3$), and the skin ($n = 4$). Cytologic preparations from each metastatic lesion underwent May-Grünwald-Giemsa (MGG) staining for routine diagnostic cytology as well as HER-2/*neu* evaluation by FISH. In addition, 17 corresponding primary breast tumors were evaluated by FISH (13 paraffin-embedded histologic sections and 4 destained archival cytolgic smears).

HER-2/*neu* Fluorescence In Situ Hybridization on Cytologic Smears

Sample collection

Cytologic smears from skin metastases were obtained by FNAB using a 22-gauge needle and a 20-mL syringe, whereas liver metastases were sampled by ultrasound-guided FNAB. The aspirated material obtained by FNAB from superficial or deep metastatic lesions was smeared on glass slides and air dried. Cellular suspensions obtained from pleural or ascitic fluids were cytocentrifuged and air dried. At least two slides were stained with MGG for routine cytology. The remaining slides were kept unstained at room temperature until assay. After cytologic diagnosis of malignancy, one representative slide was submitted to HER-2/*neu* FISH.

Pretreatment of cytologic slides

Unstained slides were fixed in absolute methanol, air dried, incubated in wash buffer (0.3% NP-40, 2 × saline-sodium citrate buffer [pH 7.0–7.5]) at 37 °C for 30 minutes, dehydrated through gradients of 70%, 85%, and 100% ethanol, air dried, and processed by FISH. Archival cytologic slides, stained with MGG, were treated with xylene to remove the coverslip and rinsed twice for 10 minutes each in clean xylene. After washing three times for 10 minutes each in Carnoy solution, the slides were dehydrated gradually in ethanol and processed by FISH.

An alternative pretreatment with proteinase K was required for smears demonstrating considerable thickness and overlapping cells. In this case, unstained or destained slides were dehydrated through an ethanol gradient and incubated for 5 minutes at 37 °C in 20 μg/mL proteinase K. After washing in water and ethanol dehydration, the slides were air dried before being evaluated by FISH.

Fluorescence in situ hybridization

After pretreatment, 10 μL of the hybridization solution containing both a Spectrum Green fluorophore-labeled α-satellite DNA probe for chromosome 17 and a Spectrum Orange fluorophore-labeled DNA probe for the HER-2/*neu* gene locus (Vysis PathVysion HER-2 DNA probe kit, Vysis, Downers Grove, IL) was applied to the cells. Samples were denaturated at 70 °C for 5 minutes and hybridized overnight at 37 °C in a HYBrite denaturation/hybridization system for FISH (Vysis). The following day, slides were incubated in wash buffer at 72 °C for 2 minutes, air dried in the dark, and counterstained with 4,6-diamidino-2-phenylindole (DAPI). A positive control slide was included in each run and consisted of a cytologic slide known to be amplified for the HER-2/*neu* gene because it was assayed by FISH in a previous run.

Slides were viewed at a magnification of × 1250 on an Olympus CX40 fluorescence microscope (Olympus, Inc., Melville, NY) using a triple excitation/emission filter for simultaneous detection of Spectrum Orange, Spectrum Green, and DAPI. At least 50 evaluable nuclei for each case were scored visually. Results were expressed as a ratio of the number of copies of the HER-2/*neu* gene to the number of chromosome 17

centromeric markers. A ratio of greater than 2 indicated amplification. Samples were classified as unamplified when two copies of HER-2/*neu* and two copies of chromosome 17 were found in the majority of cells and anyway when the HER-2/*neu*-to-chromosome 17 ratio was less than 2.0. Specimens were classified as poorly amplified when the number of HER-2/*neu* signals ranged from 5–10, moderately amplified when the number of signals ranged from 11–20, and highly amplified when there was a consistent presence of signal clusters or $>$ 20 signals per cell. Cases with an equal number of HER-2/*neu* and centromere 17 signals but more than 2 in greater than 10% of cells were classified as polysomic. Aneuploidy of chromosome 17 was excluded as a source of increased HER-2/*neu* copy number. Cases with fewer HER-2/*neu* copies than centromere 17 copies were classified as deleted.

HER-2/*neu* Fluorescence In Situ Hybridization on Paraffin Sections

Formalin-fixed, paraffin-embedded tissue sections were cut into 4-μm–thick sections that were incubated overnight at 56 °C. Deparaffinization, pretreatment, enzyme digestion, and fixation of slides were performed using the Vysis paraffin pretreatment kit (Vysis) according to the manufacturer's recommended protocol. Denaturation and hybridization were performed in a HYBrite denaturation/hybridization system for FISH (Vysis). A HER-2/*neu* probe mix (10 μL) was applied to tissue sections that were denaturated at 72 °C for 2 minutes and hybridized overnight at 37 °C. The slides were then washed in wash buffer at 72 °C for 2 minutes and counterstained with DAPI. Control slides (Vysis) were included in each assay run.

For each specimen, at least 100 cells were scored for both HER-2/*neu* and chromosome 17 signals by image analysis. FISH images were processed at a magnification of $\times$ 1250 utilizing an Olympus MX60 fluorescence microscope with a 100-W mercury lamp. Separate narrow band pass filters were used for the detection of Spectrum Orange, Spectrum Green, and DAPI. Images were processed using Quips software (Applied Imaging, New Castle, UK; Olympus distributor).

Amplification of the HER-2/*neu* gene was indicated by a ratio of HER-2/*neu* to chromosome 17 copy number greater than 2. For amplified, polysomic, and deleted cases, the same criteria of cytologic samples were applied.

RESULTS

HER-2/*neu* results obtained by FISH in primary breast tumors and corresponding distant metastatic lesions are shown in Table 1. The time period between a primary tumor diagnosis and a cytologic diagnosis of metastatic lesions ranged from 1 month to 13 years.

HER-2/*neu* was assessed on 22 cytologic samples from metastatic sites (14 slides were unstained and 8 were MGG-destained smears). Of the 22 metastases, 7 (32%) were amplified. Amplification was observed in 4 of the 12 liver metastases, in one of the three ascitic fluid specimens, and in two of the four skin metastases. Among amplified cases, one liver and one skin metastasis resulted in a classification of poorly amplified. In all the three pleural fluid specimens, HER-2/*neu* was unamplified. One of the 12 liver and one of the four skin metastases were polysomic.

HER-2/*neu* was assessed on the primary tumor in 17 of the 22 metastatic cases. Thirteen of the 17 cases were evaluated by FISH on archival histologic sections. Four cases underwent HER-2/*neu* evaluation on MGG-destained cytologic smears obtained at the time of diagnosis. HER-2/*neu* was not evaluable in 3 of 17 (18%) cases (1 histologic sample because of technical pitfalls and 2 samples [1 cytologic and 1 histologic] because of a lack of hybridization). Among the 14 evaluable primary tumors, 5 were amplified, 8 were unamplified, and 1 was polysomic. Among the five amplified cases, one was poorly amplified. In 5 of the 22 primary tumors, neither histologic nor cytologic specimens were retrieved because patients had been treated for their primary breast carcinoma elsewhere.

Paired FISH results on primary and corresponding metastatic tumors were obtained in 14 cases. All five matched, amplified metastatic lesions also showed amplification in the primary tumors. Of the two polysomic metastases, one had a primary polysomic tumor and one was unamplified. In the remaining seven cases, no amplification was detected in either the metastatic site or in the corresponding primary tumor. Overall, HER-2/*neu* evaluation on primary and metastatic lesions yielded concordant results in all cases that were compared.

DISCUSSION

Trastuzumab is a novel anti–HER-2/*neu* antibody that targets metastatic breast carcinoma. HER-2/*neu* status is usually assessed by IHC or FISH technique and is generally performed only on primary tumors because recurrent tumors are rarely removed or undergo biopsy. However, HER-2/*neu* positivity of primary tumors incompletely predicts the response to trastuzumab, possibly because of a change of HER-2/*neu* status during metastatic progression. It has been demonstrated previously that recurrent breast carcinoma is actually a heterogeneous disease in which a biologically dominant clone could eventually overcome the others and evolve independently in both primary can-

TABLE 1
HER-2/*neu* Status Assessed by Fluorescence In Situ Hybridization on Primary Breast Carcinoma and on Cytologic Samples from Metastatic Sites

Case	HER-2/*neu* primary tumor	Date of primary/metastasis	Metastatic site	HER-2/*neu* metastatic site
1	Not evaluable	May 1986/June 1998	Liver	Amplified (low level)[a]
2	Amplified	May 1996/August 1998	Liver	Amplified[a]
3	Amplified	June 1997/March 2002	Liver	Amplified[a]
4	Polysomic	March 2000/August 2002	Liver	Polysomic
5	Amplified[a]	November 2001/October 2002	Liver	Amplified
6	Unamplified	March 2000/February 2001	Liver	Unamplified[a]
7	Unamplified	May 1995/June 2000	Liver	Unamplified
8	Unamplified	March 2000/May 2002	Liver	Unamplified
9	Unamplified[a]	September 2002/October 2002	Liver	Unamplified
10	Unamplified	April 1989/June 2001	Liver	Unamplified
11	Unavailable	February 1994/June 2001	Liver	Unamplified[a]
12	Unavailable	June 1988/February 2002	Liver	Unamplified
13	Unavailable	June 1997/April 2002	Pleura	Unamplified
14	Unavailable	November 1995/July 2002	Pleura	Unamplified
15	Not evaluable[a]	February 1998/January 2002	Pleura	Unamplified[a]
16	Amplified	January 1993/December 2002	Peritoneum	Amplified
17	Not evaluable	December 1997/December 2002	Peritoneum	Unamplified[a]
18	Unamplified	September 1999/January 2003	Peritoneum	Unamplified[a]
19	Unamplified[a]	April 1995/May 2001	Skin	Unamplified
20	Unamplified	May 1997/July 2002	Skin	Polysomic
21	Unavailable	June 1998/March 2002	Skin	Amplified
22	Amplified (low level)	February 1993/November 2002	Skin	Amplified (low level)

[a] Fluorescence in situ hybridization on destained cytologic smears.

cer and metastases.[3] Although generally well tolerated, trastuzumab-based therapy is correlated with a significant risk of cardiotoxicity. To select patients who will derive the maximum benefit from this treatment, it would be worthy to ascertain the concordance of HER-2/*neu* status between the primary and metastatic lesions. Nevertheless, few reports concern HER-2/*neu* assessment on distant metastatic lesions, whereas the majority of studies compare HER-2/*neu* status in primary tumors and in regional concurrent lymph node metastases. Among the more recent published data, Cardoso et al.[6] and Simon et al.[9] found that the HER-2/*neu* status of primary tumors was maintained in the majority of concurrent, ipsilateral axillary lymph node metastases. However, concurrent regional lymph node metastases should not be assumed to be equivalent to distant metastases. There is actually a genetic heterogeneity that underlies the development of a distant lesion, years after the primary cancer. Moreover, cells that metastasize via lymphatics could display different biologic properties compared with cells that travel to distant sites, due to blood vessel invasion.

Few studies have compared HER-2/*neu* status in primary breast carcinoma with paired distant metastases. In a preliminary study from Edgerton et al.,[13] an overall 25% discordance was found in HER-2/*neu* status between 193 primary tumors and their recurrences (68 paired local recurrences, 32 lymph node and 93 distant metastases). A note of caution should be applied to that study due to the lack of further details. Conversely, other studies based on FISH or IHC assessments and performed on small series, ranging from 11 to 30 paired retrieved samples, have reported a stable, although not always complete, HER-2/*neu* status congruence between primary and distant metastases.[8,11,14,15] A bigger series, recently published by Gancberg et al.,[12] found 94% and 93% concordance between paired primary tumors and distant metastatic lesions when analyzed by IHC and FISH, respectively.

Our data suggest that HER-2/*neu* status is mostly stable in primary breast carcinoma and in the corresponding distant metastatic sites. In the current analysis, one case of skin metastasis was polysomic, but was unamplified on the paired primary histologic sample. Wang et al.[19] reported that in primary breast carcinoma, aneusomy 17 is a common feature that occurs in the absence of HER-2/*neu* amplification. Even high polysomy 17 was not sufficient to produce a significant increase in gene trascription, eventually leading to HER-2/*neu* protein overexpression.

It is noteworthy that a low-level gain of only a few

extra copies of the HER-2/*neu* gene was found in one case, both in the primary and in the metastatic lesions. This finding must be distinguished from extra gene copies due to the formation of sister chromatids in S-phase or G_2-phase cells, mostly arranged in pairs. Criteria for a low-level gene copy number increase are the presence of extra signals in a major subpopulation and their random distribution in the nuclei. The biologic and clinical significance of a gain of a few gene copies is still unclear.

In our opinion, to test HER-2/*neu* status exclusively on primary breast carcinoma specimens could be a safe policy for the use of trastuzumab in metastatic disease, especially when results are obtained by FISH methodology. Reasons other than a possible change in HER-2/*neu* status could be advocated to explain the lack of response to trastuzumab therapy. Many methodologic limitations may interfere with HER-2/*neu* assessment either by IHC or FISH in primary breast carcinoma, particularly when using archival samples collected years ago. Previous studies have indicated that, in terms of feasibility and accuracy, FISH provides a tempting alternative to HER-2/*neu* evaluation by IHC, whose specificity and sensitivity problems have been described.[20–22] Conversely, hybridization may be eventually compromised by Bouin fixation in archival histologic samples subsequently assessed by FISH. This may account for the Gancberg et al.[12] finding that a high number of samples were not evaluable by FISH. Similarly, in the current study, 2 of the 13 histologic samples and 1 of the 12 cytologic-destained smears in the archival series were not assessable by FISH.

We suggest that FNAB, performed on a suspicious breast carcinoma metastatic lesion, may provide fresh cytologic material for an updated characterization of relevant predictive factors and a real-time HER-2/*neu* assessment for trastuzumab-based therapy. Furthermore, to retest HER-2/*neu* status on metastatic lesions may be worthwhile when a negative score is obtained by IHC performed on the primary tumor sample resected many years before.

To our knowledge, none of the studies that evaluated HER-2/*neu* status on metastases from breast carcinoma patients were performed on cytologic material. In our experience, FNAB coupled with ultrasound methodology for hepatic lesions is a relatively easy and safe method to obtain cellular material for FISH analysis. This procedure has been proved to circumvent many of the shortcomings due to molecular, immunocytochemical, and IHC techniques both on frozen tissue sections and on fresh aspirates.[18,20] FISH on cytologic specimens, including smears, fine-needle aspirates, and imprint preparations, allows for a rapid and reproducible quantitative analysis of DNA alterations in single cells,[17,23,24] thus avoiding many of the drawbacks of tissue sections.

The results of the current study underline the feasibility and the advantages of two rapid and very informative techniques, such as FNAB and FISH, performed to ascertain the malignant nature of a suspicious lesion and to obtain predictive markers for response. Since the advent of trastuzumab, the characterization of the molecular profile in metastatic disease has become increasingly important for targeted therapy selection.

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