Original article

Annals of Oncology 13: 1398–1403, 2002
DOI: 10.1093/annonc/mdf217

HER-2/neu amplification detected by fluorescence *in situ* hybridization in fine needle aspirates from primary breast cancer

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Received 26 October 2001; revised 22 January 2002; accepted 7 March 2002

Background: The *HER-2/neu* gene is amplified in 20–30% of human breast cancers and has been shown to have prognostic and predictive value for treatment with chemotherapy, hormone therapy and antibodies against the *HER-2/neu* domain (trastuzumab). The aim of our study was to evaluate the reliability of *HER-2/neu* determination by fluorescence *in situ* hybridization (FISH) on fine-needle aspirates (FNAs) from primary breast cancer patients by comparison with the results obtained by FISH and immunohistochemistry (IHC) on the corresponding histological sections.

Materials and methods: *HER-2/neu* amplification was determined by FISH on 66 breast cancer FNAs. Twenty-three and 36 corresponding formalin-fixed, paraffin-embedded sections were assayed by FISH and by IHC, respectively, in order to detect *HER-2/neu* amplification and *HER-2/neu* protein expression.

Results: Twenty-seven per cent (18/66) of breast cancer FNAs showed amplification of *HER-2/neu* by FISH. Paired results by FISH cytology and FISH histology were available in 22 cases. Concordance was 91% (20/22). Paired results by FISH cytology and IHC were available in 36 cases. Concordance was 92% (33/36). Eighteen of 66 breast cancer FNAs were also submitted to flow cytometric DNA analysis. None of the diploid cases showed *HER-2/neu* amplification by FISH. Six out of the eight aneuploid cases were amplified and two were polysomic.

Conclusions: *HER-2/neu* gene amplification can be reliably estimated by FISH on breast cancer FNAs and a good correlation has been found between FISH and IHC results from the corresponding histological sections.

Key words: breast cancer, fluorescence *in situ* hybridization, *HER-2/neu*

Introduction

Amplification and overexpression of the *HER-2/neu* proto-oncogene has been described in ~25% of breast carcinomas. These alterations have been associated with an increased risk of recurrent disease or a shorter survival.

For this reason, an innovative therapeutic approach targeting the receptor by monoclonal antibodies (MAb) to the *HER-2/neu* protein product has been promoted. Trastuzumab is a high-affinity anti-*HER-2/neu* MAb developed by Genentech (San Francisco, CA). The results of the initial trastuzumab trials have clearly shown that this agent represents a new specific therapy active in patients with advanced *HER-2*-positive breast cancer either alone or in combination with anthracycline- and taxane-based chemotherapy.

In spite of these results, the method of choice to determine whether a tumor is *HER-2/neu* positive is still under debate. *HER-2/neu* gene amplification is usually analyzed by fluorescence *in situ* hybridization (FISH) and *HER-2/neu* overexpression by immunohistochemistry (IHC) on tissue sections. Immunohistochemistry is relatively simple and inexpensive, but the use of different antibodies as well as interpretative difficulties prevents this technique being highly reproducible. Gene-based techniques, such as FISH, have less variability, but require dedicated equipment and may be more expensive. Nevertheless, FISH seems to be the more powerful technique. In some clinical situations, such as cases of preoperative chemotherapy or unavailability of archival samples in metastatic patients, the determination of *HER-2/neu* status by FISH on cytological material may be helpful in order to choose the best treatment approach.

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In this context, reliable information on *HER-2/neu* status, in addition to other biological markers, may be a determinant in both predicting prognosis and the planning of treatment for breast cancer patients. The biology of *HER-2/neu* and its use as a target for antibody-based therapeutics, as well as current methodologies for *HER-2/neu* testing and its evolving role in the management of breast cancer have been recently reviewed [1, 2].

Fine-needle aspiration, a well-established method in the diagnosis of breast cancer, has recently been successfully combined with molecular and cytogenetic analysis. Only a few studies concerning the feasibility of dual color FISH on cytological material from breast cancer fine-needle aspirates (FNAs) have been published [3–6].

This study was carried out to determine the feasibility of detecting *HER-2/neu* in breast carcinoma FNAs using the FISH technique. Moreover, the results of FISH cytology have been compared with those obtained by both FISH and IHC on the corresponding histological sections. Flow cytometric DNA analysis was also performed when sufficient cytological material was available.

Materials and methods

Fine needle aspirates from 66 breast cancer patients were submitted for routine diagnostic cytology as well as for *HER-2/neu* evaluation by FISH. After surgery, the corresponding sections of archival formalin-fixed, paraffin-embedded tissue were evaluated for *HER-2/neu* both by FISH and by IHC. According to a procedure in use in our laboratory, cellular material obtained by scraping an unstained cytological slide was utilized for flow cytometric DNA analysis.

HER-2/neu FISH on cytological smears

Sample collection. All samples were collected by fine-needle aspiration biopsy using a 22-gauge needle and 20 ml syringe.

The aspirated material was smeared on a glass slide and air dried. At least two slides were stained with May–Grunwald Giemsa stain for routine cytology. The remaining slides were kept unstained. After cytological diagnosis of malignancy, one representative slide was assessed for *HER-2/neu* status by FISH.

Pretreatment of cytological 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 min, gradually dehydrated in alcohol, air dried and processed by FISH.

An alternative pretreatment with proteinase K was required for smears showing considerable thickness and overlapping cells. In this case, unstained and unfixed slides were dehydrated through an ethanol gradient and incubated for 5 min at 37°C in 20 µg/ml proteinase K. Following H_2O washing and ethanol dehydration, the slides were air dried prior to being evaluated by FISH.

Fluorescence in situ hybridization (FISH). After pretreatment, cytological slides were incubated in a denaturating solution (70% formamide, 2 × saline–sodium citrate buffer, pH 7.0–8.0) at 70°C for 2 min, dehydrated through a 70, 80 and 100% ethanol gradient and air dried. 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, USA) was applied to the denaturated cells. A glass coverslip was placed over the probe mix and the slides were incubated in a pre-warmed humidified hybridization chamber overnight at 37°C. The following day, slides were incubated in wash buffer at 72°C for 2 min, air dried in the dark and counterstained with DAPI.

Slide evaluation. Slides were viewed at 1250× magnification on a Olympus CX40 Fluorescence Microscope using a triple excitation/emission filter for simultaneous detection of Spectrum Orange, Spectrum Green and DAPI. At least fifty evaluable nuclei for each case were visually scored. Only single, non-overlapping and intact nuclei were examined. Nuclei lacking hybridization signals were excluded from the evaluation. Split centromere signals were counted as one. As the level of gene amplification was heterogeneous among the nuclei of the same specimen, an average *HER-2/neu* gene copy number and an average centromere 17 copy number were determined for each preparation. 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, with a ratio of greater than two being considered amplified. 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 lower than 2.0. Specimens were classified as 'poorly amplified' when the number of *HER-2/neu* signals ranged from 5 to 10, 'moderately amplified' when the number of signals ranged from 11 to 20 and as 'highly amplified' when there was a consistent presence of signal clusters or >20 signals/cell. Cases with an equal number of *HER-2/neu* and centromere 17 signals but greater than two in >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 FISH on paraffin sections

Formalin-fixed, paraffin-embedded tissue was 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 carried out in a HYBrite Denaturation/Hybridization System for FISH (Vysis). Tissue sections were denaturated at 72°C for 2 min, *HER-2/neu* probe mix (10 µl) was added and hybridization took place at 37°C for 14–18 h. The slides were then washed in post-hybridization wash buffer at 72°C for 2 min and counterstained with DAPI.

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 1250× magnification 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 the software Quips (Applied Imaging, Newcastle, 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 two. For polysomic and deleted cases the same criteria of cytological samples were applied.

HER-2/neu immunohistochemistry

Sections of archival formalin-fixed, paraffin-embedded tissue (5 μm) were placed on slides coated with poly-L-lysine. After deparaffinization and blocking of endogenous peroxidase, *HER-2/neu* immunostaining was performed using rabbit anti-human c-erbB-2 oncoprotein as primary antibody (Dako, Copenhagen, Denmark) at 1/100 dilution. Binding of the primary antibody was revealed by means of the Dako Quick-Staining, Labelled Streptavidin–Biotin System (LSAB; Dako), followed by the addition of diaminobenzidine (DAB) as a chromogen.

HER-2/neu immunoreactivity was evaluated in a semiquantitative way. The tumor samples were scored as 3+ when >10% of the cells showed a specific dark brown border associated with the cell membrane. Scores of 0, 1+ and 2+ where assigned to negative, weak or moderate membrane staining, respectively.

Flow cytometric DNA analysis

Cells from an unstained smear were solubilized with propidium iodide solution and then scraped in order to obtain a cell suspension for flow cytometric DNA analysis. Staining was performed according to a one-step protocol as previously described [7]. Flow cytometric data analysis was performed using the Multicycle Cell Cycle Analysis Software (Phoenix Flow System, San Diego, CA, USA).

Results

HER-2/neu was evaluated by FISH on 66 primary breast cancer FNAs. Twenty-three paired paraffin sections were tested by FISH and 36 by immunohistochemistry. Figure 1 shows an example of a highly amplified and overexpressed primary breast cancer.

HER-2/neu FISH on cytological smears

FISH was performed on 66 breast cancer FNAs. Forty-eight (73%) of 66 cases were unamplified and 18 cases (27%) were amplified. In unamplified cases, the most frequent pattern consisted of two red and two green signals; one case (1.5%) was polysomic and seven cases (11%) showed a *HER-2/neu* deletion. Of the 18 amplified tumors, one, six and 11 cases showed low, medium and high levels of amplification, respectively. Highly amplified tumors showed *HER-2/neu* spot numbers ranging from 20 to 100, and often distributed in clusters. Samples with gene amplification showed centromere 17 disomy in nine cases and chromosome 17 polysomy in nine cases, with a number of centromere 17 signals up to 10.

With regard to the feasibility of FISH on cytology, hybridization was successful in 80% of samples pretreated with wash buffer only. Twenty per cent of samples were submitted to a further digestion with proteinase K.

HER-2/neu FISH on paraffin sections

FISH was performed on 23 paraffin-embedded sections. One case was not evaluable because of the lack of hybridization. Five of 22 evaluable cases (23%) were amplified and 17 (77%) unamplified. Among the unamplified cases, four were classified as polysomic. Amplified tumors showed a percentage of amplified cells ranging between 50% and 100%, and often distributed in clusters.

HER-2/neu immunohistochemistry

Thirty-six of 66 samples tested by FISH on cytology were evaluated by immunohistochemistry on formalin-fixed, paraffin-embedded sections. Nine cases (25%) showed overexpression: eight cases had a percentage of intensively stained (3+) tumor cells ranging between 90% and 100% and one case showed moderate (2+) staining intensity. The remaining 27 cases (75%) showed normal HER-2 protein expression.

FISH cytology versus FISH histology

The relationship among *HER-2/neu* results evaluated by FISH on cytological specimens and both by FISH and by immunohistochemistry on corresponding histological sections is shown in Table 1. Among 66 cases examined, matched results from FISH cytology and FISH histology were obtained in 22 cases. Five cases were amplified and 15 unamplified according to FISH both on cytological and histological samples. Four of five amplified cytological specimens showed a high level and one a medium level of amplification. Two cases, moderately amplified on FISH cytology, were classified as polysomic on FISH histology. Concordance between FISH cytology and histology was 91%.

FISH cytology versus immunohistochemistry

Paired results according to cytology by FISH and histology by immunohistochemistry were obtained in 36 cases (Table 1). Eight paired cases were both amplified and overexpressed. All of them were strongly immunostained; four were moderately and four highly amplified. Twenty-five cases were negative for both amplification and overexpression. Three of 36 cases were discordant. Two of them, moderately amplified on FISH cytology, were not overexpressed and were polysomic by FISH histology. The other discordant case, unamplified on FISH cytology, showed a medium level of overexpression. Concordance between FISH cytology and immunohistochemistry was 92%.

HER-2/neu FISH and ploidy

Eighteen of 66 cases evaluated by FISH cytology were submitted to flow cytometric DNA analysis. The results are shown in Table 2. Eight cases were aneuploid and 10 diploid. Six of eight aneuploid cases were amplified and two were polysomic by FISH. None of the diploid cases showed amplification by FISH cytology.

Discussion

In the next few years, there is likely to be an expanding role for *HER-2/neu* assessment, either in the selection of chemotherapy (antracycline or not), or in the selection of patients for

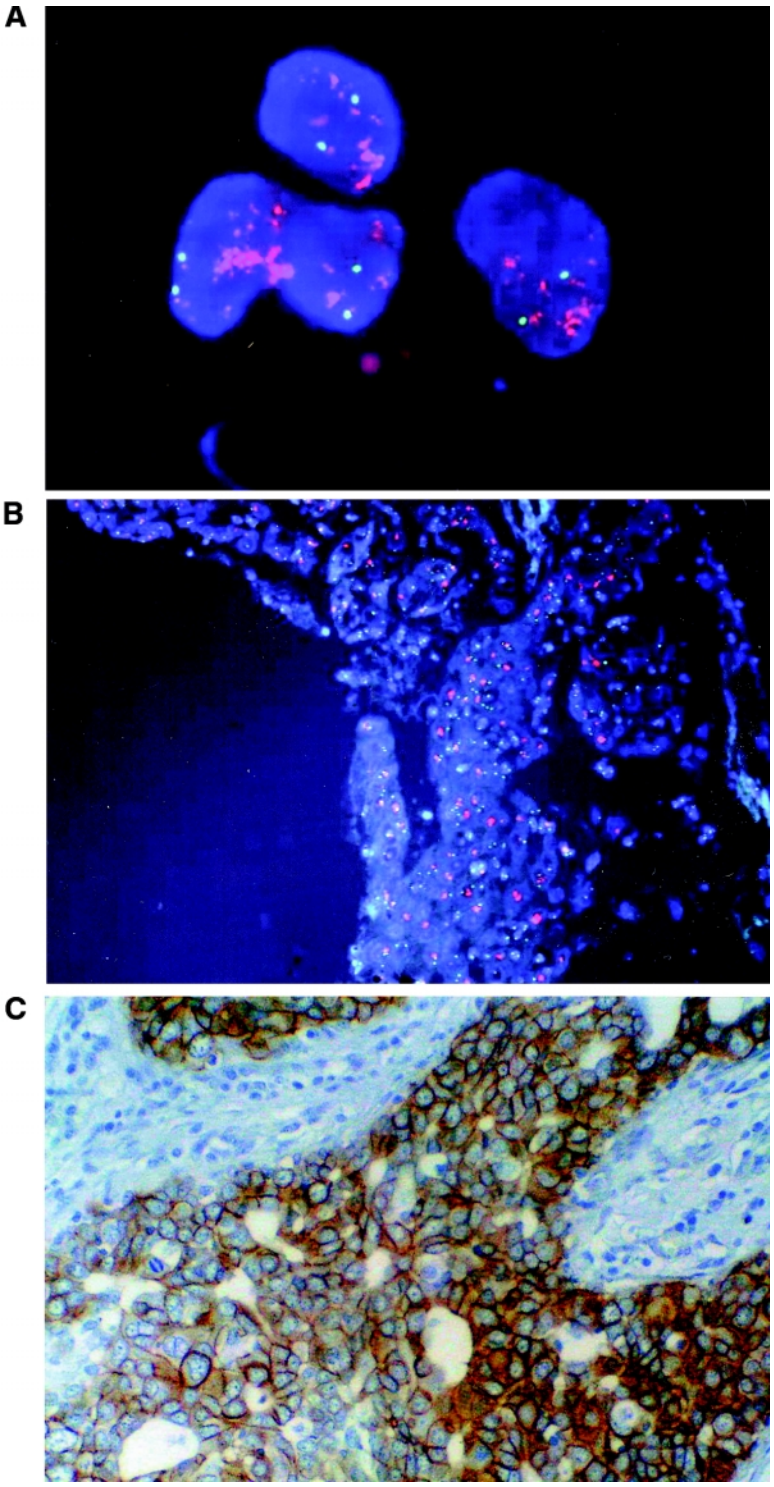

Figure 1. (**A**) Example of a breast cancer FNA hybridized with a *HER-2/neu* oncogene probe: multiple red signals in a cluster pattern indicate *HER-2/neu* amplification; (**B**) corresponding paraffin section: amplification is expressed in each cell as a red spot, instead of a cluster pattern, because of the low magnification; (**C**) corresponding *HER-2/neu* positively-immunostained paraffin section. Original magnification: (A) ×1250; (B) ×100; (C) ×400.

trastuzumab treatment; so far, different testing methods will be developed. The possibility of determining *HER-2/neu* status not only from histological samples but also on cytological specimens may help the clinician in treatment decisions.

Patients that overexpress *HER-2/neu*, who seem to be less responsive to cyclophosphamide, methotrexate and fluorouracil (CMF)-containing adjuvant therapy regimens, may benefit from high-dose anthracycline-based adjuvant chemo-

Table 1. *HER-2/neu* results comparing fluorescence *in situ* hybridization (FISH) on cytological samples with FISH and immunohistochemistry (IHC) on the corresponding histological sections

FISH cytology	Histology			
	FISH		IHC	
	Amplified	Unamplified	Overexpressed	Normal
Amplified	5	2	8	2
Unamplified	0	15	1	25
Concordance	20/22 (91%)		33/36 (92%)	

therapy and, when receptor positive, may be unresponsive to tamoxifen. The association described between *HER-2/neu* positivity and prognosis in human breast cancer is somewhat controversial. In lymph node-positive patients, most studies indicate that *HER-2/neu* is associated with decreased relapse-free survival and/or overall survival in univariate analyses. These findings suggest that *HER-2/neu* may be a biological marker of tumor aggressiveness. However, in multivariate analyses, *HER-2/neu* is found to be an independent prognostic factor in several studies, although not in others. In the case of lymph node-negative patients, most studies did not find *HER-2/neu* to be a prognostic indicator. In recent clinical trials, trastuzumab, a high-affinity humanized anti-HER-2 antibody, has shown benefit in the therapy of patients with metastatic *HER-2/neu* overexpressing breast cancer. Clinical trials with trastuzumab have recently been reviewed by Baselga [8].

Up to now, few studies have evaluated the feasibility of detecting *HER-2/neu* amplification by FISH in breast cancer FNAs [3–5]. *HER-2/neu* results obtained by FISH on FNAs from surgical samples or on FNAs from breast cancer patients have been correlated with the results obtained by immunocytochemistry on the corresponding frozen sections or on cytological smears. To our knowledge, only one report has correlated the detection of *HER-2/neu* amplification by FISH both on breast cancer FNAs and on the corresponding frozen section [6].

In our study, *HER-2/neu* has been evaluated by FISH on breast cancer FNAs and both by FISH and IHC on the corresponding paraffin sections. A good concordance was obtained between FISH cytology and FISH histology (91%) as well as between FISH cytology and IHC (92%). In our opinion, the discrepancies between FISH cytology and FISH histology, could mainly be ascribed to the fact that FISH spots on cytological preparations are more easily visualized than those on the corresponding tissue section, as previously reported by Klijanienko et al. [6]. The presence of monolayered and isolated cells allows for a more accurate signal enumeration on FNAs than on histological sections where fluorescence signal clusters are often prevalent. For this reason, while cytological samples are classified by visual evaluation only, for histological samples the use of image analysis is recommended. On the other hand, difficulties in detecting and counting the signals on cytological samples, which showed considerable thickness and overlapping cells, could be overcome by proteinase K pretreatment. FISH on FNAs allows for the visualization of *HER-2/neu* on a cell-by-cell basis. Counterstaining with DAPI permits the recognition of some nuclear details for the differentiation between epithelial cells and host elements. The application of a dual-color FISH technique, using a probe for the *HER-2/neu* gene together with a probe for a pericentromeric region of chromosome 17, has the advantage of providing an accurate evaluation of gene copy number alterations, allowing one to distinguish between tumors with normal *HER-2/neu* gene content, tumors with a gain of only a few extra copies of the *HER-2/neu* gene, tumors highly amplified and tumors with a *HER-2/neu* deletion.

Table 2. *HER-2/neu* results comparing fluorescence *in situ* hybridization (FISH) on cytological samples with ploidy

FISH cytology	Ploidy	
	Diploid	Aneuploid
Amplified	0	6
Unamplified	10	0
Polysomic	0	2

With respect to the correlation between FISH cytology and IHC, two cases that were amplified on cytology, were classified as polysomic on histology and not overexpressed by IHC. One of these cases showed an aneuploid DNA pattern.

In agreement with previous studies [6, 9], *HER-2/neu* status was found to be strongly correlated with ploidy. Among the 18 cases submitted for flow cytometry, all unamplified cases were diploid and all amplified or polysomic cases were aneuploid.

In conclusion, our data suggest that FISH may be a reliable procedure for *HER-2/neu* assessment on cytological specimens. FISH testing is suitable on unstained smears for patients who are candidates for preoperative chemotherapy, as well as on smears already stained for patients whose paraffin blocks of the primary tumor are not available [5]. Moreover, since some differences in *HER-2/neu* expression between primary tumors and their lymph node metastases have been described [10],

suggesting that *HER-2/neu* status of the primary tumor may differ from that of the metastases, FISH on FNAs from metastatic sites could not only allow for a better definition of *HER-2/neu* status, but also aid in the subsequent treatment decisions.

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