HER-2/*neu* Gene Amplification Characterized by Fluorescence In Situ Hybridization: Poor Prognosis in Node-Negative Breast Carcinomas

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Purpose: **The HER-2/*neu* gene codes for a membrane receptor protein that is homologous, but distinct from the epidermal growth factor receptor. This investigation was performed to validate fluorescence in situ hybridization (FISH) as a sensitive and specific method for assessing HER-2/*neu* gene amplification in archival tissue and to test whether this alteration is associated with poor prognosis.**

Materials and Methods: **HER-2/*neu* gene amplification was determined by FISH in 140 archival breast cancers, previously characterized for gene amplification by Southern hybridization or dot-blot hybridization, and for gene expression by Northern hybridization, Western immunoblot, or immunohistochemistry. A separate cohort of 324 node-negative breast cancers was assessed for amplification by FISH to determine the utility of HER-2/*neu* gene amplification.**

Results: **Relative to solid-matrix blotting procedures, FISH analysis of HER-2/*neu* gene amplification showed a sensitivity of 98% and a specificity of 100% in 140 breast cancers. Among patients treated by surgery only, the relative risks (relative hazard) of early recurrence (recurrent disease within 24 months of diagnosis), recurrent disease (at any time), and disease-related death were statistically significantly associated with amplification. The prognostic information contributed by HER-2/*neu* amplification was independent of the other markers studied.**

Conclusion: **FISH was an alternative technique for determining gene amplification and had some distinct advantages over Southern hybridization. Our results demonstrate that HER-2/*neu* gene amplification in the absence of adjuvant therapy is an independent predictor of poor clinical outcome and is a stronger discriminant than tumor size. Women with small tumors that had gene amplification were at increased risk of recurrence and disease-related death.**

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AMPLIFICATION AND OVEREXPRESSION of the HER-2/*neu* proto-oncogene has been described in approximately 25% of breast, ovarian, endometrial, gastric, and salivary gland carcinomas.[1-5] These alterations have been associated with an increased risk of recurrent disease or shorter overall survival in patients with each of these cancers.[1-6] HER-2/*neu* gene amplification is routinely analyzed by Southern or slot-blot hybridization. Although these are well-established and accepted methods, they require relatively large specimens, are subject to dilutional artifacts from inclusion of normal cells, and, because they depend on nondegraded DNA, results are normally obtained from frozen tissue. Based on the need to analyze small, paraffin-embedded tissues rapidly on a cell-by-cell basis, we investigated the use of fluorescence in situ hybridization (FISH) as a method for analysis of HER-2/*neu* gene amplification using a series of archival tumor specimens previously characterized molecularly for HER-2/*neu* gene amplification and expression. Finally, 324 axillary lymph node-negative breast cancers treated with surgical resection at three different institu-

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The Oncor HER-2/neu DNA Probe is for investigational use only.

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tions were analyzed to confirm that HER-2/*neu* gene amplification by FISH was a marker of poor prognosis.

MATERIALS AND METHODS

This study was performed in two phases. Phase I was performed to validate FISH as a method capable of assessing amplification in paraffin-embedded tissue. In phase II, paraffin-embedded tissue sections from sequentially diagnosed retrospectively identified invasive node-negative breast cancer patients were used to determine whether gene amplification, assayed by FISH, was prognostically useful.

Phase I: Validation of FISH Using Molecularly Characterized Breast Cancers

Breast cancer specimens with known HER-2/*neu* gene copy and expression levels were selected for study as archival tissue specimens. Amplification had been previously determined by Southern blot using DNA extracted from frozen tumor specimens and expression had been determined by Northern hybridization, Western immunoblotting, and immunohistochemistry using total RNA, total protein, and histologic sections, respectively, from frozen tumor tissue.[5] Amplification had been previously analyzed by slot-blot analysis and expression by immunohistochemistry alone in 19 cases.[6] To minimize inclusion of cases that were misclassified due to dilutional artifacts associated with solid-matrix blotting methods, samples selected as representative of gene amplification were required to show both amplification and overexpression, while cases selected as representative of nonamplified samples were required to show no gene amplification or overexpression. One hundred forty cases were included as validation samples for this phase of the study, with 50 showing both amplification and overexpression and 90 showing no amplification or overexpression by solid-matrix blotting.

Paraffin-embedded tissue sections. The breast cancer specimens used for the validation phase of the study were contained in two multitumor paraffin-embedded tissue blocks.[7] These blocks, prepared according to the methods of Battifora and Mehta[8] from paraffin-embedded tissue blocks obtained from institutional archives, contained small strips of each breast cancer specimen. This methodology permits the use of small quantities of reagents and, more importantly, assures equal exposure of each breast cancer to all of the reagents in the assay system.

FISH. HER-2/*neu* gene copy level was determined in paraffin-embedded tissue sections. Since the HER-2/*neu* gene is located on chromosome 17,[9] an alpha satellite (pericentromeric) DNA probe (Oncor, Inc, Gaithersburg, MD) for chromosome 17 was selected as an internal control for chromosomal aneuploidy. By comparing the number of copies of these two chromosomal markers, aneuploidy of chromosome 17 was excluded as a source of increased HER-2/*neu* gene copy number. The alpha-satellite DNA was also used as an internal control to correct for differences that might arise due to tissue sectioning artifact in paraffin-embedded sections. FISH was performed as previously described.[3,5] The nuclei were routinely counterstained with 4'-6'-diamidino-2'-phenylindole (DAPI). Some cases were also counterstained with acridine orange. Staining was visualized with a Zeiss fluorescence microscope. SK-BR-3 human breast cancer cells, known to have HER-2/*neu* gene amplification, and MDA-MB-468 human breast cancer cells, known to lack HER-2/*neu* gene amplification, were used as control cells (HTB30 and HTB132; American Type Culture Collection, Rockville, MD)[10] (Zhou and Press, unpublished data, July 1993).

If a total of 100 nuclei could be identified and scored for both HER-2/*neu* and chromosome 17 centromere, the sample was included in the validation study. Based on the analysis of these results, scoring fewer nuclei was acceptable for the clinical outcome phase of the study (see Results). A mean HER-2/*neu* gene copy per tumor-cell nucleus and a mean chromosome 17 centromere count per tumor-cell nucleus were determined for each preparation. Using criteria established for Southern hybridization,[3,5,11] HER-2/*neu* gene amplification was defined as a HER-2/*neu*-to-chromosome 17 ratio greater than 2.0.

Phase II: Clinical Study of Axillary Lymph Node-Negative Breast Cancers

A total of 382 archival breast cancer specimens were identified in the records of the M.D. Anderson Cancer Center (107 cases), the University of Wisconsin (116 cases), and the University of Iowa (159 cases), and retrieved for analysis by FISH to assess the relationship between HER-2/*neu* gene amplification and three clinical outcomes. Inclusion in this study was based on a diagnosis of invasive breast cancer made before June 1, 1990, lack of tumor in axillary lymph nodes, availability of tissue blocks, primary treatment by surgery alone without radiation therapy, adjuvant chemotherapy, or hormone therapy except after development of recurrent disease, and availability of at least 24 months of clinical follow-up information unless recurrent disease was identified. Cases were disqualified for lack of tumor or insufficient tissue in blocks (nine cases), failure to locate paraffinized tissue blocks in the institutional archives (eight), a technically inadequate test result (two), ineligible tumor type (eg, noninvasive breast carcinoma [DCIS], nonprimary breast carcinoma) (26 cases), involved axillary lymph nodes (eight), and duplicate specimens (five), which left 324 invasive node-negative primary tumor specimens with FISH results. Additional cases were eliminated from the analysis of early recurrent disease because of adjuvant treatment with chemotherapy, radiation therapy or tamoxifen (71 cases) and lack of sufficient clinical follow-up information (11 cases) for a total of 242 cases assessable for early recurrent disease (recurrence within 24 months of diagnosis). The evaluation of recurrence was restricted to 234 patients with recurrence at any time or, if recurrence-free, who had at least 36 months of follow-up data. Evaluation of disease-related death was restricted to 232 patients known to have died with disease or who had at least 36 months of follow-up data.

Routine paraffin-embedded tissue sections of each breast cancer were analyzed for HER-2/*neu* gene copy level at three study sites (University of Iowa, University of Wisconsin, and University of Southern California). In addition to information on dates of recurrence, follow-up evaluation, and death, data on age at diagnosis, tumor size, and estrogen receptor (ER) and progesterone receptor (PR) status were obtained from medical records.

FISH of node-negative breast cancers. FISH analysis of the invasive node-negative breast carcinomas was performed in a fashion similar to that used in phase I except that only the biotinylated-HER-2/*neu* probe (Oncor, Inc) was used. This change was made because the use of chromosome 17 probe in the 140 phase I validation cases did not provide additional discrimination between amplified and nonamplified tumor above that obtained using a cut-off of more than four copies of HER-2/*neu* per cell (see Results). Gene copy level was assessed in at least two sets of 20 nonoverlapping

nuclei, rather than in 100 nuclei per case because correct classification could be made in all validation cases with this smaller number of nuclei (see Results). As in phase I, the nuclei were selected for assessment purely on the basis of nuclear morphology with tumor-cell nuclei assessed with a DAPI filter under a low- (4 ×) and/or intermediate-power (10 ×) microscope objective.

Assessment of tumor grade. The Scarff-Bloom-Richardson grading system, which is based on a numerical score that represents the sum of scores assigned to each of three independently evaluated histologic features, was used to grade each tumor specimen.[12] The histologic features used in this system are (1) the degree of architectural differentiation, (2) the extent of pleomorphism or anisonucleosis, and (3) the mitotic rate.

Hematoxylin-and-eosin–stained slides were requested from each of the three institutions that contributed cases to this phase of the study. At the time the request was made, slides for 197 of the 242 cases were provided; however, the slides provided for 13 cases did not contain the invasive breast carcinoma and were excluded from further evaluation. A slide from each of 184 cases was coded, relabeled, and independently reviewed and graded by three board-certified pathologists. A consensus tumor grade, defined as assignment of the same grade to a breast cancer by two or more pathologists, was achieved for 182 of 184 cases. The remaining two cases were reviewed by a fourth board-certified pathologist to achieve consensus. Fifty-seven breast cancers were grade 1, 70 were grade 2, and 57 were grade 3.

Statistical analysis. For the phase I validation of FISH, we determined whether a particular sample was amplified or nonamplified by examining 100 cells and calculating 99% confidence intervals (CIs) for the ratio of the mean number of HER-2/*neu* signals to the mean number of chromosome 17 centromeres using the delta method.[13] Samples were considered ambiguous if the CI included the value 2, nonamplified if the upper confidence limit was less than 2.0, and amplified if the lower confidence limit exceeded 2.0. Similar analyses were conducted using the results from fewer sets of cells and all results were compared with those based solely on the number of HER-2/*neu* gene copies per cell. The sensitivity and specificity of the FISH procedure was determined relative to solid-matrix blotting results.

For the study of node-negative breast cancer, we used standard life-table and Cox proportional hazards methods to examine the risk of early recurrence, recurrence, and disease-related death associated with HER-2/*neu* status, age, ER status, PR status, tumor grade, and tumor size.[14] Multivariate relationships were assessed using Cox proportional hazards methods. Analyses were performed that included a missing category for ER status, PR status, tumor grade, and tumor size, as well as excluded subjects with missing information. Since the results obtained were consistent with regard to the effects of amplification on early recurrence, recurrence, and disease-related death, the data presented here are for those analyses in which a category for missing data was included for each of the relevant covariates. In the results that follow, we use the term relative risk to represent the relative hazard.

RESULTS

FISH Analysis of Molecularly Characterized Breast Cancers

Fluorescent signals for HER-2/*neu* gene and chromosome 17 alpha-satellite DNA were readily identified in tumor-cell nuclei (Fig 1) and in the normal non–tumor-cell nuclei of paraffin-embedded tissue sections. In cases in which HER-2/*neu* gene amplification was identified (see following), only tumor-cell nuclei showed this alteration. Normal inflammatory connective tissue and vascular cells in those cases did not show increased numbers of HER-2/*neu* gene signals.

Of 140 cases analyzed in the phase I validation study, all had known amplification levels, determined by Southern or dot-blot analysis, and known expression levels, determined by Northern hybridization, Western immunoblot analysis, and/or immunohistochemistry.

Successful probe hybridization was obtained in paraffin-embedded tissue sections for all 140 cases in the validation study. When hybridization was achieved, intranuclear signals were observed in a relatively uniform fashion throughout the tissue section. The DNA in these tissues appeared to be stable. Forty-nine of 50 cases with known gene amplification and overexpression had a HER-2/*neu*-to-chromosome 17 ratio greater than 2.0. One case with gene amplification by Southern hybridization was not identified as amplified by FISH. All 90 cases known to lack gene amplification and to have low HER-2/*neu* expression had a HER-2/*neu*-to-chromosome 17 ratio of less than 2. Among these 90 nonamplified, low-expression cases, the mean HER-2/*neu*-to-chromosome 17 ratio was 1.1 (99% CI, 1.0 to 1.2), which indicates that the ratio of 2.0 used to identify gene amplification was valid. The sensitivity of FISH for HER-2/*neu* gene amplification was 98% and the specificity was 100% relative to solid-matrix blotting techniques (Table 1).

Number of cells needed for assessment of gene amplification. Although analysis of HER-2/*neu* gene amplification by FISH correlated strongly with gene amplification determined by Southern hybridization, analysis of 100 tumor-cell nuclei by microscopic inspection was time-consuming and tedious. It became subjectively apparent during the course of the analysis that an accurate assessment of gene amplification could be made with analysis of fewer cells.

Based on the examination of 100 cells and calculation of 99% CIs for the ratio of the mean number of HER-2/*neu* signals to the mean number of chromosome 17 centromeres, three cases (2.3%) were considered ambiguous (CIs included 2). To determine the number of tumor cells required to achieve a consistent assessment of gene amplification status, we assumed that the first nucleus entered in our data file for each case was the first nucleus scored in each of a series of progressively larger samples beginning with 10 nuclei. Excluding the three ambiguous

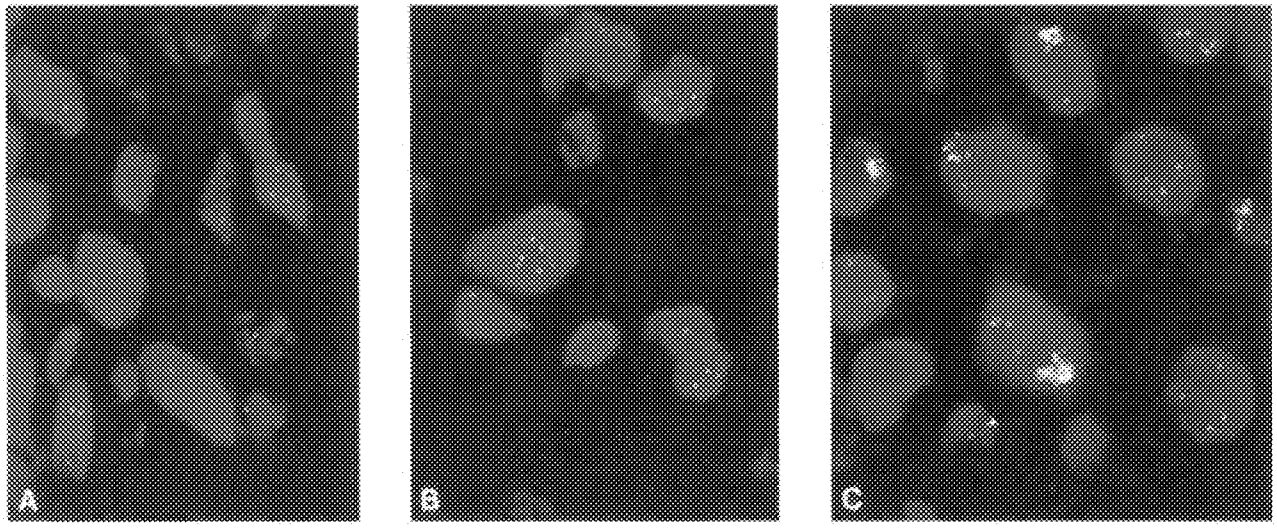

Fig 1. HER-2/*neu* gene, localized by green fluorescent signals, and alpha-satellite DNA of chromosome 17, localized by red fluorescent signals, in interphase nuclei of breast carcinoma cells (blue). (A) Breast cancers lacking HER-2/*neu* gene amplification demonstrate the normal diploid content of HER-2/*neu* signals per nucleus. (B) Breast cancers with low levels of HER-2/*neu* gene amplification show an increased number of HER-2/*neu* gene copies with HER-2/*neu*-to-chromosome 17 signal ratios > 2.0. (C) Breast cancers with high levels of HER-2/*neu* gene amplification also show an increased number of HER-2/*neu* gene copies with HER-2/*neu*-to-chromosome 17 signal ratios > 2.0.

cases, 53% of cases could be unambiguously assigned as amplified or nonamplified after scoring 10 nuclei, 71% after scoring 15 nuclei, 84% after 20 nuclei, 93% after 40 nuclei, 98% after 60 nuclei, and 99% after 80 nuclei. No differences in classification occurred when HER-2/*neu* average counts in the first 20 tumor-cell nuclei were compared with counts in the last 20 nuclei and with counts of the entire set of 100 nuclei. Furthermore, after scoring 20 tumor-cell nuclei, all cases were correctly categorized as amplified or nonamplified if the CI was ignored and only the comparison of the ratio to the value 2.0 was considered.

Gene amplification by FISH with and without chromosome 17 centromere counts. During FISH assessment of the 140 validation cases, we observed relatively little variation in the average number of chromosome 17 centromeres per tumor-cell nucleus. The range of chromosome 17 centromere averages in tissue sections was 0.5 to 3.2 with more than 96% averaging 2 or fewer chromosome 17 centromeres per tumor-cell nucleus. Unexpectedly, none of the cases in this group of breast cancers demonstrated a parallel increase in both chromosome 17 centromere and HER-2/*neu* copies, which suggests that none of the increased HER-2/*neu* copies was due to aneuploidy alone. Since chromosome 17 centromeres contributed little and since HER-2/*neu* counts contributed greatly to the assessment of amplification, analysis of HER-2/*neu* gene alone appeared to provide an accurate assessment of gene amplification. During the G2 phase of the cell cycle, cells will replicate each gene. Therefore, a gene that is present in the cell at 2 or fewer copies would be increased to three or four only during these phases of the cell cycle. A cell with an average of more than four copies of HER-2/*neu* would be expected to have an abnormally increased number of copies, ie, gene amplification. We reanalyzed our data for HER-2/*neu* copy number without chromosome 17 centromere copy number using 4.0 as the point of separation for nonamplified and amplified cases to determine if this simpler approach provided accurate classification of samples. All validation cases were classified the same as when using chromosome 17. Since chromosome 17 centromere counts provided no additional infor-

Table 1. Her-2/*neu* Amplification Assessed by Solid-Matrix Blotting Techniques Compared With FISH

	Southern or Dot-Blot Hybridization		
FISH	Amplified	Not Amplified	Total
Amplified	49	0	49
Not amplified	1	90	91
Total	50	90	140

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