HER-2/neu gene amplification in stages I–IV breast cancer detected by fluorescent *in situ* hybridization

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Purpose: Approximately 25–30% of breast and ovarian carcinomas have amplification of the HER-2/neu oncogene. The aim of the present study was to focus on HER-2/neu gene amplification in different clinical stages of breast cancer in order to (1) determine if fluorescent *in situ* hybridization (FISH) can be used to detect HER-2/neu gene amplification in different clinical stages of breast cancer, (2) establish whether HER-2/neu gene amplification characterizes a subset of breast cancer in each of these stages, and (3) determine whether a trend for correlation of amplification with the clinical stage of the disease can be detected using the FISH technology. **Methods:** A total of 40 specimens of formalin-fixed, paraffin-embedded breast cancer tissues were analyzed cytogenetically, in a blinded fashion, for HER-2/neu gene amplification using FISH and the Vysis LSI HER-2/neu Orange and CEP 17 Green DNA dual color probe. The criterion for "high amplification" was an amplification ratio of >4.0, that for "moderate amplification" a ratio between 2.1 and 4.0, and that for "low amplification" a ratio of 1.5–2.0. **Results:** Using a cutoff point of ≥1.5, the overall frequency of HER-2/neu gene amplification among stage I tumors was 30% (3 out of 10). Of these, one-third (1 out of 3) showed low amplification, one-third (1 out of 3) were moderately amplified, and one-third (1 out of 3) were highly amplified. The overall frequency of HER-2/neu gene amplification among stage II tumors was 0% (0 out of 10). The overall frequency of HER-2/neu gene amplification among stage III tumors was 10% (1 out of 10). The sole tumor found positive was classified as moderately amplified by our criteria. The overall frequency of HER-2/neu gene amplification among stage IV tumors was 50% (5 out of 10). Four of the 5 tumors found positive were highly amplified. The overall frequency of gene amplification in the 40 cases studied was 22.5% (9 out of 40 tumors studied). **Conclusion:** Although a linear correlation between HER-2/neu amplification and clinical stage cannot be established at this time, it is interesting to note that when stages I and II, and when stages III and IV are combined, respectively, the latter category has a higher amplification frequency than the former. Furthermore, stage IV has the highest frequency (5 out of 10) of HER-2/neu gene amplification than all three lower stages combined (4 out of 30). This is no doubt due to the high frequency of gene amplification observed in stage IV tumors, which, interestingly, also demonstrate high level amplification of HER-2/neu gene copy numbers. Although the biologic and clinical basis for gene amplification is not clear, given the observation that the most aggressive disease stage is associated with the highest frequency of gene amplification and the most high level amplification, further exploration of HER-2/neu as a prognostic marker of poor outcome using FISH is warranted. ***Genetics in Medicine*, 1999; 1(3):98–103**

Key words: *Breast cancer, fluorescent in situ hybridization, HER-2/neu gene amplification, infiltrating ductal carcinoma of the breast, stage I breast cancer, stage II breast cancer, stage III breast cancer, stage IV breast cancer*

Previous research in this laboratory has focused on molecular cytogenetic markers in breast cancer.[1–4] The mapped location of the HER-2/neu (erb B-2) oncogene is on human chromosome 17q21. HER-2/neu gene amplification has been reported in a number of cancers, including breast, ovarian, endometrial, and salivary gland tumors.

The present study focuses on the exploration of HER-2/neu amplification in breast cancer using FISH as an alternative or adjunct to the traditional methodologies of immunohistochemistry (IHC) and molecular biology. We aim to establish whether HER-2/neu amplification as detected by FISH characterizes a subset of breast cancer, and whether this is correlated with the clinical stage of the disease.

MATERIALS AND METHODS

Breast cancer cases

Ten cases of stage I (Figure 1a), 10 cases of stage II (Figure 1b), 10 cases of stage III (Figure 1c), and 10 cases of stage IV (Figure 1d) infiltrating ductal breast cancer were identified from the database at Rhode Island Hospital. Formalin-fixed,

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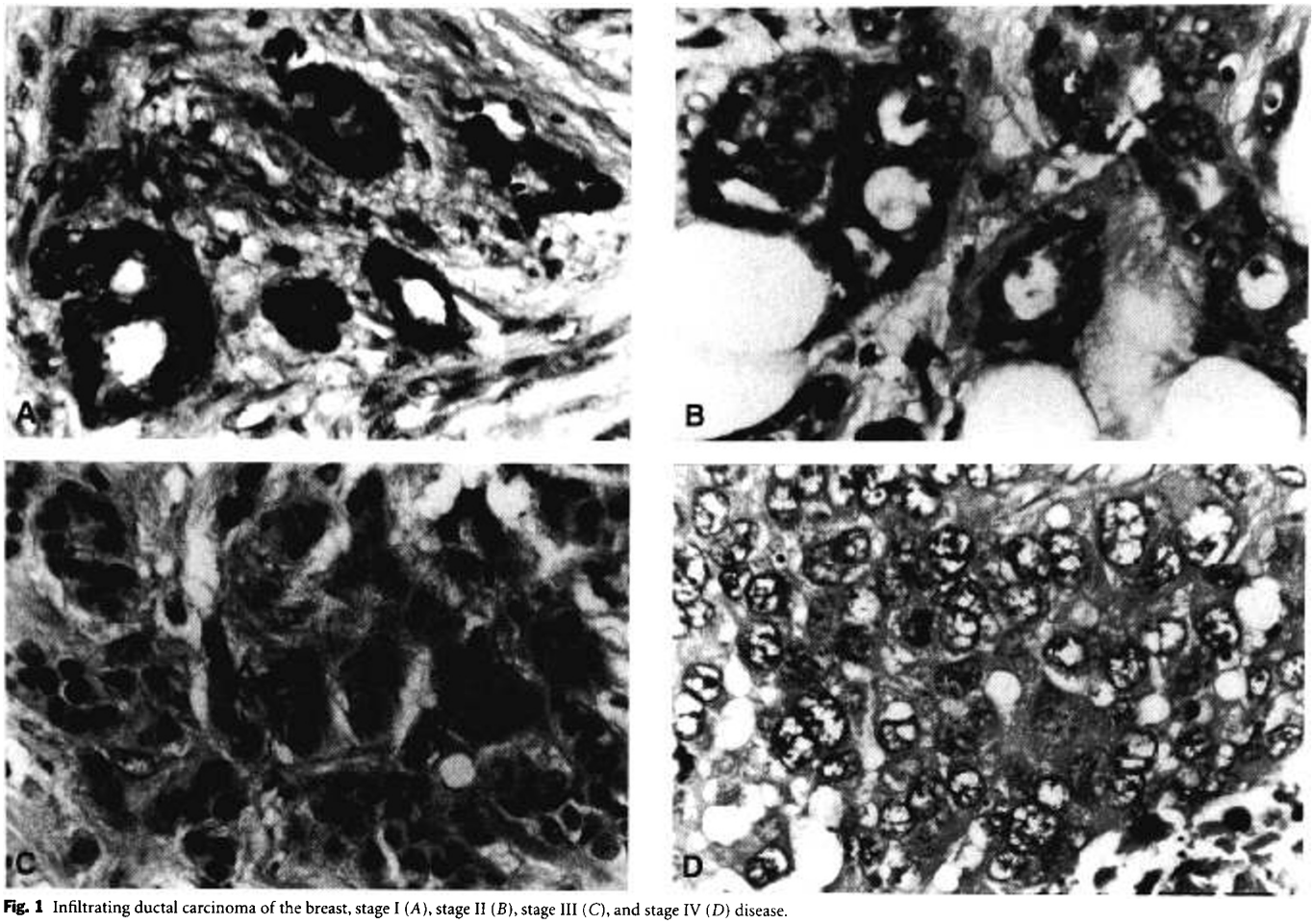

Fig. 1 Infiltrating ductal carcinoma of the breast, stage I (*A*), stage II (*B*), stage III (*C*), and stage IV (*D*) disease.

paraffin-embedded tissue blocks of the cases were retrieved from the routine surgical pathology files of the Department of Pathology. For each specimen, 6-micron thick consecutive sections were cut. At least one slide from consecutive sections was stained by H & E, the diagnosis confirmed and the location of the tumor cells identified. The tumors were also evaluated for histological grade according to a modified Bloom and Richardson grading system,[5] although we did not segregate the tumor specimens in the present study according to pathologic grade. The sections adjacent to the H & E stained section were then processed for FISH. FISH assays were performed and HER-2/neu and CEP 17 signals were enumerated in the target areas where tumor cells were located. Extra sections, wherever available, were prepared for possible repeat of the assay.

Fluorescent in situ hybridization

For fluorescent *in situ* hybridization (FISH),[6–8] manufacturer's instructions (Vysis, Downers Grove, IL) were followed. Briefly, formalin-fixed, paraffin-embedded, 6-micron thick tissue sections were applied to silanized glass slides. Slides were baked at 60°C for 60 minutes. Specimens were deparaffinized by immersing them three times in Histoclear (National Diagnostics, Atlanta, GA) for at least 10 minutes each at room temperature, dehydrated, and air-dried. Slides were pretreated by immersing in 0.2 N HCl for 20 minutes, washed and treated with a Pretreatment Solution composed of 1 N sodium thiocyanate (NaSCN) at 80°C for 30 minutes. After washing with distilled water and a Wash Buffer (2×SSC, pH 7.4), the slides were treated for 10 minutes at 37°C in a Protease Solution (2500–3000 U/mg Protease, lyophilized, 0.9% NaCl, pH 2.0). The slides were subsequently washed twice in Wash Buffer for 5 minutes, and air-dried. They were then placed in 10% buffered formalin at room temperature for 10 minutes, washed twice in Wash Buffer for 5 minutes and air-dried. Thereafter, the protocol for FISH on regularly prepared cytogenetic slides were followed.

Specimen slides were denatured in 70% formamide/2×SSC (pH 7.0–8.0) at 72°C and immediately washed in 70%, 85%, and 100% ethanol rinses. Ten microliters of the pre-mixed, pre-denatured HER-2/neu probe was applied to the target area on each slide. A 22 mm × 22 mm glass coverslip was applied to the slide. The coverslip was sealed with rubber cement. Slides were then incubated overnight in a 37°C incubator.

The Vysis (Downers Grove, IL) LSI HER-2/neu DNA probe is a SpectrumOrange directly labeled fluorescent DNA probe specific for the HER-2/neu gene locus (17q11.2–q12). The CEP 17 DNA probe is a SpectrumGreen directly labeled fluorescent DNA probe specific for the α-satellite DNA sequences at the centromeric region of chromosome 17 (17p11.1–q11.1).

Fluorescent signal scoring

Hybridization signals were enumerated among tumor cells. The target areas were identified by H & E stain on every tenth slide of the same tissue block. As a quality control measure a reproducibility study (data not shown) was performed to ensure repeatability of results and reliability of data, details of which will be described elsewhere (Masood et al., unpublished data). Once the quality control/quality assurance measures have been satisfied, scoring was performed under 1000× magnification independently by operators without any prior clinical information or knowledge of other histological and pathological results. A standardized signal enumeration guide was followed, in which non-overlapping nuclei with well-defined nuclear outlines were chosen for scoring fluorescent signals, as adopted by Mark et al.,[9–14] Kim et al.,[15] and Hopman et al.[16] Only bright, unambiguous signals were scored as positive. Slides with suboptimal quality hybridizations were repeated and reanalyzed. Because the nuclei are usually present in slightly different planes of the section, the focus of the microscope was constantly adjusted to enable all positive signals to be visualized. Except for one tumor with 42 scorable cells and one case with 46 scorable cells, all other 38 tumors had 99–133 evaluable cells for FISH analysis.

Calculation of amplification ratios

The raw data on the number of HER-2/neu gene copy number and CEP 17 copy number were entered in a 2-way table. The average copy numbers for both HER-2/neu and CEP 17 were calculated separately using the marginal totals in the 2-way table. For example, if for HER-2/neu, there were 10 cells with 5 copies, 10 with 7 copies, 5 with 11–15 copies, and 5 cells with ≥20 copies in a sample of 30 cells, the average copy number would be calculated as follows:

HER-2/neu copy number = $[10 \times 5 + 10 \times 7 + 5 \times 13^{*} + 5 \times 25^{**}]/[30] = [310]/[30] = 10.33$, where * represents the midpoint for 11–15 copies and ** represents an estimated value for ≥ 20 copies. CEP copy number was calculated analogously.

The amplification ratio is then calculated by dividing the HER-2/neu copy number by the CEP copy number to correct for copy number. For example, for the above illustration, if the CEP 17 copy number were found to be 2, then the amplification ratio would be 10.33 divided by 2 or 5.165.

Slides with amplification ratios of less than 1.5 were classified as "nonamplified." Slides with amplification ratios equal to or greater than 1.5 were classified as "amplified." Furthermore, ratios between 1.5 and 2.0 were considered as "low amplification." Ratios between 2.1 and 4.0 were considered as "moderate amplification." Ratios exceeding 4.0 were considered as "high amplification." An analogous classification system was used by Young et al. (1996)[17] in ovarian cancer, although the protocols for amplification ratio calculations in our two studies are not completely equivalent.

RESULTS

A total of 40 specimens of formalin-fixed, paraffin-embedded breast cancer tissues were analyzed cytogenetically, in a blinded fashion, for HER-2/neu gene amplification using a previously optimized FISH protocol (Masood et al., 1998).[18] The results of the present FISH study are given in Table 1.

Table 1

Results of scoring for HER-2/neu gene amplification using FISH with a HER-2/neu probe (orange) and a chromosome 17 specific α-satellite control probe (green) on formalin-fixed, paraffin-embedded tumor tissue

	Number of cells counted	HER-2/neu: CEP 17 ratio
Stage I		
96–6773–A3	100	1.32
97–151–A4	100	3.91
96–913–A1	100	1.47
97–3934–A1	100	1.19
96–7158–A2	100	7.38
97–5058–B1	100	1.29
96–1310–A3	100	1.47
97–2942–A5	100	1.50
96–4826–B29	100	1.18
S96–7053–B1	100	1.18
Stage II		
97–4222–A8	105	1.10
97–4033–A1	105	0.98
97–4826–A4	100	1.19
97–5821–A8	106	0.98
96–6510–A1	108	1.06
97–6040–B10	101	1.11
97–1025–A2	103	0.99
97–163–A5	110	1.07
97–9493–B1	100	0.79
97–2788–A2	100	0.93
Stage III		
97–4409–A9	42	0.94
S96–5187–A1	100	1.28
96–5028–A1	100	1.06
96–6727–A5	46	1.01
96–5613–A3	99	3.21
96–4520–A2	100	1.09
97–66–B1	100	1.04
97–5007–A4	100	1.00
97–4909–A3	100	0.42
97–6896–A1	100	0.97
Stage IV		
96–4979–A1	133	1.28
97–187–A5	126	2.16
S96–7267–A2	100	10.00
95–2580–A6	100	6.31
96–8448–B5	100	1.01
96–12148–A2	120	1.36
96–13089–A4	100	4.52
96–15015–A1	100	1.35
95–11771–A1	100	1.12
95–280–A1	100	4.69

Of the 40 cases studied, 10 were stage I, 10 were stage II, 10 were stage III, and 10 were stage IV.

Using the criteria defined above, the frequency of HER-2/neu gene amplification among stage I tumors was 30% (3 out of 10). Of these, one-third (1 out of 3) showed low amplification, one-third (1 out of 3) were moderately amplified, and one-third (1 out of 3) were highly amplified. The frequency of HER-2/neu gene amplification among stage II tumors was 0% (0 out of 10). The frequency of HER-2/neu gene amplification among stage III tumors was 10% (1 out of 10). The sole tumor found positive was classified as moderately amplified by our criteria. The frequency of HER-2/neu gene amplification among stage IV tumors was 50% (5 out of 10). Four of the 5 tumors found positive were highly amplified. These results are summarized in Table 2. Representative fields illustrating HER-2/neu non-amplification and gene amplification are given in Figures 2 and 3.

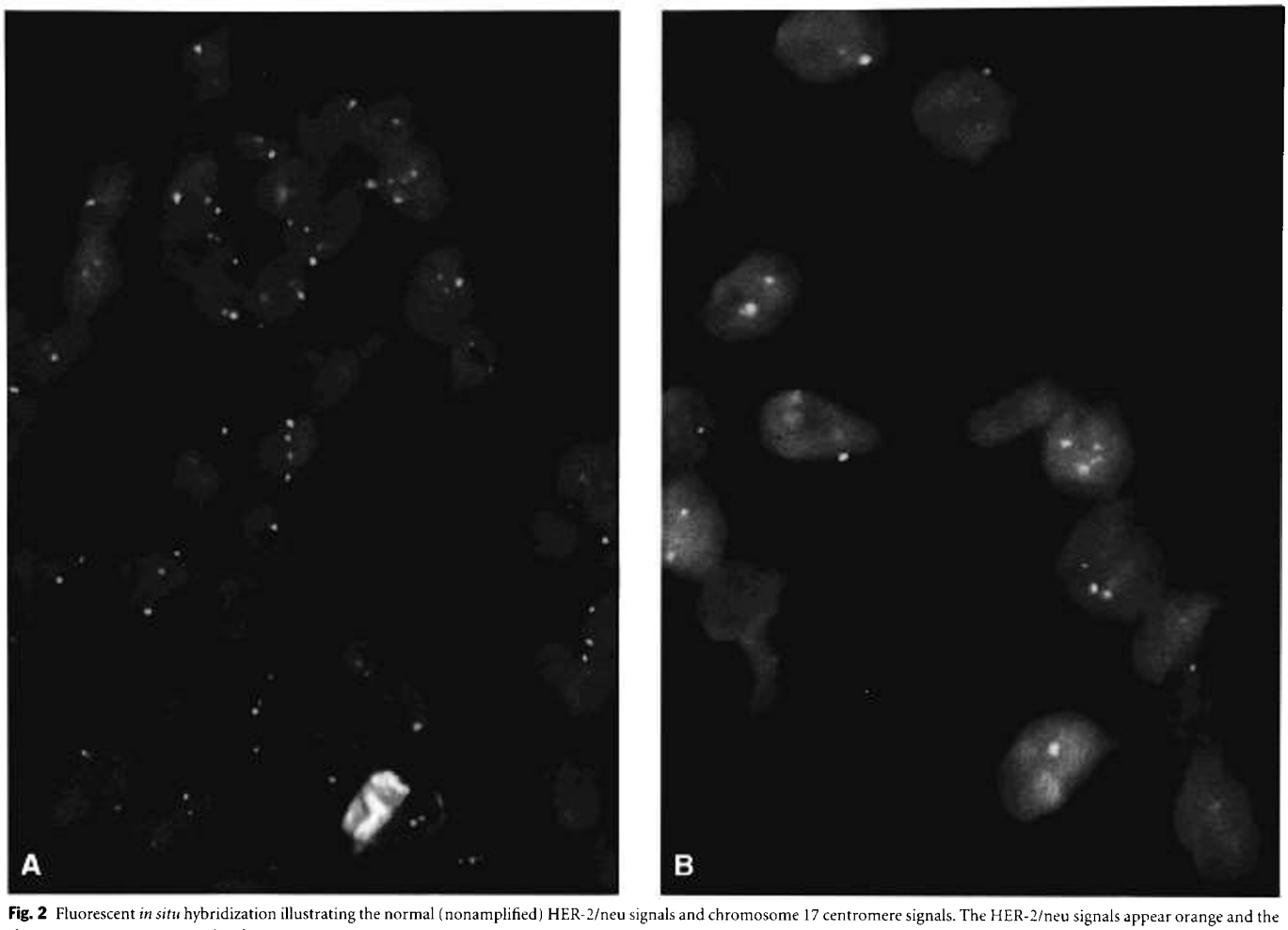

Fig. 2 Fluorescent *in situ* hybridization illustrating the normal (nonamplified) HER-2/neu signals and chromosome 17 centromere signals. The HER-2/neu signals appear orange and the chromosome 17 centromere signals appear green.

DISCUSSION

The HER-2/neu oncogene codes for a 185 kilodalton transmembrane oncoprotein. Approximately 25–30% of breast and ovarian carcinomas have amplification of the HER-2/neu gene.[19–21] Gene amplification is a characteristic of cancer cells that allows increased production of specific proteins used for the acquisition and maintenance of the malignant phenotype. Amplification of oncogenes may play important roles in the progression of many tumors.

Table 2
Summary of HER-2/neu gene amplification results

	Clinical stage				
Amplification status	Stage I	Stage II	Stage III	Stage IV	Total
Nonamplified*	7	10	9	5	31
Low amplification**	1	0	0	0	1
Moderate amplification***	1	0	1	1	3
High amplification****	1	0	0	4	5
Total	10	10	10	10	40

*Amplification ratio <1.5.
**Amplification ratio 1.5–2.0.
***Amplification ratio 2.1–4.0.
****Amplification ratio >4.0.

FISH is a useful technique for quantitation of HER-2/neu gene amplification because it can assess the level of HER-2/neu gene amplification directly in the tumor cells while retaining the characteristic morphology of the tissue studied. FISH assay is rapid, non-radioactive, requires very little material, and is capable of detecting lower level amplification (2–8 gene copies) of the oncogene. Preliminary data indicate that FISH is probably superior to Southern blot or immunohistochemistry analysis (Vysis, unpublished data). In a recent study, Persons, Borelli, and Hsu[22] found that the immunoperoxidase procedure was less reproducible than FISH in touch preparations from pulverized tumors. They postulated that several factors most likely contributed to their findings. First, the increased reproducibility of FISH might be related to the objectivity associated with FISH analysis (i.e., counting fluorescent signals) compared with the subjectivity involved with analyzing immunoperoxidase stained cells or tissue (i.e., visually estimating the degree of intensity of staining and percentage of nuclei stained). A second factor that might contribute to the discrepancies between the two procedures may be the relative stability of DNA compared with protein, especially in paraffin-embedded samples.

FISH may also overcome many of the inherent technical limitations and difficulties in interpretation associated with other techniques. In the present study, FISH assessment of HER-2/neu amplification was achieved with the LSI HER-2/neu Orange and CEP 17 Green DNA dual color probe. Fluorescent signals in interphase nuclei on formalin-fixed paraffin-embedded archival slides were enumerated. The use of chromosome 17 copy number to derive the amplification ratio served as an internal control to distinguish between polysomy chromosome 17 and oncogene amplification.

As can be seen by examining the data presented in Tables 1 and 2, the overall frequency of gene amplification was 22.5%. This is comparable to the 25–30% frequency generally quoted in the literature for breast and ovarian cancers.[19–21]

From Table 2, it can be seen that 1 out of 3 tumors showed low amplification, 1 out of 3 showed moderate amplification, and 1 out of 3 showed high amplification in Stage I disease. The overall frequency of HER-2/neu gene amplification among stage I tumors was 30% (3 out of 10 tumors studied). The overall frequency of HER-2/neu gene amplification among stage II tumors was 0% (0 out of 10 tumors studied). Among stage III tumors, the one positive tumor was moderately amplified. The overall frequency of HER-2/neu gene amplification among stage III tumors was 10% (1 out of 10 tumors studied). The highest frequency of HER-2/neu gene amplification occurred in stage IV disease. Among 5 amplified tumors, 1 was moderately amplified and 4 were highly amplified by our criteria. The overall frequency of HER-2/neu gene amplification among stage IV tumors was 50% (5 out of 10 tumors studied).

No apparent linear relationship could be seen between HER-2/neu gene amplification frequency and clinical stage of the disease in this pilot sample. However, when the data are examined as to the levels of gene amplification (low, moderate, or high amplification) as defined, it can be seen not only that the stage IV tumors had the highest frequency of HER-2/neu gene amplification, but also that 4 out of 5 amplified tumors were highly amplified.

Although a correlation between the overall frequency of HER-2/neu amplification and clinical stage cannot be established at this time because of the size of this sample, it is interesting to note that when stages I and II, and when stages III and IV are combined, respectively, the higher tumor stages have a higher amplification frequency than the lower ones. The combined frequency of amplification in stages III and IV tumors was 30% (6 out of 20 tumors studied) versus 15% (3 out of 20 tumors studied) for stages I and II tumors. Furthermore, stage IV has the highest frequency (5 out of 10) of HER-2/neu gene amplification than all three lower stages combined (4 out of 30). This is no doubt due to the high frequency of gene amplification observed

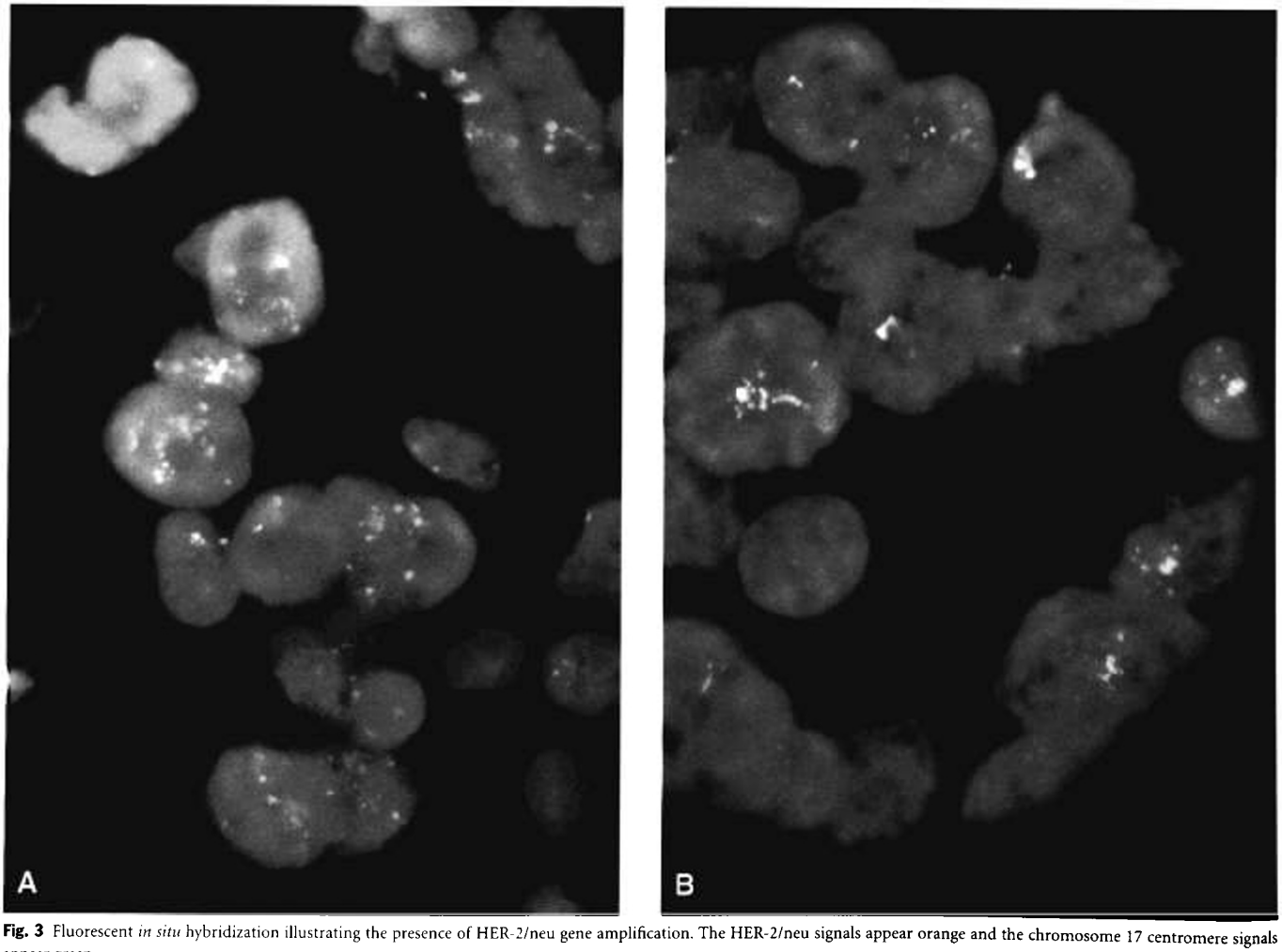

Fig. 3 Fluorescent *in situ* hybridization illustrating the presence of HER-2/neu gene amplification. The HER-2/neu signals appear orange and the chromosome 17 centromere signals appear green.

in stage IV tumors, which, interestingly, also demonstrates the most highly amplified HER-2/neu gene copy numbers.

Recent studies showed that an overexpression or amplification of HER-2/neu in breast tumor tissue is associated with aggressive disease (Zhou et al., 1987),[23] disease recurrence (Press et al., 1993),[20] poor overall survival (Slamon et al., 1987),[19] and differential drug responsiveness (Muss et al., 1994).[24] However, these results were not consistently observed in the literature. Apart from disease stage, clinical information such as ER/PR positivity is not complete. Available information indicates that this cohort of patients ranged from 34 to 89 years of age, with means of 61, 60, 65, and 65, for stages I, II, III, and IV, respectively. The size of the tumors ranged from 0.6 cm to 4.5 cm, with the larger tumors found in the higher stages. Pathological grade ranged from Grades I to III in stages I, II and III, with the highest frequency of grade III being found in clinical stage III disease. Based on flow cytometric information available, 63% of the clinical stage I tumors were aneuploid, compared to 71% in clinical stage II tumors and 43% in clinical stage III tumors. The only clinical stage IV tumor with flow cytometry results was aneuploid (100%).

To conclude, although the biologic and clinical basis for gene amplification is not totally clear at this time, given the observation that the most aggressive disease stage is associated with the highest frequency of gene amplification, further exploration of HER-2/neu as a prognostic marker of poor outcome is warranted, as it may have significant clinical utility such as in selecting patients for adjuvant therapies (Slamon, 1987; Press, 1993).[19,20]

Acknowledgments

We would like to thank Dr. Roger Mark for reading the manuscript. The continued support of the dedicated staff of the Lifespan Academic Medical Center Cytogenetics Laboratory, which celebrates its third decade of service, is also acknowledged. This study is partially funded by Vysis, Inc.

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