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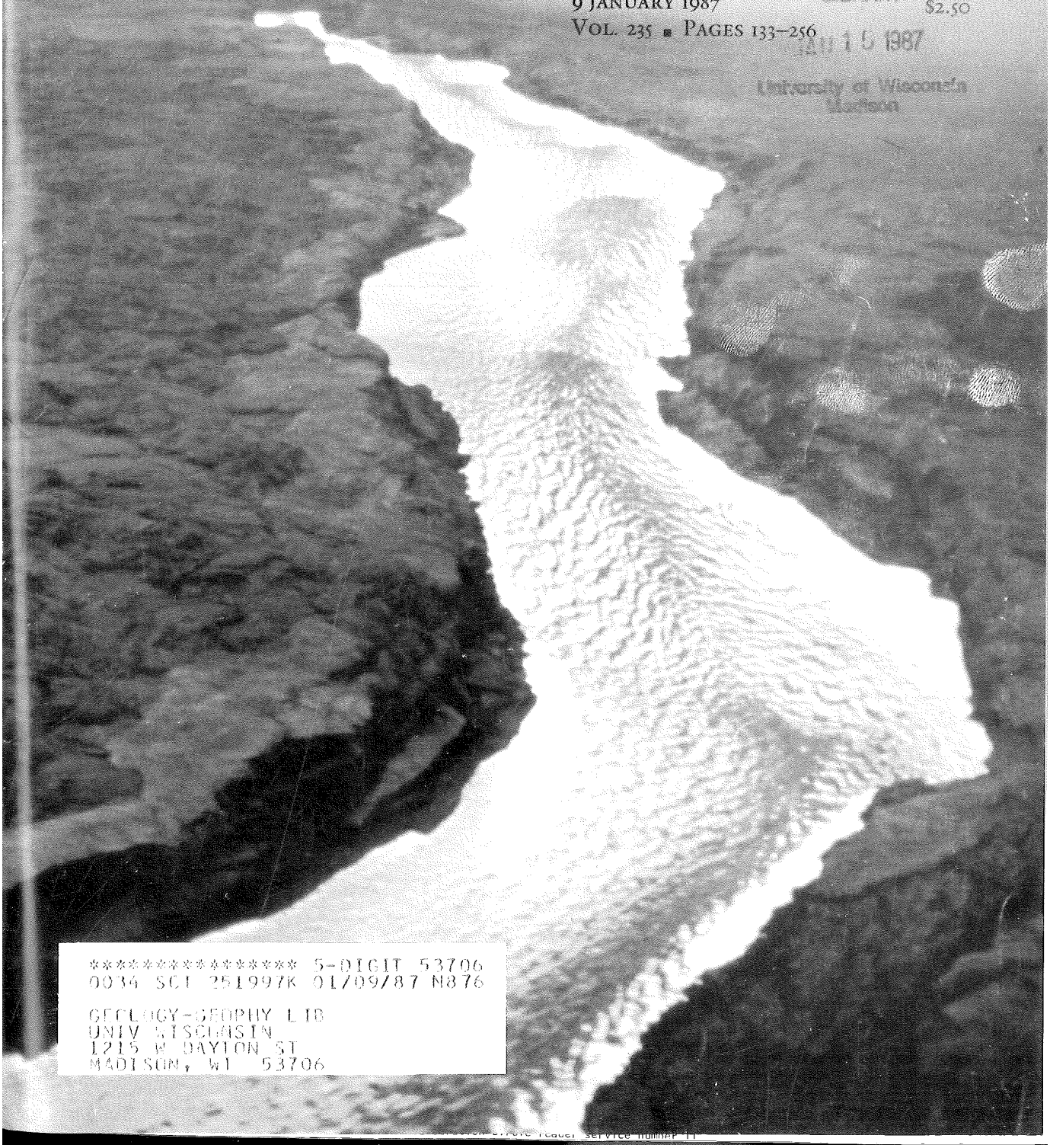

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# Human Breast Cancer: Correlation of Relapse and Survival with Amplification of the HER-2/*neu* Oncogene

DENNIS J. SLAMON,* GARY M. CLARK, STEVEN G. WONG, WENDY J. LEVIN, AXEL ULLRICH, WILLIAM L. MCGUIRE

**The HER-2/*neu* oncogene is a member of the *erb*B–like oncogene family, and is related to, but distinct from, the epidermal growth factor receptor. This gene has been shown to be amplified in human breast cancer cell lines. In the current study, alterations of the gene in 189 primary human breast cancers were investigated. HER-2/*neu* was found to be amplified from 2- to greater than 20-fold in 30% of the tumors. Correlation of gene amplification with several disease parameters was evaluated. Amplification of the HER-2/*neu* gene was a significant predictor of both overall survival and time to relapse in patients with breast cancer. It retained its significance even when adjustments were made for other known prognostic factors. Moreover, HER-2/*neu* amplification had greater prognostic value than most currently used prognostic factors, including hormonal-receptor status, in lymph node–positive disease. These data indicate that this gene may play a role in the biologic behavior and/or pathogenesis of human breast cancer.**

THE EVIDENCE LINKING PROTO-ONCOGENES TO THE INDUCtion or maintenance of human malignancies is largely circumstantial, but has become increasingly compelling. This circumstantial evidence is derived from studies of animal models, tumor cell lines, and actual human tumors. Data from animal models and cell lines include: (i) sequence homology between human proto-oncogenes and the viral oncogenes of transforming retroviruses that are known to be tumorigenic in some species (*1, 2*); (ii) transfection studies showing the transforming potential of proto-oncogenes in NIH 3T3 cells and primary embryo fibroblasts (*3–5*); and (iii) the central role of certain proto-oncogenes in tumorigenesis by chronic transforming retroviruses such as avian leukosis virus (*6*). Data from human tumors include: (i) increased expression of specific proto-oncogenes in some human malignancies (*7, 8*); (ii) localization of proto-oncogenes at or near the site of specific, tumor-associated chromosomal translocations (*9*); and (iii) amplification of proto-oncogenes in some human tumors (*10, 11*).

Additional data linking proto-oncogenes to cell growth is their expression in response to certain proliferation signals (*12, 13*) and their expression during embryonic development (*14, 15*). More direct evidence comes from the fact that, of the 20 known proto-oncogenes, three are related to a growth factor or a growth factor receptor. These genes include c-*sis*, which is homologous to the transforming gene of the simian sarcoma virus and is the β chain of platelet-derived growth factor (PDGF) (*16, 17*); c-*fms*, which is homologous to the transforming gene of the feline sarcoma virus and is closely related to the macrophage colony-stimulating factor receptor (CSF-1R) (*18*); and c-*erb*B, which encodes the EGF receptor (EGFR) and is highly homologous to the transforming gene of the avian erythroblastosis virus (*19*). The two receptor-related proto-oncogenes, c-*fms* and c-*erb*B, are members of the tyrosine-specific protein kinase family to which many proto-oncogenes belong.

Recently, a novel transforming gene was identified as a result of transfection studies with DNA from chemically induced rat neuroglioblastomas (*20*). This gene, called *neu*, was shown to be related to, but distinct from, the c-*erb*B proto-oncogene (*21*). By means of v-*erb*B and human EGFR as probes to screen human genomic and complementary DNA (cDNA) libraries, two other groups independently isolated human *erb*B–related genes that they called HER-2 (*22*) and c-*erb*B-2 (*23*). Subsequent sequence analysis and chromosomal mapping studies revealed all three genes (*neu*, c-*erb*B-2, and HER-2) to be the same (*22, 24, 25*). A fourth group, also using v-*erb*B as a probe, identified the same gene in a mammary carcinoma cell line, MAC 117, where it was found to be amplified five- to ten-fold (*26*).

This gene, which we will call HER-2/*neu*, encodes a new member of the tyrosine kinase family; and is closely related to, but distinct from, the EGFR gene (*22*). HER-2/*neu* differs from EGFR in that it is found on band q21 of chromosome 17 (*22, 24, 25*), as compared to band p11–p13 of chromosome 7, where the EGFR gene is located (*27*). Also, the HER-2/*neu* gene generates a messenger RNA (mRNA) of 4.8 kb (*22*), which differs from the 5.8- and 10-kb transcripts for the EGFR gene (*28*). Finally, the protein encoded by the HER-2/*neu* gene is 185,000 daltons (*21*), as compared to the 170,000-dalton protein encoded by the EGFR gene. Conversely, on the basis of sequence data, HER-2/*neu* is more closely related to the EGFR gene than to other members of the tyrosine kinase family (*22*). Like the EGFR protein, HER-2/*neu* has an extracellular domain, a transmembrane domain that includes two cysteine-rich repeat clusters, and an intracellular kinase domain (*21*), indicating

D. J. Slamon, S. G. Wong, and W. J. Levin are in the Division of Hematology-Oncology, Department of Medicine and Jonsson Comprehensive Cancer Center, UCLA School of Medicine, Los Angeles, CA 90024. G. M. Clark and W. L. McGuire are in the Division of Oncology, Department of Medicine, University of Texas Health Science Center at San Antonio, San Antonio, TX 78284. A. Ullrich is in the Department of Molecular Biology, Genentech, Inc., South San Francisco, CA 94080.

*To whom correspondence should be addressed.

that it too is likely to be a cellular receptor for an as yet unidentified ligand.

As a result of the published data showing amplification of HER-2/*neu* in a human mammary carcinoma cell line, and as part of an ongoing survey in our laboratory of proto-oncogene abnormalities in human tumors, we evaluated alterations of the HER-2/*neu* gene in a large series of human primary breast cancers. Our results show that amplification of this gene occurs relatively frequently in breast cancer, and that it is associated with disease relapse and overall patient survival.

Factors that are known to be important in the prognosis of breast malignancies in individual patients include: size of the primary tumor, stage of disease at diagnosis, hormonal receptor status, and number of axillary lymph nodes involved with disease (positive nodes) (*29*). The current study, which was conducted in two parts, involved the evaluation of tissue from 189 separate breast malignancies that were part of a breast cancer study ongoing at the University of Texas, San Antonio. This cohort of tumors was of interest because considerable information was available on the majority of the specimens including size of the primary tumor, estrogen receptor status, progesterone receptor status, age of patient, disease stage, and status of the axillary lymph nodes.

In the initial survey, tissue from 103 primary breast cancers was evaluated for alterations in the HER-2/*neu* gene. DNA from individual tumors was prepared as described (*30*), digested with Eco RI, and subjected to Southern blot analysis with a $^{32}$P-labeled HER-2/*neu*-1 probe, which is known to detect a 13-kb hybridizing band in human DNA (*22*). Examples of tumors from the initial survey are shown in Fig. 1. Of the 103 samples examined, 19 (18%) showed evidence of HER-2/*neu* gene amplification. The degree of amplification in individual cases was determined by dilution analysis (Fig. 2A), as well as soft laser densitometry scanning. To determine that the amount of DNA loaded in each lane was equivalent, all filters were washed and rehybridized with a $^{32}$P-labeled arginase gene probe (*31*). This probe identifies a 15-kb hybridizing band on Eco RI–digested human DNA, and was selected as a control because it more appropriately assesses the relative amount and transfer of high molecular weight species than a probe hybridizing with low molecular weight species, which transfer more readily on Southern blotting. All lanes were shown to contain equivalent amounts of high molecular weight DNA (Fig. 2B). Individual tumors were assigned to groups containing a single copy, 2 to 5 copies, 5 to 20 copies, and greater than 20 copies of the HER-2/*neu* gene (Fig. 1). Assignment of tumors to the various groups was done

**Table 1.** Association between HER-2/*neu* amplification and disease parameters in 103 breast tumors.

| Factor* | Number of tumors | | | | | *P*† |
|---|---|---|---|---|---|---|
| | Single copy | 2 to 5 copies | 5 to 20 copies | >20 copies | Total | |
| *Hormonal receptor status* | | | | | | |
| ER+ | 53 | 2 | 9 | 1 | 65 | 0.99 |
| ER− | 31 | 1 | 2 | 4 | 38 | |
| PgR+ | 42 | 2 | 6 | 2 | 52 | 0.85 |
| PgR− | 42 | 1 | 5 | 3 | 51 | |
| *Tumor size (centimeters)* | | | | | | |
| ≤2 | 13 | 1 | 1 | 0 | 15 | 0.82 |
| 2–5 | 34 | 1 | 5 | 1 | 41 | |
| >5 | 17 | 1 | 2 | 2 | 22 | |
| Unknown | 20 | 0 | 3 | 2 | 25 | |
| *Age at diagnosis (years)* | | | | | | |
| ≤50 | 21 | 1 | 2 | 1 | 25 | 0.83 |
| >50 | 52 | 2 | 7 | 4 | 65 | |
| Unknown | 11 | 0 | 2 | 0 | 13 | |
| *Number of positive lymph nodes* | | | | | | |
| 0 | 30 | 0 | 3 | 1 | 34 | 0.11 |
| 1–3 | 20 | 0 | 1 | 1 | 22 | |
| >3 | 17 | 2 | 4 | 2 | 25 | |
| Unknown | 17 | 1 | 3 | 1 | 22 | |

*Receptor status was analyzed as described (*39*). ER, estrogen receptor: + and − refer to the presence or absence of ≥3 fmol of receptor per milligram of protein. PgR, progesterone receptor: + and − refers to the presence or absence of ≥5 fmol of receptor per milligram of protein. †Statistical analyses for correlation of HER-2/*neu* amplification with disease parameters were performed by the $\chi^2$ test. *P* values were computed after combining the cases with 5 to 20 and >20 copies.

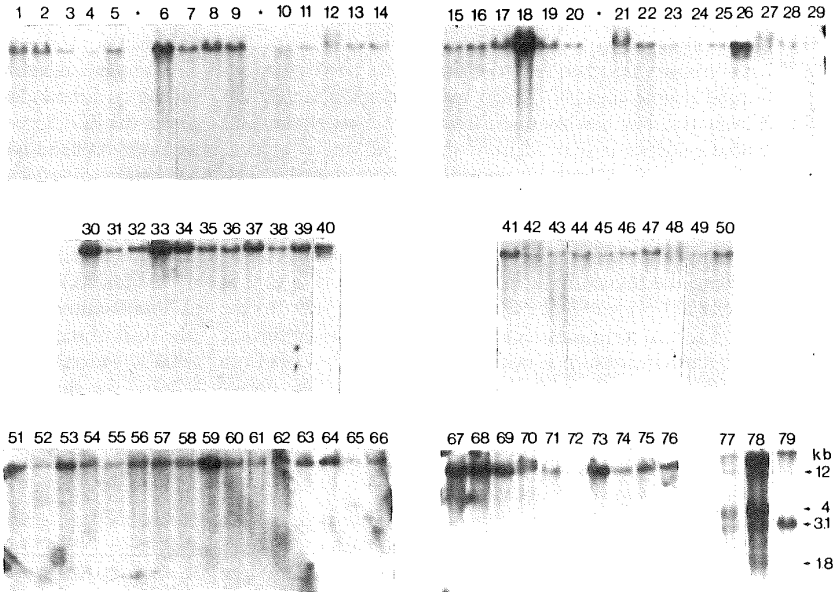


**Fig. 1.** Analysis of alterations of the HER-2/*neu* gene in human breast cancer. Shown are 79 of the 189 breast tumors used in this analysis. Tumors with a single copy of HER-2/*neu*: 3, 4, 10 to 15, 20, 23 to 25, 27 to 29, 31, 38, 42 to 46, 48, 49, 52, 55, 61, 65, 66, 71, 72, and 74. Tumors with two to five copies of HER-2/*neu*: 1, 2, 5, 7, 9, 16, 17, 19, 21, 22, 32, 35, 36, 47, 50, 54, 56 to 58, 60, 62, 70, and 75 to 77. Tumors with 5 to 20 copies of HER-2/*neu*: 6, 8, 26, 34, 37, 39 to 41, 51, 53, 63, 64, 67, 69, 73, and 79. Tumors with more than 20 copies of HER-2/*neu*: 18, 30, 33, 59, 68, and 78. Examples of tumors 77 to 79 have rearrangements in the HER-2/*neu* gene. DNA was extracted from tissues and digested with Eco RI as described (*30*). A total of 12 μg of Eco RI–digested DNA was loaded onto 0.8% agarose gels, separated by electrophoresis, and transferred onto nylon filter papers (Biodyne) (*30*). All filters were baked in a vacuum oven for 3 hours at 80°C, prehybridized in 5× SSC (standard saline citrate) containing 50% formamide, 10% dextran sulfate, 0.1% SDS, denatured salmon sperm DNA (1 mg/ml), and 4× Denhardts solution for 12 hours, then hybridized in the same solution containing $^{32}$P-labeled nick-translated HER-2 probe (*21*) specific activity of $1 \times 10^8$ cpm per microgram of DNA; $2 \times 10^6$ cpm/ml. Hybridization occurred at 42°C for 48 hours, followed by washing of filters under the following conditions in succession: 2× SSC for 20 minutes at room temperature; two washes of 30 minutes each in 2× SSC, 0.1% SDS at 65°C; one wash of 30 minutes in 0.5× SSC, 0.1% SDS at 65°C. Filters were then exposed to XAR-5 x-ray film (Kodak) for autoradiography.

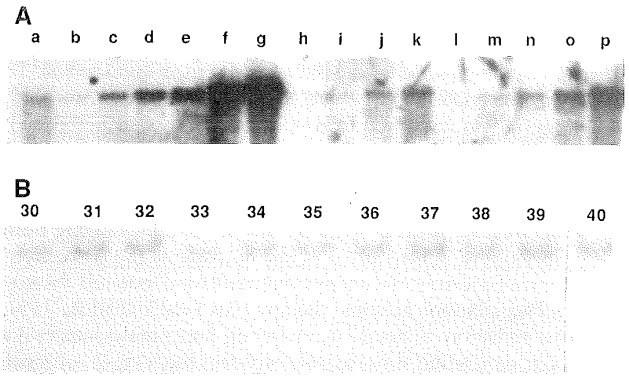


**Fig. 2.** (**A**) Example of dilutional analysis to assess degree of HER-2/*neu* gene amplification. Lanes a, g, k, and p were loaded with 12 μg of Eco RI–digested breast tumor DNA. Lane a is DNA from tumor 31 (Fig. 1), which represents a tumor with a single copy of the HER/2-*neu* gene. Lane g is DNA from tumor 33, which represents a tumor with >20 copies of the HER-2/*neu* gene. Lanes b to f are serial dilutions (1:100, 1:20, 1:10, 1:5, and 1:2, respectively) of the DNA sample in lane g. Lane k is DNA from tumor 35 (Fig. 1), which represents a tumor containing two to five copies of the HER-2/*neu* gene. Lanes h to j are serial dilutions (1:10, 1:5, and 1:2, respectively) of the DNA sample in lane k. Lane p is DNA from tumor 34 (Fig. 1), which represents a tumor with 5 to 20 copies of the HER-2/*neu* gene. Lanes l to o are serial dilutions (1:20, 1:10, 1:5, and 1:2, respectively) of the DNA sample in lane p. The filter was prepared and hybridized with a $^{32}$P-labeled HER-2 probe as in Fig. 1. (**B**) Example of arginase probe hybridization to demonstrate that equivalent amounts of tumor DNA were loaded into each lane. Rehybridization of filter containing lanes 30 to 40 (Fig. 1). The filter was first stripped of label by washing in a buffer made up of 50% formamide, 3× SSC, and 0.1% SDS at 65°C for 20 minutes, following by three successive washes of 5 minutes each in 0.1× SSC at room temperature. Filters were exposed overnight on XAR-5 film (Kodak) to ensure removal of all radioactive probe, then rehybridized as in Fig. 1 with a $^{32}$P-labeled human arginase gene probe (*31*).

in a blinded fashion, in that they were made without knowledge of disease parameters. Analysis of the data for association between gene amplification and a number of disease parameters was then performed.

Of 103 tumors evaluated in the initial survey, there was essentially no correlation between gene amplification and estrogen receptor status, progesterone receptor status, size of tumors, or age at diagnosis (Table 1). However, when analysis was performed for association between HER-2/*neu* amplification and number of positive lymph nodes, a trend was noted. This analysis showed that 4/34 (11%) of patients with no involved nodes, 2/20 (10%) with 1 to 3 involved nodes, and 8/25 (32%) with >3 involved nodes had gene amplification ($P = 0.11$). If these data were examined by comparing 0 to 3 positive nodes versus >3 positive nodes, the correlation with gene amplification became more significant ($P < 0.05$). Thus, there was a significant increase in incidence of HER-2/*neu* gene amplification in patients with >3 axillary lymph nodes involved with disease. A multivariate regression analysis to correlate HER-2/*neu* amplification with various disease parameters identified the number of positive nodes as the only significant factor, either alone or in combination, to correlate with amplification.

This initial study indicated that it might be possible to discriminate among node-positive patients on the basis of HER-2/*neu* gene amplification. It is well known that the number of positive nodes is the best prognostic factor for disease recurrence and survival in patients with breast cancer (*29*). Given the correlation between number of nodes positive and HER-2/*neu* amplification, one might predict that amplification of this gene might also have some prognostic value. No long-term follow-up data, however, were available on the 103 patients analyzed in the initial study. For this reason, a second study was conducted on 100 breast cancer samples from patients with positive axillary lymph nodes. All of the information available for the first group of 103 patients was available for these patients. In addition, relapse and survival information was available, since these cases had a median follow-up of 46 months (range 24 to 86 months). Of these 100 samples, 86 yielded sufficient DNA for study. Amplification of the HER-2/*neu* gene was measured as in the initial survey, and examples of tumors from this study are shown (Fig. 1). Amplification was found in 34/86 (40%) of these patients. For this larger sample of node-positive patients, several statistically significant or nearly significant relationships were observed. In agreement with the preliminary survey, there was an association between number of involved lymph nodes and HER-2/*neu* amplification (Table 2). In addition, the presence of gene amplification was correlated with estrogen receptor status and size of primary tumor (Table 2). Together, these two surveys yielded data on 189 patients and the association of HER-2/*neu* amplification with various disease parameters in the combined group is shown in Table 3.

While these correlations were of interest, the strong relationship

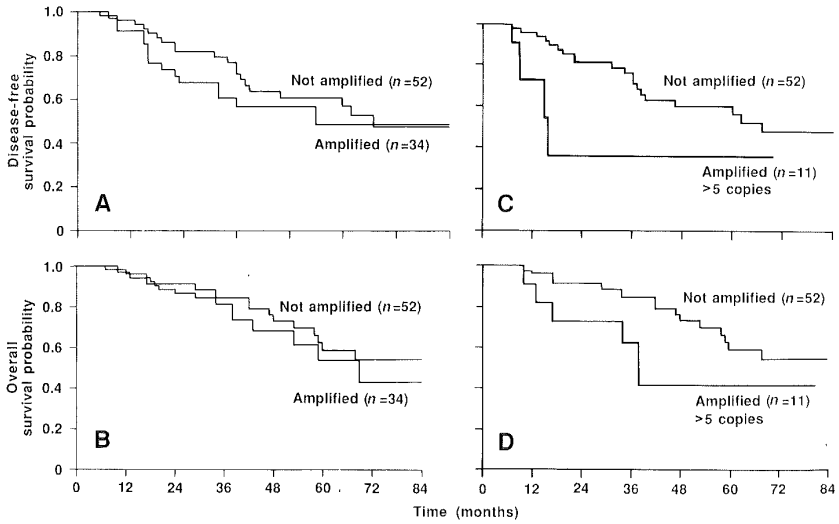


**Fig. 3.** Actuarial curve for relapse in (**A**) node-positive patients with no amplification versus node-positive patients with any amplification (>2 copies) of HER-2/*neu* and (**C**) node-positive patients with no amplification versus node-positive patients with greater than 5 copies of HER-2/*neu*. Actuarial curve for overall survival in (**B**) node-positive patients with no amplification versus node-positive patients with any amplification (>2 copies) of HER-2/*neu* and (**D**) node-positive patients with no amplification versus node-positive patients with greater than 5 copies of HER-2/*neu*. Actuarial curves for both relapse and overall survival were computed by the method of Kaplan and Meier (*44*) and compared by the log rank test (*42–44*).

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