Second, in our study there is no evidence of an association between the copy number of c-erbB-2 and the number of positive lymph nodes either by the $\chi^2$ test or by the more powerful Kendall's correlation ($\rho = \pm 0.087 \pm 0.083$) (12). Neither of the two separate groups of patients examined by Slamon et al. (5) provides strong evidence of an association. The reported strong association ($P = 0.002$) obtained by combining data from their two groups poses two major problems. (i) The importance of the $P$ value (0.002) is diminished by the fact that the analysis of the first group was used to formulate the association hypothesis as well as to help confirm it; and (ii) the above analysis of Table 1 shows that the proportions with the amplified gene are quite different in their two groups. Hence their combination is of questionable validity for studying the association between the amplified c-erbB-2 gene and the number of positive lymph nodes. The inconsistency between our analysis and that of Slamon et al. could be due to the small proportion of patients with amplified c-erbB-2 gene in our study (or to the large proportion in their second group). It is, however, not due to the original size of the group, as comparable numbers of patients were available in all groups.

Third, the analysis of survival information on the patients in our study does not confirm the importance of c-erbB-2 amplification in breast tumors in predicting overall survival or time to relapse. The only variables important for our study in predicting disease-free and overall survival, both in univariate and multivariate Cox regression analyses (12) as well as a robust log rank analysis (8), were the number of positive lymph nodes and progesterone receptor status (Table 2). These are the same two variables identified previously by Clark et al. (14).

At present there cannot be a simple explanation for these contradictory findings. The discrepancies between our results and those of Slamon et al. or between the results of various groups of patients studied in the same or different laboratories could reflect differences in genetic background, geographical location, or other nutritional and environmental factors. In this respect, breast cancer patients in the French population seem to have longer disease-free and overall survival periods compared with the American patients (8). Our results therefore advise caution in the preliminary assignment of c-erbB-2 amplification as an indicator for breast tumor aggressiveness and poor disease prognosis. Moreover, an association of a particular genetic alteration with disease prognosis requires analysis of expanded numbers of tumors from patients representing different geographical and genetic compositions.

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REFERENCES
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Response: The association between amplification of the HER-2/neu gene, also called c-erbB2, and poor prognosis in human breast cancer was first reported approximately 18 months ago (1). Since that report, a number of studies regarding amplification of this gene in primary human breast cancers have been published (2–7). Ali et al. present results indicating that there is "no evidence of an association between increased copy number of c-erbB2 and the biological characteristics of tumors that may be indicative of their degree of malignancy. Among these parameters are numbers of involved lymph nodes, hormone receptor status, histopathologic grading, age at diagnosis, and menopausal status" (8). They go on to say that...
Table 1. Incidence of gene amplification in breast cancer patients.

<table>
<thead>
<tr>
<th>Reference</th>
<th>All patients</th>
<th>Node-positive patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. %</td>
<td>No. %</td>
</tr>
<tr>
<td>Berger et al. (4)</td>
<td>13/51 25</td>
<td>7/17 41</td>
</tr>
<tr>
<td>Venter et al. (6)</td>
<td>12/36 33</td>
<td>3/15 20</td>
</tr>
<tr>
<td>Varley et al. (7)</td>
<td>7/37 19</td>
<td>4/10 22</td>
</tr>
<tr>
<td>Zhou et al. (3)</td>
<td>15/86 17</td>
<td>8/37 22</td>
</tr>
<tr>
<td>Vijver et al. (2)</td>
<td>16/95 17</td>
<td>6/41 15</td>
</tr>
<tr>
<td>Cline et al. (5)</td>
<td>8/53 15</td>
<td>8/35 23</td>
</tr>
<tr>
<td>Mean</td>
<td>19</td>
<td>27</td>
</tr>
<tr>
<td>Ali et al. (8)</td>
<td>12/122 10</td>
<td>8/75 11</td>
</tr>
<tr>
<td>Slamon et al. (1)</td>
<td>53/189 28</td>
<td>34/86 40</td>
</tr>
</tbody>
</table>

reflect variations due to sample size. They further state that combining a group of tumors (those without follow-up) in which we first saw an apparent association between HER-2/neu amplification and positive lymph nodes with a group of tumors, all having lymph node positivity and follow-up, is of “questionable validity,” since “the first group was used to formulate the association hypothesis as well as to help confirm it.” This is a valid criticism.

Another possible explanation for a potential overestimate of signal intensity and thus an overestimate of the true amplification rate in a cohort of tumors is chromosomal duplication. Ali et al. correctly make the point that “chromosomal duplication rather than gene amplification, at least in some cases, might give rise to an exaggerated frequency of tumors with c-erbB2 amplification.” This was a potential source of error in our initial study, since we used the human arginase gene probe as a single copy control to determine that equivalent amounts of DNA were loaded into each lane (1). The rationale behind using this probe was clearly stated (1), however: the arginase gene is located on chromosome 6 (9) as opposed to chromosome 17, where the HER-2/neu gene is found (10). The possibility existed that some of the tumors which show increased HER-2/neu signal do so as a result of chromosomal 17 duplication rather than true gene amplification. To address this issue we rehybridized all filters from our initial study sequentially with a p53 gene probe and a myeloperoxidase probe. The p53 gene is found on the short arm of chromosome 17 (11), while the myeloperoxidase gene is found on the long arm (12), which is where HER-2/neu resides. This latter probe was used to evaluate the tumors for the occurrence of isochromosomy 17, a condition reported in some human malignancies in which only the long arm is duplicated (13). This abnormality occurs most frequently in hematopoietic cancers (13). Although isochromosomy 17 has yet to be reported in breast cancer, it has been seen in other solid tumors, such as colon cancer (14, 15). In order to evaluate our tumors for any duplication of chromosome 17, we used both probes. In this fashion, we could assess them for duplication of either all or part of chromosome 17 and thus address any increase in signal resulting from duplication of the chromosome. In no case that we had called amplified did we find evidence for chromosomal duplication; thus, the incidence for gene amplification presented in our initial cohort was correct for this group of tumors. Ali et al. point out the difference between our incidence of amplification (30%) and that found in their study (10%); however, they do not point out the discrepancy between their data and those published by a number of other investigators (2–7).

When one examines the published reports it becomes evident that, while there is considerable variability in the incidence of gene amplification reported between groups, no one has observed an incidence as low as that found by Ali et al. (Table 1). An argument can be made that some of the large discrepancies between studies may be due to the small numbers analyzed. This issue can only be addressed by a large study.

A possible technical problem that could result in difficulties in assessing gene copy number in human tumor DNA is the integrity of the DNA. The way tumor specimens are handled varies considerably between the time of surgical removal and DNA analysis. Small amounts of DNA degradation can lead to significant differences in signal intensity on Southern blot analysis (Fig. 1, a and b), and may result in inclusion of samples that incorrectly appear to have low or single gene copy intensity on blots. It is important to carefully assess the integrity of the DNA before performing an analysis for gene copy number. This is particularly true for genes such as HER-2/neu that migrate as high-molecular-weight species on a Southern blot when cut with Eco RI (1).

In addition, two statistical issues may explain the differences between our initial study and the results of Ali et al. The first is the issue of the statistical power to detect a significant difference between two groups of patients within a given study. If one uses the guidelines of George and Desu (16) a minimum of 26 deaths or relapses in each of two groups would be required in a group of 122 patients such as that presented by Ali et al. in order for there to be an 80% probability of detecting a two-fold increase in median survival time. Given that only 12 patients (8 who were node-positive) in the study of Ali et al. had tumors with amplified HER-2/neu, it is likely that large differences between survival distributions might not be detected. Thus the low incidence of gene amplification reported in their study could have had an impact on how Ali et al. assessed the association of gene amplification with survival differences. Second, the relative statistical weights given to HER-2/neu copy number are different in the two studies. The weights used by Ali et al. are 1 for single copy; 2 for 2 to 5 copies; 3 for 6 to 15 copies; and 4 for more than 15 copies. The weights used in our analysis were 1 for single copy; 2 for 2 to 4 copies, 5 for 5 to 20 copies and 20 for more than 20 copies. The weights used by Ali et al. would minimize any differences in patient outcome that might be due to large copy numbers. Also of note is the fact that the most significant differences we found in our initial study

![Fig. 1. Analysis of genomic DNA obtained from human breast tumors.](http://science.sciencemag.org/)

**A** 1 2 3 4 5 6 7 8 9

**B** 1 2 3 4 5 6 7 8 9

**C** 1 2 3 4 5 6 7 8 9

**D** 1 2 3 4 5 6 7 8 9

**E** 1 2 3 4 5 6 7 8 9

**F** 1 2 3 4 5 6 7 8 9

**G** 1 2 3 4 5 6 7 8 9

**H** 1 2 3 4 5 6 7 8 9

**I** 1 2 3 4 5 6 7 8 9

**J** 1 2 3 4 5 6 7 8 9

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were between patients with a single copy of the HER-2/neu gene and those with more than five copies (1). The combination of a low estimate of copy number because of technical differences, a number of cases that was too small to allow detection of a difference with high probability, and statistical weighting of samples with a high copy number could lead to a lack of association between gene amplification and clinical outcome.

Two separate groups have recently found an association between HER-2/neu amplification and poor prognosis 

indicated that amplification of overexpression of the gene results in increased transformation (17). Moreover in the study using gene amplification to overexpress HER-2/neu, not only was there an increase in transformation rate, but the transformed cells were tumorigenic in the nude mouse. These data lend further credence to the concept that amplification or overexpression of the gene, or both, can lead to more aggressive biologic behavior of the cells containing such alterations.

Human breast cancer is a heterogeneous disease with regard to its clinical behavior. Some women with breast cancer have short survivals indicating aggressive clinical disease, while others with the same diagnosis can survive with active disease for many years. On the basis of our previous analysis as well as a repeat analysis with additional controls and updated follow-up, we are convinced that the data presented in our initial study are correct, and give an accurate assessment of the association between HER-2/neu amplification and poor clinical outcome for the group of tumors studied (1). In addition, we feel that there are sufficient independent clinical and biological data from other groups to support a potential role for this gene in the pathogenesis of some human breast cancers. However, even though our initial study and those of others have evaluated significant numbers of tumors (between 30 and 189), a true idea of the prognostic role of amplification of the HER-2/neu gene can only be gained by studying a large series of patients with adequate long-term clinical follow-up.

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REFERENCES
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