c-erbB-2 Amplification in Node-negative Human Breast Cancer

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ABSTRACT

c-erbB-2 gene analysis by Southern and DNA dot blot methods was done in 66 tumor samples from patients with histologically node-negative breast cancer. The c-erbB-2 gene was amplified 2- to >8-fold in 13 tumors (20%). None of 59 tumors that were examined by the Southern method showed c-erbB-2 gene rearrangement. c-erbB-2 amplification was analyzed in relation to other prognostic factors. The c-erbB-2 gene was amplified in five of 36 (14%) diploid and eight of 30 (27%) aneuploid tumors. Thirteen of 54 (24%) tumors with nuclear Grade 1 or 2 displayed c-erbB-2 amplification, whereas none of 12 tumors with nuclear Grade 3 did. No correlation was observed with estrogen receptor content, tumor size, histological type, or age of patients. The median follow-up date for these patients was 85+ mo. Of 13 patients whose tumors showed c-erbB-2 amplification, six patients (46%) developed recurrence, and five patients (38%) died of metastatic disease. In contrast, of 53 patients whose tumors did not show c-erbB-2 amplification, 15 patients (28%) developed recurrence, and seven patients (13%) died of disease.

In conclusion, our results show that c-erbB-2 gene amplification was more frequent in aneuploid tumors and tumors with poor nuclear grade. c-erbB-2 amplification may be considered a possible prognostic factor in node-negative breast cancer.

INTRODUCTION

Breast cancer is the most frequently occurring cancer among women in the United States. Although the prognosis for patients with Stage I/II node-negative breast cancer is excellent, approximately one-third of the patients develop recurrent disease and eventually die of metastatic disease (1, 2). Therefore, studies of prognostic factors in patients with node-negative breast cancer to identify high-risk and low-risk groups of patients for relapse remain one of the major research tasks in breast oncology. In node-negative breast cancer, no single prognostic factor appears to be superior nor is any one factor a good enough discriminant of outcome (3).

We performed Southern and DNA dot blot analysis for c-erbB-2 gene amplification in 66 tumor samples from patients with histologically node-negative breast cancer to determine its value as a prognostic indicator. Further, we evaluated other prognostic factors on these cases, and the results were correlated with c-erbB-2 amplification and clinical outcome.

MATERIALS AND METHODS

Samples from 66 consecutive cases of node-negative breast cancer, which have been stored in liquid nitrogen since 1978, were examined for c-erbB-2 amplification by DNA dot (n = 66) and Southern blot (n = 59) methods. Because the samples were the remaining portions from well-selected tumor areas originally collected for ER1 assay, normal tissue did not grossly contaminate them. Twenty-nine tumors (44%) were ≤2 cm, and 35 (53%) were >2 to ≤5 cm. All tumor samples had been assayed for ER content by the dextran-coated charcoal method at the time of surgical resection. A pathological review was made by one of the authors (J. Y. R.) for histological type and modified Black's NG, with NG 1 being most anaplastic (4). Comparative, well-kept paraffin blocks, one to two per case, were selected for analysis by DNA flow cytometry. No patients had received adjuvant chemotherapy or hormonal treatment. Twenty patients had received postoperative radiotherapy. The median age of the patients was 58 yr (range, 36 to 83 yr). The median clinical follow-up date was 85 mo (range, 36 to 120+ yr). Twenty-one patients (32%) developed tumor recurrence, and 12 patients (18%) died of metastatic disease.

DNA Analysis of the c-erbB-2 Gene. High-molecular-weight DNAs were extracted as previously described (5). A total of 16 μg of DNA from each of 66 tumors was denatured, neutralized, serially diluted, and spotted onto nylon filters using a dot blot apparatus. This serial dilution method was used to determine the presence of and degree of c-erbB-2 amplification. For Southern transfer, 10 μg of DNA from each of 59 tumors were digested with restriction endonucleases, size fractionated by electrophoresis in a 0.8% agarose gel, denatured, neutralized, and transferred to nylon filters (6). Hybridization to oligo-primed3P-labeled probes was done at 42°C for 48 h. The filters were washed at 60°C for 60 min in 0.1× standard saline citrate solution containing 0.1% sodium dodecyl sulfate and autoradiographed at −70°C using intensifying screens.

Probes Used. Molecular cloning of the probes used for the analyses in this study has been described as follows: c-erbB-2, a 1-kilobase BamHI cDNA fragment (7); c-myc, a 1.3-kilobase Clal-EcoRI genomic clone from the 3' end of the gene (8); HER-A64-3, a 0.7-kilobase EcoRI subclone of human EGF-r cDNA (HER-A64) (9); and chick β actin, a 1.8-kilobase Prsl cDNA (10). The actin probe was used to show that an equal amount of tumor DNA was loaded into each agarose gel slot.

DNA Flow Cytometry. Paraffin-embedded tissue sections 50 μm thick were processed by step-wise dewaxing, rehydration, and dispersion into a single cell suspension (11, 12). The cell number per milliliter was adjusted using a Coulter Counter, and a minimum of 10,000 cells per specimen was stained with the intercalating dye propidium iodide and then analyzed. The DNA ploidy pattern and cell cycle analyses were performed using a laser-based EPICS Profile flow cytometry (Coulter Corporation), with specific excitation and emission filters. Reference standard DNA content of age-matched, paraffin-embedded nonneoplastic lymph nodes was used as a diploid control. A “boxogram” gating procedure was used to analyze different cell compartments (13). DI in aneuploid specimens was calculated by establishing the ratio of the relative DNA content of the abnormal population to the diploid population and normal human lymphocytes from lymph nodes. The mean coefficient of variation of the diploid population was 4.2 ± 1.5% and of the aneuploid population was 5.5 ± 1.0%.

Statistical Analysis. Correlations between c-erbB-2 amplification with other factors were made using the χ2 test. Kaplan-Meier plots were prepared to show the influence of c-erbB-2 amplification and other prognostic factors on disease-free survival, and the statistical significance was assessed using the Gehan-Breslow test.

RESULTS

Thirty-six of 66 tumors (20%) showed c-erbB-2 amplification. Two tumors showed 2-fold amplification; 5, 4-fold; 4, 8-fold; and 2, >8-fold amplification (Fig. 1). None of the 59 tumors that were examined by the Southern blot method showed c-erbB-2 amplification in 66 tumors from patients with histologically node-negative breast cancer. The c-erbB-2 gene was amplified 2- to >8-fold in 13 tumors (20%). None of 59 tumors that were examined by the Southern method showed c-erbB-2 gene rearrangement. c-erbB-2 amplification was analyzed in relation to other prognostic factors. The c-erbB-2 gene was amplified in five of 36 (14%) diploid and eight of 30 (27%) aneuploid tumors. Thirteen of 54 (24%) tumors with nuclear Grade 1 or 2 displayed c-erbB-2 amplification, whereas none of 12 tumors with nuclear Grade 3 did. No correlation was observed with estrogen receptor content, tumor size, histological type, or age of patients. The median follow-up date for these patients was 85+ mo. Of 13 patients whose tumors showed c-erbB-2 amplification, six patients (46%) developed recurrence, and five patients (38%) died of metastatic disease. In contrast, of 53 patients whose tumors did not show c-erbB-2 amplification, 15 patients (28%) developed recurrence, and seven patients (13%) died of disease.

In conclusion, our results show that c-erbB-2 gene amplification was more frequent in aneuploid tumors and tumors with poor nuclear grade. c-erbB-2 amplification may be considered a possible prognostic factor in node-negative breast cancer.

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erbb-2 gene rearrangement. After the filters were washed, they were reprobed with c-myc, which was amplified in 3 of 56 tumors. The same filters were also hybridized with an EGF-r probe, and the EGF-r gene was amplified in one of 66 tumors. Neither the c-erbB-2 nor c-myc gene was amplified in this one tumor.

Relation of Disease-free Survival and Patients’ Survival to c-erbB-2 Amplification and Other Prognostic Factors. Of 13 patients with c-erbB-2-amplified tumors, 6 patients (46%) developed recurrence, and 5 patients (38%) died of metastatic disease (Table 1). In contrast, of 53 patients who had tumors without c-erbB-2 amplification, 15 tumors (28%) recurred, and 7 patients (13%) died of metastatic disease. The actuarial disease-free survival rate was 61% at 84 mo for patients with c-erbB-2 amplification and 71% in patients without c-erbB-2 amplification (P = 0.230). The actuarial survival rate at 84 mo was 68% for patients with c-erbB-2 amplification and 90% for patients without c-erbB-2 amplification (P = 0.021). One of 6 patients (17%) with c-erbB-2-amplified tumors developed chest wall recurrence; 6 of 15 patients (40%) without c-erbB-2-amplified tumors did (P = 0.3). DNA histograms revealed that 36 tumors were diploid and 30 were aneuploid. Eighteen of 30 aneuploid tumors were hyperdiploid (DI, 1.05 to 1.90); 5, tetraploid (DI, 1.90 to 2.10); 4, hypertetraploid (DI, >2.10); and 3, multiclonal. For 11 tumors, we had 2 histograms from 2 different tissue blocks, and these showed similar DNA indices with very little intersample variability. Twelve of 36 patients (33%) with diploid tumors and 9 of 30 patients (30%) with aneuploid tumors developed recurrences. There was no significant correlation between DNA ploidy and survival of patients.

While there was no difference in recurrence or survival between patients with tumors of NG 1 and 2, none of 12 patients with tumors of NG 3 developed recurrence or died of disease. There was also no difference in disease-free survival or survival between groups of patients with ER of <20 fmol/mg and ≥20 fmol/mg. However, of 17 patients with tumor estrogen receptor ≥100 fmol/mg, only 2 (12%) developed recurrence, and one died of disease. There was no significant correlation between recurrence or survival and tumor size. Eleven of 18 patients <50 yr of age developed recurrence, and 10 of 48 patients ≥50 yr developed recurrence. This was statistically significant (P = 0.006). Five of 18 patients <50 yr died of disease, and 7 of 48 patients ≥50 yr died of disease. The difference was not statistically significant (P = 0.535).

Relation of c-erbB-2 Amplification to Individual Prognostic Factors. Table 2 shows the correlation of c-erbB-2 amplification with DNA ploidy, nuclear grade, and other prognostic factors in these 66 patients with node-negative breast cancer. Aneuploid tumors showed c-erbB-2 amplification more often than diploid tumors (27% versus 14%). Twenty-eight tumors were NG 1; 26, NG 2; and 12, NG 3. Although there was no difference between NG 1 and 2 in relation to c-erbB-2 amplification, none of 12 tumors with NG 3 had c-erbB-2 amplification. ER content, tumor size, histological type, and age of patients did not appear to correlate with c-erbB-2 amplification in this population.

DISCUSSION

The prognosis for patients with breast cancer largely depends on the number of tumor-bearing lymph nodes at the time of diagnosis (1). Several studies have shown that ER or progesterone receptor status (14, 15), nuclear or histological grade (16), and, more recently, DNA flow cytometry counts (17-20) and protooncogene alteration (21, 22) might be pertinent prognostic factors in node-negative breast cancer. Our study focused on the prognostic significance of c-erbB-2 amplification in patients with node-negative breast cancer who had a long clinical follow-up. Furthermore, these data were analyzed and correlated with other known prognostic factors.
Altering the specific protooncogenes that have been reported to be associated with clinically aggressive tumors (23, 24). In primary human breast cancer, alterations of several protooncogenes have been implicated as prognostic factors (21, 25). In node-positive breast cancer, the incidence of c-erbB-2 amplification varies from 11 to 41%, and in node-negative breast cancer, the incidence is somewhat lower (26). Thus far, the question as to whether c-erbB-2 amplification is a poor prognostic factor has not been consistently answered (25). Slamon et al. (24) reported that the presence of the c-erbB-2 amplification in node-positive breast cancer was the most significant prognostic factor. Cline et al. (21) studied a number of oncogenes in breast cancer and found the most frequent abnormality was c-myc and c-erbB-2 amplification and that any protooncogene alteration was a significantly poor prognostic factor (21, 26). However, no study has been done exclusively for node-negative breast cancer, partly because it would require rather long-term clinical follow-up for valid data. Our data revealed that, in patients with c-erbB-2 amplification, disease-free survival was not significantly shorter, but overall survival time was significantly reduced. A possible explanation is that, in the c-erbB-2-amplified group, a significant portion of patients who developed recurrence died of disease because of its aggressive clinical behavior. Recently, van de Vijver (27) reported neuroprotein overexpression in Stages I and II breast cancer in which such expression appeared to be a prognostic factor for overall survival. Although one-third of the sample population received postoperative adjuvant chemotherapy, the survival result was comparable to ours.

Studies with DNA flow cytometric methods utilizing stored paraffin blocks showed that there was close correlation of DNA indices and percentages of time in S phase between paraffin-embedded tissues and fresh samples (11, 12). A higher proportion of tumors from node-negative breast cancer patients had diploid DNA content as shown in our data, whereas tumors from breast cancer patients with involvement of ≥10 nodes were mainly aneuploid (28–30). In this population of node-negative breast cancer patients, there was no significant correlation between DNA ploidy and disease-free survival or overall survival.

Since nuclear grade was reported to be a significant prognostic factor in node-negative breast cancer by Fisher et al. (5), we correlated our NG findings with tumor recurrence and patient survival. Our results showed that only good NG (NG 3) was a prognostic indicator for low risk of relapse.

Because measurement of ER content has been commonly used for predicting recurrence or survival in node-negative breast cancer (4, 21), we analyzed our data for clinical correlation with ER content. Patients with tumors of ER content ≥100 fmol/mg had longer disease-free and overall survivals than patients with tumors of low and intermediate ER content.

When c-erbB-2 amplification was analyzed in correlation with DNA ploidy, NG, and other prognostic factors, its presence was more frequent in aneuploid tumors and in tumors with poor NG (NG 1 and 2). No other factors were significantly correlated. The parameters in this study, c-erbB-2 amplification, DNA flow cytometric results, and nuclear grade, are different biological or morphological markers of tumor differentiation and aggressiveness; and our data demonstrate a positive, although not significant, correlation between these three parameters. In addition, these data suggest that c-erbB-2 amplification is a possible prognostic factor for node-negative breast cancer. Further confirmation of these results in node-negative breast cancer would require a larger series of cases.

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REFERENCES

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