Cyclin D1 Amplification Is Not Associated with Reduced Overall Survival in Primary Breast Cancer but May Predict Early Relapse in Patients with Features of Good Prognosis

Ram Seshadri, Christine S. L. Lee, Rina Hui, Kieran McCaul, David J. Horsfall, and Robert L. Sutherland

ABSTRACT

Amplification of chromosome 11q13 is frequently observed in human malignancies, including breast cancers. A candidate oncogene at this locus is the CCND1 gene, which encodes the cell cycle regulatory protein cyclin D1. Because published data on the relationship between 11q13 amplification and prognosis in breast cancer have been controversial, we investigated the clinical significance of CCND1 amplification and its association with established clinicopathological features of prognosis in 1014 primary breast cancer patients. Amplification of the CCND1 gene and the INT-2/FGF-3 gene, which also maps to 11q13, was 10% and 17%, respectively. There were no associations between CCND1 or INT-2 amplification and patient age, tumor size, tumor grade, axillary lymph node status, HER/neu amplification, MIB-1 monoclonal antibody to Ki67 antigen count, or p53 expression. CCND1 amplification was predominantly observed in hormone receptor-positive tumors; at a copy number ≥3, CCND1 amplification was significantly correlated with both estrogen receptor (ER; \( P = 0.036 \)) and progesterone receptor (\( P = 0.012 \)) positivity. After a median follow-up period of 66 months, CCND1 or INT-2 amplification was not associated with significant increases in relapse or death from breast cancer. However, in the node-negative and ER-positive subgroups, there was a trend for an increased relapse rate in patients with INT-2 or CCND1 amplification. Thus, in this study, assessment of CCND1 or INT-2 amplification at 11q13 by slot-blot hybridization was of little use in determining phenotype or disease outcome in the whole group of patients but had a potential role in identifying a subset of poor-prognosis patients within the node-negative or ER-positive, good-prognosis groups. Because the prevalence of CCND1 amplification is much lower than the reported prevalence of cyclin D1 overexpression, additional studies are required to determine the true prognostic significance of altered cyclin D1 expression in breast cancer.

INTRODUCTION

The recent identification of alterations in several oncogenes and tumor suppressor genes associated with the development and progression of breast cancer raises the possibility that recognition of these alterations may be useful in predicting disease outcome and response to therapy (1, 2). Because some of these gene alterations may be observed independent of established features of prognosis (such as the axillary lymph node status), several workers are attempting to construct a prognostic index using these new biological features to predict outcome in individual patients (3). If such a prognostic index was to be established independent of axillary lymph node status, it may obviate the need for axillary clearance in a significant proportion of patients. Unfortunately, the independent prognostic relevance of many of the new biological features of breast cancer is unclear and controversial (2). There are several possible reasons for this continuing controversy, but major limitations include the small number of patients studied in many published reports and the short duration of follow-up (1). This deficiency has been addressed in recent studies from one of our laboratories in which a large cohort of >1000 patients has been followed for >5 years (4–6). The current study has used the same patient set to investigate the prognostic significance of gene amplification at the 11q13 locus.

Research on mammary carcinoma induced by the mouse mammary tumor virus initially identified the FGF-3 and FGF-4 genes as major targets of transcriptional activation by proviral insertion (7, 8) and ultimately led to the identification of an amplified region of DNA on human chromosome 11 band q13 harboring the human homologue of INT-2/FGF-3 (9). This locus is commonly rearranged in B-cell lymphomas and is amplified in a number of carcinomas, including breast cancer (10–12). Depending on the gene probe and the criterion used to define amplification, the reported prevalence of 11q13 amplification in breast cancer has ranged from 5–25%: a recent review of 13 published studies, however, indicates a mean incidence of amplification of 13% (10). Although some early studies associated INT-2 amplification with poor prognosis (9, 13, 14), it subse-
consequently became clear that int-2 was not expressed in human breast cancer (8), and therefore, the clinical relevance of INT-2 amplification was questioned. More recently, in addition to INT-2, several candidate oncogenes including HST-1 (FGF-4), EMS-1, and CCND1 have been mapped to the 11q13 amplicon. Of these genes, there is accumulating evidence for a potential functional role for cyclin D1 and EMS-1 (10–12).

In contrast to int-2, cyclin D1 is overexpressed in primary breast cancer (15–18), and there is now convincing evidence that cyclin D1 plays a pivotal role in mammary gland development, control of cell cycle progression in mammary epithelial cells, and mammary tumorigenesis. Transgenic mice expressing cyclin D1 under the control of a mammary gland-specific promoter have premature development of the lobular alveoli and epithelial hyperplasia, and they develop adenocarcinomas after a long latent period (19). Conversely, cyclin D1 knockout mice fail to develop lobular alveoli during pregnancy, supporting an essential role for this gene in mammary gland development (20, 21). Cyclin D1 is also intimately involved in cell cycle control of mammalian cells, including normal and neoplastic breast epithelial cells (22). Recent data indicate that cyclin D1 is a key regulator of mitogenic responses to steroids and growth factors in these cells (22, 23). Furthermore, overexpression of cyclin D1 in growth-arrested breast cancer cells was sufficient to allow cells to reenter and progress through the cell cycle in the absence of growth factors (24). Such data imply that overexpression of cyclin D1 may lead to a loss of normal growth constraints, e.g., a loss of a requirement for steroids or growth factors, resulting in autonomous growth. These data suggest that dysregulated expression and function of cyclin D1 in mammary epithelial cells could contribute to the development and progression of breast cancer.

To develop a more detailed understanding of the potential involvement of cyclin D1 in human breast cancer, we determined the prevalence of amplification of the CCND1 gene and compared it with INT-2 amplification in tumors from a cohort of patients analyzed previously in a large prospective study designed to systematically characterize the association between a variety of molecular alterations and prognosis (4–6).

MATERIALS AND METHODS

Clinicopathological Features. The clinical details of the cohort of patients included in this study have been described previously (4). In the present study, slot-blot filters containing DNA from 1014 tumors used in our previous study of HER-2/new oncogene amplification were suitable for reprobing with cyclin D1 and int-2 cDNA probes (see below). All patients had their diagnosis confirmed by biopsy and were treated by either total (74%) or partial (26%) mastectomy, with axillary lymph node clearance. Adjuvant postoperative radiotherapy was not administered to patients who underwent total mastectomy, whereas 67% of patients who underwent partial mastectomy received it. Adjuvant chemotherapy with six cycles of cyclophosphamide, methotrexate, and fluorouracil or tamoxifen was administered to axillary node-positive, premenopausal (15%) or post-menopausal (24%) women. The pathological characteristics of the primary tumors (including tumor size, histological type, and grade) were determined as described previously (4). ERα and PgR concentrations were determined in tumor cytosol fractions by saturation analysis as reported previously (25). Tumor grading was performed according to the Bloom and Richardson grading method by the pathologists who had reported the initial histological findings. Patients were followed-up at 6–12 monthly intervals by their treating surgeons, and information (such as the time and type of recurrence) was documented prospectively.

Data on the amplification of the HER-2/neu oncogene (c-erbB2), the tumor cell proliferation fraction determined by MIB-1 count, and the expression of the tumor suppressor gene, p53, were available from previously published studies on this series of tumors (4, 6).

Analyses of CCND1 and INT-2 Gene Amplification. The details of DNA extraction from tumors and slot-blot hybridization to determine HER-2/neu amplification have been described previously (4). After the study of HER-2/neu amplification, the HER-2/neu probe was removed from the slot-blot filters by boiling in 0.1 × SSC (15 mm sodium chloride, 1.5 mm sodium citrate) and 0.1% SDS for 5 min. Filters were then rehybridized with labeled cyclin D1 cDNA probe (26) as described by Buckley et al. (15). After strip-washing the filters to remove the cyclin D1 probe, filters were rehybridized with a PgR cDNA (27); the PgR gene localized to 11q22 (28). This allowed correction for differences in the amount of DNA immobilized in each slot of the filter and allowed amplification to be distinguished from chromosome 11 polysomy. In addition to tumor DNA, each filter also contained four slots with DNA samples from normal human peripheral blood lymphocytes as controls. The CCND1 signal was quantitated by densitometric analysis of autoradiographs, using a Bio-Rad Model 620 video densitometer and Bio-Rad 1-D Analyst software. In all cases, the hybridization and densitometric analysis were completed in one laboratory, and the subsequent calculations of gene amplification were completed in another. No prior knowledge of the samples in each slot was available. The ratio of CCND1 signal: PgR signal was used to compare tumor samples with control samples (i.e., CCND1 copy number = tumor DNA CCND1 signal/tumor DNA PgR signal divided by the mean of four control DNA CCND1 signal/control DNA PgR signal). In each batch of slot-blot analysis, tumor DNA samples showing signal ratio ≥2 × the mean signal ratio observed in normal DNA were considered to be amplified. A similar procedure was performed for the determination of INT-2 amplification, using an int-2 cDNA kindly provided by Dr. Clive Dickson (Imperial Cancer Research Fund Laboratories, London, United Kingdom), comprising a 0.9 kb sac-1 fragment of the human INT-2 gene.

Statistical Analysis. All data were censored in May 1995, including patients who were lost to follow-up, or who died of disease other than breast cancer. Both systemic and local relapses were documented as events. Sample size calculations based on the number of events and the prevalence of tumors with CCND1 or INT-2 gene amplification were performed using the method described by Schoenfeld (29) for a two-sided 5%
significance level and 90% statistical power. Using relapse-free survival or overall survival as end points, univariate Cox analysis was performed to determine the association between various parameters and prognosis. The association between gene amplification and other pathological variables was determined by \( \chi^2 \) analysis or Fisher's exact test. All tests were two-tailed, and \( P < 0.05 \) was considered to be significant. Relapse-free survival and overall survival were also estimated for CCND1 and INT-2 groups by using the Kaplan-Meier method, and the statistical significance was determined by log-rank test.

### RESULTS

CCND1 amplification (defined as gene copy number \( \geq 2 \)) was detected in 103 (10%) of the 1014 tumor DNA samples available for study. In 771 samples, the amplification of INT-2 was also determined, and using \( \geq 2 \) gene copy number as the cutoff, 131 samples (17%) showed amplification. Of the 1014 tumors, 488 were \( < 21 \) mm in diameter, and 526 were \( \geq 21 \) mm in diameter, whereas 573 (57%) had no evidence of metastatic disease in the axillary lymph nodes. Using ER and PgR concentrations of \( \geq 10 \) fmol/mg cytosol protein, 704 tumors (69%) were classified as ER positive, and 668 tumors (66%) were PgR positive. A total of 861 tumors were invasive ductal carcinomas, and 103 tumors were nonductal carcinomas. Tumor grading was available for 579 tumors, of which 88 (15%), 258 (44.5%), and 233 (40%) were grade I, II, or III tumors, respectively. Amplification of the HER-2/neu oncogene was available from a previous study (4) on all samples, and 114 samples (11%) had gene copy numbers of \( \geq 3 \). Immunohistochemical analysis of p53 expression and MIB-1 counts has been presented previously (6); 29% of the samples were positive for p53, and 33% were positive for MIB-1.

Table 1 shows the association between CCND1 and INT-2 gene amplification and the various clinical and pathological parameters examined. There were no associations between gene amplification and patient age, tumor size or grade, axillary lymph node status, histological subtype, HER-2/neu amplification, MIB-1 count, or p53 expression. CCND1 amplification was more often associated with ER- or PgR-positive tumors, but this association was not statistically significant when a gene copy number of \( \geq 2 \) was used as the cutoff. However, at a CCND1 gene copy number of \( \geq 3 \), CCND1 amplification was
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Univariate Cox analyses of relapse-free survival and overall survival

<table>
<thead>
<tr>
<th>Variable</th>
<th>Relative risk</th>
<th>95% Confidence interval</th>
<th>P</th>
<th>Relative risk</th>
<th>95% Confidence interval</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor size</td>
<td>2.0</td>
<td>1.6-2.5</td>
<td>0.0001</td>
<td>2.3</td>
<td>1.7-3.0</td>
<td>0.0001</td>
</tr>
<tr>
<td>Nodes 1-3</td>
<td>1.2</td>
<td>0.9-1.6</td>
<td>0.2</td>
<td>1.5</td>
<td>1.1-2.2</td>
<td>0.02</td>
</tr>
<tr>
<td>Nodes 4-9</td>
<td>2.2</td>
<td>1.6-3.0</td>
<td>0.0001</td>
<td>2.6</td>
<td>1.7-3.7</td>
<td>0.0001</td>
</tr>
<tr>
<td>Nodes ≥10</td>
<td>6.2</td>
<td>4.4-8.6</td>
<td>0.0001</td>
<td>8.4</td>
<td>5.8-12.2</td>
<td>0.0001</td>
</tr>
<tr>
<td>ER</td>
<td>1.6</td>
<td>1.2-2.0</td>
<td>0.0002</td>
<td>1.9</td>
<td>1.5-2.5</td>
<td>0.0001</td>
</tr>
<tr>
<td>HER-2/neu</td>
<td>2.1</td>
<td>1.4-3.1</td>
<td>0.0002</td>
<td>2.1</td>
<td>1.4-3.4</td>
<td>0.0009</td>
</tr>
<tr>
<td>MIB-1</td>
<td>2.1</td>
<td>1.5-2.7</td>
<td>0.0001</td>
<td>2.6</td>
<td>1.8-3.6</td>
<td>0.0001</td>
</tr>
<tr>
<td>p53</td>
<td>1.5</td>
<td>1.1-2.0</td>
<td>0.008</td>
<td>1.6</td>
<td>1.2-2.3</td>
<td>0.004</td>
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<tr>
<td>CCND1 (≥2)</td>
<td>1.1</td>
<td>0.8-1.6</td>
<td>0.6</td>
<td>1.0</td>
<td>0.6-1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>CCND1 (≥3)</td>
<td>1.0</td>
<td>0.6-1.8</td>
<td>0.9</td>
<td>1.0</td>
<td>0.5-2.0</td>
<td>0.9</td>
</tr>
<tr>
<td>INT-2 (≥2)</td>
<td>1.2</td>
<td>0.9-1.7</td>
<td>0.2</td>
<td>1.1</td>
<td>0.7-1.6</td>
<td>0.8</td>
</tr>
<tr>
<td>INT-2 (≥3)</td>
<td>1.3</td>
<td>0.9-2.0</td>
<td>0.2</td>
<td>1.0</td>
<td>0.6-1.8</td>
<td>0.9</td>
</tr>
</tbody>
</table>

- CCND1 (n = 1014): <2 vs. ≥2 gene copy number.
- CCND1 (n = 1014): <3 vs. ≥3 gene copy number.
- INT-2 (n = 771): <2 vs. ≥2 gene copy number.
- INT-2 (n = 771): <3 vs. ≥3 gene copy number.

As of March 1995, the median follow-up duration of patients still alive was 66 months (range 39–97 months). At the time of analysis, 305 patients had relapsed outside the breast, and 212 patients had died of breast cancer. Table 2 shows the Cox univariate analyses of several pathological features, using relapse-free survival and overall survival as endpoints. In these analyses, large tumor size (≥21 mm), the number of axillary lymph nodes involved (≥4), ER and PgR negativity, HER-2/neu amplification, p53 expression, and MIB-1 positivity were all associated with increases in risk for both relapse and death from breast cancer. In contrast, INT-2 and CCND1 amplification were not associated with increases in risk for relapse or death from breast cancer. Because almost equal numbers of patients with INT-2 or CCND1 amplification were node negative and node positive in this patient cohort, and because others have suggested different effects of INT-2 or CCND1 amplification on relapse-free survival and overall survival in these two groups (30), we assessed the effect of INT-2 or CCND1 amplification on relapse-free survival and overall survival in these two groups. In the node-negative subgroup, the patients with INT-2 amplification had a significant increase in risk of relapse as compared to those without amplification (P = 0.02). This was not seen in the node-positive subgroup. Although it did not reach significance, there was also a trend for a shorter relapse-free survival in the node-negative group with CCND1 amplification (P = 0.09; Fig. 3). In addition, we attempted to distinguish the effects of INT-2 amplification on the outcome of patients with ER-positive and ER-negative tumors. Although there was a trend for increased relapse in the ER-positive subgroup with either INT-2 or CCND1 (Fig. 4) amplification, this was not significant. There was no effect of CCND1 and INT-2 amplification on overall survival in these two subgroups.

DISCUSSION

There are now at least 15 published studies determining associations between INT-2 amplification and clinicopathological features of breast cancer and patient outcome (9, 13, 14, 30–40). Earlier studies suggested that INT-2, HST-1, BCL-1, EMS-1, and CCND1 are all closely linked on chromosome 11q13 and are coamplified in breast cancer. However, there is accumulating evidence for separate regions of amplification within this large amplicon (9, 13, 14, 30–40), including different frequencies of EMS-1 and INT-2/HST-1 amplification in breast cancer (41). Our data showing a highly significant correlation between INT-2 and CCND1 amplification would be expected from their close localization at 11q13 and are supported by other published studies (37, 41). Both INT-2 and HST-1 encode members of the fibroblast growth factor family (42, 43), but they show low or no expression in breast tumors even when amplified (8), suggesting that they are unlikely to contribute to the breast cancer phenotype. BCL-1 corresponds to a breakpoint locus in genomic DNA (44) observed frequently in B-cell neoplasms, and there is now strong evidence that this leads to overexpression of cyclin D1 (45); a conclusion supportive of the view that CCND1 is a likely candidate oncogene at the 11q13 locus (10–12). Consequently, we used a cyclin D1 probe to assess 11q13 amplification in primary breast cancer and compared it with the more widely studied INT-2 gene, which is located within 160 kb of CCND1 at 11q13 (10, 11).

The variability in the reported prevalence of 11q13 amplification in breast cancer may be due to several factors: (a) the determination of gene amplification by either slot-blot or Southern analysis is not precise, and attention to appropriate controls is essential to determine true amplification (46); (b) the gene probe used for the study may show variability in hybridization.
In this regard, it was interesting to note that in the present study the proportion of tumors with amplified DNA was greater when INT-2 eDNA was used as a probe. Although this could be interpreted as a real difference in the frequency of amplification between CCND1 and INT-2, differences in the degree of non-specific binding between these probes could, in turn, affect assay sensitivity; and (c) depending on the source of tumor, the degree of admixture of normal and tumor cells in samples used to determine amplification may be variable; too much normal cell contamination may mask amplification of tumor DNA. In our study, we used normal DNA samples in every slot-blot filter as controls and used another chromosome 11 marker, PgR, to correct for polysomy and DNA loading. Although attempts were made to select tumor areas free of normal tissue, significant admixture with normal cells was inevitable. Therefore, the prevalence of 11q13 amplification in this study may be an underestimate but is in agreement with published data using similar methodology as outlined above (reviewed in Ref. 10). Because of the heterogeneous nature of breast cancer, these potential underestimations cannot be addressed with conventional techniques. However, recent experience with fluorescinated in situ hybridization analysis has established that HER-2/neu oncogene amplification is much more prevalent than reported with DNA slot-blot studies (47). It remains to be determined whether this and other techniques will reveal a higher prevalence of amplification of genes at the 11q13 locus.

The relationship between 11q13 amplification and other clinicopathological features (including patient age, tumor size, axillary lymph node involvement, stage of disease, and histological type) reported thus far has failed to reach a consensus. Some studies have reported relationships between 11q13 amplification and patient age (14, 32), lymph node involvement (33, 36), or tumor size (38), but neither our study nor the other large study of Berns et al. (39) support these conclusions. Because most studies had small sample size (n = 30–311), it is difficult to reconcile these results with the data reported here. A confounding factor is the patient population under study; our study contained an equal proportion of 11q13-amplified tumors (i.e., 10% in the node-negative and node-positive groups), whereas some other studies had populations with a markedly lower frequency of amplification (1.8–6.0%) in node-negative patients (36, 38). Whether the relationship of 11q13 amplification to other clinicopathological features changes with the progression of disease remains to be elucidated.

Of several published studies reporting on the prognostic significance of 11q13 amplification in breast cancer, four documented an association with ER-positive tumors (30, 31, 33, 35), two noted a trend for the association (34, 40), whereas five
reported no association (14, 36–39). The present study and that of Berns et al. (31) are the two largest series published to date, with a sample size of 1052 and 1014, respectively. In both studies, a significant positive association between 11q13 amplification and ER positivity was evident. Further independent evidence to support a relationship between cyclin D1 overexpression and ER status is provided by our previous observation that tumors expressing the highest levels of cyclin D1 mRNA are uniformly ER positive (15), and by a more recent study using RNA from 364 primary breast cancers, in which we demonstrated a significant positive correlation between ER and cyclin D1 gene expression (48). In the present study, we also documented a positive correlation between CCND1 amplification (≥3 copies) and PgR positivity; the same association was reported in a smaller study (33) but was not observed by Berns et al. (31) and five other groups (14, 36, 37, 39, 40). Thus, the relationship between cyclin D1 and PgR gene expression requires further investigation.

Several early studies (9, 13, 14) suggested an association between INT-2 amplification and reduced relapse-free survival or overall survival, and this has been supported by more recent data in which (in some instances) subgroups have also been analyzed. Tsuda et al. (32) noted a significant association with increased relapse and a trend toward increased death from breast cancer in cases carrying four or more copies of INT-2/HST-1. Similarly, Börg et al. (30) and Berns et al. (40) found INT-2/HST-1 amplification to be significantly associated with increased risk of relapse in axillary lymph node-negative or ER-positive patients but no significant association with reduced overall survival. On the contrary, Schuuring et al. (36) reported an association between 11q13 (INT-2/BCL-1) amplification and increases in both relapse and death from breast cancer in axillary lymph node-positive patients; in that study the number of node-negative cases with amplification was too small to draw any conclusions. Finally, Henry et al. (38) observed that patients with INT-2 amplification had significantly reduced probability of relapse-free and overall survival, and this was particularly so in the lymph node-positive subgroup. Again there were fewer than 2% of node-negative patients carrying amplified INT-2; too small a sample size to determine prognostic significance. Although the latter study suggested a higher proportion of patients with INT-2 amplification suffered relapse during the follow-up period (67% with amplification versus 35% without amplification relapsed), and all of these subsequently died, the absolute numbers of patients were small (8 of 12 with INT-2 amplification versus 35 of 99 with normal INT-2 gene copy number relapsed). Our study, which included 1014 patients with a median follow-up period of 66 months, failed to detect any statis-
tically significant association between CCND1 or INT-2 amplification and increases in relapse or death from breast cancer. Similarly, another more recent study, which has been presented to date only in abstract form, found no association between INT-2 amplification and increased relapse from breast cancer among 661 patients with a median follow-up of 57 months (39). However, in studies with significant numbers of node-negative patients, including our own, there is a trend for early relapse (40). A similar trend was also noted in the ER-positive subgroup. Thus, INT-2/CCND1 amplification may identify a subset of patients with a relatively increased risk of relapse who might benefit from more aggressive treatment.

In conclusion, despite earlier reports that amplification of genes at the 11q13 locus (particularly INT-2) predicts for poor prognosis in breast cancer, our large series of patients with more than 5 years follow-up fails to confirm a relationship between CCND1 or INT-2 amplification and relapse-free survival or overall survival in the whole group of patients. However, in agreement with others (30, 40), our data suggest a potential role for INT-2 or CCND1 amplification in identifying subsets of poor-prognosis patients within the node-negative or ER-positive, good-prognosis subgroups. Because there is now substantial evidence that overexpression of cyclin D1 mRNA and protein is much higher than the frequency of CCND1 amplification (i.e., >45% versus 13%, respectively; Refs. 10 and 15), mechanisms other than DNA amplification must contribute to dysregulated expression of cyclin D1. Thus, the relationship between cyclin D1 overexpression and prognosis may offer more relevant information, and this relationship is currently under investigation in this series of patients.

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REFERENCES


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