CYCLIN D1 AND RETINOBLASTOMA GENE EXPRESSION IN HUMAN BREAST CARCINOMA: CORRELATION WITH TUMOUR PROLIFERATION AND OESTROGEN RECEPTOR STATUS

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SUMMARY

Cyclin D1 (CCND1) and retinoblastoma (Rb) genes are cell cycle regulators which are altered in some breast carcinomas. However, the possible cooperation between CCND1 and Rb, as well as the influence and coincidence of their abnormalities in the proliferative capacity of mammary carcinoma cells in vivo, is still unknown. In order to assess both the significance of the CCND1 gene and Rb alterations in breast carcinomas and their relationship with the proliferative capacity of the tumours and other clinico-pathological factors, CCND1 mRNA expression was studied in 46 cases of primary breast carcinomas and matched normal tissue, 45 of which were also studied immunohistochemically. Rb expression was analysed in the same cases by immunohistochemistry, whereas the proliferative activity of the carcinomas was evaluated by flow cytometry. CCND1 mRNA was overexpressed in 19 tumours (41 per cent). Sixteen cases showed diffuse immunohistochemical expression, ten carcinomas had few positive cells, and 19 were absolutely negative. CCND1 mRNA and protein overexpression was associated with oestrogen receptor (ER) expression by the tumour. Interestingly, lack of ER expression was associated with a decreased CCND1 mRNA signal in non-overexpressed tumours. No association was observed between CCND1 mRNA or protein overexpression and tumour proliferation or other clinico-pathological parameters. Loss of Rb expression was observed in 26 per cent of the tumours. This abnormality was significantly associated with increased mean S-phase (P=0·017) and decreased CCND1 mRNA expression in non-overexpressed tumours, supporting in vivo the postulated regulatory loop between Rb and CCND1 in vitro. We conclude that CCND1 up-regulation is not associated with increased proliferative activity in breast carcinomas, whereas its expression might be regulated in vitro by hormones and Rb. Loss of Rb expression is significantly associated with an increased proliferation of tumour cells, suggesting an important role in the progression of a subset of breast carcinomas, regardless of CCND1 abnormalities.

INTRODUCTION

Deregulation of cell cycle control is now considered a key event in oncogenesis and alterations of genes involved in this control are frequent findings in different cancer models. CCND1 encodes cyclin D1, which has been drawn attention as a potential oncogene in several cancer models. CCND1 participates in the control of G1–S transition and its deregulation may lead to the acceleration of cell proliferation.1,2 Furthermore, transformation studies have demonstrated that this gene may function as a cooperating oncogene in the malignant transformation of cells.2 CCND1 was originally described as PRAD-1, a gene clonally rearranged and overexpressed in parathyroid adenomas and located on 11q13.3,4 It is also related to the bcl-1 gene, activated by t(11;14) translocation in some leukaemia/lymphomas.5–7 CCND1 seems to be involved in the development of several types of solid tumours, including head and neck,8–10 oesophagus,11–13 lung,14 liver,15 and breast carcinomas.16–30 In the latter, CCND1 amplification usually correlates with increased mRNA and protein expression, although a significant number of cases are overexpressed without amplification.33,25–29

The role of CCND1 in cell cycle control seems to be mediated by interaction of cyclin D1 with the retinoblastoma gene protein (Rb) through the cdk/cyclin D complex, which achieves its inhibitory effect by Rb phosphorylation, although other mechanisms are also likely to be involved.2,31,32 Conversely, CCND1 expression seems to be induced by a functional Rb.33 Thus, Rb and CCND1 functions are apparently very closely related, and this relationship probably plays a major role in the control of some types of solid tumours, including breast carcinomas.
role in the deregulation of proliferative activity in some cancer models. The retinoblastoma gene has also been found to be altered at the genomic and protein levels in breast carcinomas. Consistent with the reported relationship between CCND1 and Rb, initial studies have shown that inactivation of Rb in mammary cell lines is associated with exceptionally low levels of cyclin D1 mRNA and protein, and that reintroduction of functional Rb in Rb-deficient mammary cell lines induces cyclin D1 expression, suggesting the existence of a Rb–cyclin D1 pathway. However, the existence and significance of coincidental alterations of both cell cycle regulators in human breast cancers in vivo are not yet well characterized.

In the present study we investigated the existence of both CCND1 and Rb alterations in a series of primary breast carcinomas, as well as the possible influence of abnormalities of these genes in the proliferative activity and clinico-pathological factors of the tumours.

**MATERIALS AND METHODS**

**Patients and tissues**

A total of 46 consecutive breast carcinomas were selected from patients at our institution. Samples from tumour tissue and non-neoplastic areas were snap-frozen in liquid nitrogen and stored at −80°C. The remaining specimen was fixed in formalin and routinely studied. Five cases were infiltrating lobular carcinomas and the remaining 41 were infiltrating ductal carcinomas. Histological grading was performed according to Elston’s modification of Bloom–Richardson’s method only in infiltrating ductal tumours. Other clinico-pathological parameters such as tumour size and regional lymph node involvement were also recorded (Table I).

**Immunohistochemistry**

Cyclin D1, Rb, and oestrogen receptor (ER) expression was determined immunohistochemically on paraffin sections with monoclonal antibodies (anti-cyclin D1 clone DCS-6, 1/150, Neomarkers, Fremont, CA, U.S.A.; anti-Rb clone G3-245, 1/100, Pharmingen, San Diego, CA, U.S.A.; anti-estrogen receptor (ER-ICA), 1/5, Abbott Laboratories, North Chicago, IL, U.S.A., respectively) and with the streptavidin–biotin–alkaline phosphatase method (BioGenex, San Ramon, CA, U.S.A., Supersensitive Kit). The primary antibodies were incubated overnight at 4°C and were followed by

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**Table I—CCND1 mRNA overexpression, immunohistochemical positivity, and clinico-pathological features**

<table>
<thead>
<tr>
<th>mRNA overexpression</th>
<th>IHQ</th>
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<tr>
<td>n*</td>
<td>− (%)</td>
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<tr>
<td>Tumour size (TNM)</td>
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</tr>
<tr>
<td>T1</td>
<td>7</td>
</tr>
<tr>
<td>&gt;T1</td>
<td>39</td>
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<tr>
<td>Histological type</td>
<td></td>
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<tr>
<td>Ductal infiltrating</td>
<td>41</td>
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<tr>
<td>Lobular infiltrating</td>
<td>5</td>
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<tr>
<td>Tumour grade</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>4</td>
</tr>
<tr>
<td>II</td>
<td>31</td>
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<tr>
<td>III</td>
<td>6</td>
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<tr>
<td>Lymph nodes</td>
<td></td>
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<tr>
<td>Negative</td>
<td>16</td>
</tr>
<tr>
<td>Positive</td>
<td>24</td>
</tr>
<tr>
<td>ER</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>11</td>
</tr>
<tr>
<td>Positive</td>
<td>35</td>
</tr>
<tr>
<td>Rb expression</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>12</td>
</tr>
<tr>
<td>Positive</td>
<td>34</td>
</tr>
<tr>
<td>Cyclin D1 IHQ</td>
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</tr>
<tr>
<td>Negative</td>
<td>18</td>
</tr>
<tr>
<td>Positive</td>
<td>26</td>
</tr>
</tbody>
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*Totals do not match up since some cases lacked information in some parameters.
†Grade I+II vs. III; \(P=0.056\).
‡\(P=0.001\).
§\(P=0.001\).
*\(P=0.002\).
the link antibody and development system. Cyclin D1 and Rb immunostaining required 10 min of microwave pretreatment with incubation on 0.01 M citrate buffer. Quantification of CCND1 and Rb was performed by routinely evaluating the percentage of cells with intranuclear positivity in ten random high-power (×40) microscopic fields and counting at least 500 cells. Cases were considered arbitrarily negative for Rb when most areas of the tumours were completely negative or very few (<1 per cent) positive tumour cells were found.41 Cyclin D1 immunostaining was evaluated according to three patterns: no positive cells, few (<5 per cent) positive cells, and diffuse positivity.25 ER staining was performed after 5 min of pronase digestion prior to incubations. Cases were evaluated with a modified HSCORE system42 by multiplying the stratified percentage of positive cells (1–33 per cent = 1; 34–66 per cent = 2; 67–100 per cent = 3) by the average intensity of each case (1 to 3). A case was considered ER-positive when the score was 1 or higher. Normal glands and ducts for ER and stromal cells for Rb were used as internal positive controls. As a negative control, primary antibodies were replaced by unrelated monoclonal antibodies and by PBS.

Flow cytometry

Analysis of S-phase was performed with the Coulter Epics-Profile II flow cytometer on 50 µm paraffin sections,43 and using the Multicycle program (Phoenix Flow Systems, San Diego, CA, U.S.A.). Three cases were discarded because of a variation coefficient above 5 per cent. In aneuploid cases, the global (ponderated) S-phase was recorded.

RNA extraction and Northern blot analysis

Total RNA was isolated from the frozen samples by guanidine isothiocyanate extraction and caesium chloride gradient centrifugation. Eight micrograms of RNA from each sample were electrophoresed in a 1:2 per cent agarose formaldehyde gel and transferred to Hybond-N membranes (Amersham). The PRAD-1/ cyclin D1 probe used was a 1.4 kb EcoRI fragment (ΔP1–4) of the pPL-8 partial cDNA clone of the PRAD-1 gene,4 radiolabelled with [32P]dCTP. The blots were hybridized and washed as previously described.8 A 28S rRNA probe was used for RNA loading and transfer control.

Hybridization signals were quantified using a UVP-5000 video densitometer (UVP, San Gabriel, CA, U.S.A.) as described.8 The CCND1 values were normalized to the respective 28S rRNA signal. Overexpression was considered when the tumour signal was at least twice the value of the matched normal tissue.

RESULTS

CCND1 expression

Northern blot analysis of CCND1 showed two transcripts of 4.5 and 1.5 kb (Fig. 1). Nineteen (41 per cent) tumours showed 2- to 6.9-fold overexpression when compared with the matched normal tissue. A significant correlation of CCND1 overexpression with ER expression was also found (P=0.001). All overexpressed cases were ER-positive (Table I). Unexpectedly, the CCND1 mRNA signal was decreased in seven tumours when compared with normal tissue, with a tumour/normal ratio under 0.5 (Fig. 1, case 56). A correlation was found between tumour mRNA signal under 0.5 and oestrogen receptor negativity in non-overexpressed cases, since 6 out of 11 (55 per cent) ER-negative cases showed this abnormality whereas only 1 of the 16 ER-positive cases (6 per cent) had low CCND1 expression (P=0.008).

CCND1 mRNA overexpression was not associated with increased proliferative activity of the tumours.
(Student’s t-test, \( P=0.26 \)) or other clinico-pathological parameters (Table I).

To assess whether CCND1 mRNA overexpression correlated with enhanced protein expression by tumour cells, the presence of cyclin D1 was immunohistochemically evaluated in 45 cases in which adequate paraaffin material was available. Nineteen carcinomas were completely negative (42 percent); ten showed few, although definite, cells with intranuclear positivity (22 per cent); and 16 cases displayed diffuse positivity (36 per cent). Normal epithelial breast cells and stromal cells were negative (Figs 2A and 2B). Foci of in situ carcinoma showed a similar immunohistochemical pattern to the adjacent tumour when present. The best correlation between mRNA overexpression and immunohistochemical expression of the protein was obtained when all cases with any number of positive cells were considered positive. All but two cases with mRNA overexpression also expressed the protein (\( P=0.002 \)). However, ten immunohistochemically positive cases showed no CCND1 mRNA overexpression (Table I). All cases with decreased tumour/normal mRNA expression were also immunohistochemically negative. Immunohistochemically expression of cyclin D1 correlated significantly with oestrogen receptor positivity (\( P=0.001 \)) and showed a tendency to associate with tumour differentiation, since all grade I tumours and only one out of five poorly differentiated tumours (grade III) were positive (\( P=0.056 \)) (Table I). No association was found with other clinico-pathological parameters, including S-phase.

**Retinoblastoma gene product expression**

The immunohistochemical expression of Rb was mainly intranuclear, although cytoplasmic positivity was found in some cells undergoing mitosis. Rb-positive cells were observed in normal breast acini and stroma. The distribution and intensity of immunostaining were usually heterogeneous among tumours and even within a given case. Thirty-four tumours (74 per cent) were positive (Fig. 2C). The remaining cases (26 per cent) had no Rb-positive cells, or very few (Fig. 2D). Loss of Rb expression was significantly associated with increased mean S-phase (Fig. 3). A striking CCND1 mRNA down-regulation (tumour/normal ratio<0.5) was observed in 4 out of 6 (67 per cent) non-overexpressed Rb-negative tumours (Fig. 1), whereas this occurred in only 3 out of 21 (14 per cent) Rb-positive cases (\( P=0.023 \)). This association could not be evaluated with CCND1 immunohistochemical expression, since normal breast tissue was consistently negative, indicating the
inadequacy of this technique for evaluating basal levels of the protein both in normal tissue and in non-overexpressed tumours. No association with other tumour features was observed.

DISCUSSION

In this study we analysed the status of two cell cycle regulators, CCND1 and Rb, in a series of human breast carcinomas and we observed alterations in one or both in a significant proportion of cases.

It is now well established that cyclin D1 is up-regulated in a subset of breast carcinomas, either by gene amplification and mRNA overexpression, mRNA overexpression alone, or protein overexpression due to post-transcriptional events, indicating that a variety of mechanisms are involved in the regulation of this protein. In our study, a significant number of cases had mRNA overexpression, but an even higher number of immunohistochemically positive cases were also observed. Previous reports on the immunohistochemical evaluation of cyclin D1 show a range of positivity varying between 20 and 81 per cent of breast carcinomas, which might be largely due to the variety of antibodies, antigen-retrieval techniques (microwave, digestion), and scoring systems. No protein expression was observed in two mRNA overexpressed cases. Concordant with the latter finding, previous observations suggest that overexpression of CCND1 does not always lead to increased cyclin D1 protein.

Contradictory information exists regarding the correlation of CCND1 amplification and overexpression and clinico-pathological parameters. An association has been reported between amplification of the 11q13 region and poor prognosis in breast tumours, whereas this has not been observed in other recent studies. In the present study, a tendency for correlation was observed between cyclin D1 expression and differentiation, since all grade I (well-differentiated) tumours were positive and only one out of five grade III showed this expression. These results differ from those of Zhang et al., who reported an inverse tendency to what was observed here, in that cyclin D1 tended to associate with poor differentiation. Unfortunately, these authors did not describe the grading system used in their study, which might explain the discordance.

The tendency observed in our study might be indirectly attributed to oestrogen receptor expression, which is usually observed in carcinomas with better differentiation. Indeed, we have confirmed here the previously observed association of CCND1 overexpression with oestrogen receptor positivity. Interestingly, besides this association, we report here an association between ER negativity and decreased CCND1 mRNA expression (tumour/normal ratio <0·5) in non-overexpressed tumours. The exact nature of the relationship between steroid influence and CCND1 expression is not yet clear. It has been shown that steroids stimulate breast carcinoma cell proliferation and that this occurs at a point in the early G1 phase. Thus, our results support in vivo the hypothesis that one of the mechanisms by which steroids stimulate breast carcinoma cell proliferation in vitro may be through cyclin D1 induction.

Since cyclin D1 is involved in G1–S phase transition and its overexpression accelerates G1 progression in vitro, we also investigated the possible association of CCND1 overexpression with increased tumour cell proliferation. In our study, no significant difference was observed between the proliferative activity of breast carcinomas, measured by S-phase flow cytometry, with and without CCND1 mRNA or protein overexpression. This is consistent with our results in mantle cell lymphomas and with those obtained by Zukerberg et al. in a series of breast tumours by bromodeoxyuridine labelling. A toxic effect for an excess of cyclin suggested by some authors might explain this finding. Alternatively, CCND1 overexpression might only be important in the first steps of mammary carcinogenesis, being clonally inherited and observed in clinically evident tumours. Indeed, the observation of CCND1 protein expression in pre-invasive lesions, also observed in this study, led Bartkova et al. to postulate this alteration as an early event in breast carcinogenesis. Moreover, the observation of mammary hyperplasia induced by
CCND1 overexpression in transgenic mice supports this hypothesis. Nevertheless, it is likely that other factors cooperate with this gene in the pathogenesis of these neoplasms.

Since involvement of CCND1 in cell cycle control seems to be mediated by interaction with the retinoblastoma gene protein, we also investigated Rb protein status in our series of tumors. Abnormal loss of Rb expression was found in 26 per cent of our cases, which is in accordance with the reported incidence in other series. Consistent with its proposed role as a gene controlling cell cycle progression, loss of normal Rb nuclear expression correlated significantly with enhanced proliferative activity, suggesting that loss of the regulatory activity of Rb might be an important mechanism by which breast cancer cells acquire enhanced replicative capacity. In our limited series of breast carcinomas, we could not confirm the association of Rb loss with other clinicopathological factors reported by others.

Interestingly, Rb loss also correlated with low CCND1 expression in non-overexpressed tumors. This finding supports the results of recent studies in which cyclin D1 expression has been found to be modulated in cell lines by the retinoblastoma protein. Thus, Rb might regulate cyclin D1 and, in turn, might be an object of regulation by cyclin D1, creating an autoregulatory loop. With the present observations, this is the first time, to our knowledge, that such basal CCND1 regulation by Rb has been supported in breast cancer in vivo.

When considering other cancer models, two mechanisms have been proposed for abrogation of the inhibitory role of Rb in cell cycle progression: first, increased levels of cyclin D1 by amplification and overexpression, with normal Rb expression; second, loss of Rb expression together with low CCND1 expression. In our study of breast carcinomas both situations were found, but only Rb-impaired expression was associated with increased proliferation in breast tumors, and this capacity did not increase when coincidental CCND1 overexpression was present. Since the cyclin D1 inhibitory function on cell cycle progression is mediated by phosphorylation of Rb, it could be argued that only carcinomas with CCND1 overexpression and functional Rb can have an increased proliferative activity, whereas in those in which Rb expression is impaired, CCND1 overexpression is ineffective. In our study there was not a significant difference in the proliferative activity of CCND1 overexpressed tumours with and without loss of Rb expression. Neither was a significant difference observed between CCND1 overexpressed and non-overexpressed Rb-positive tumors. Therefore, abrogation of Rb regulatory activity might be a mechanism causing breast carcinoma cell proliferation regardless of CCND1 status.

In summary, D1 overexpression is a common event in breast cancers. The role of this abnormality as an inducer of increased proliferative capacity is not supported by our results in the invasive stage of breast carcinomas. In breast neoplasms, CCND1 mRNA up-regulation, together with basal expression, seems to be associated with steroid modulation. CCND1 basal expression may also be modulated in breast cancer in vivo by Rb, the down-regulation of which could be an important oncogenic mechanism in the breast by mediating increased proliferative capacity. Further studies with a larger number of cases and appropriate follow-up are necessary to elucidate the prognostic and therapeutic usefulness of cyclin D1 and Rb evaluation in breast neoplasms.

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