

# Retinoblastoma (RB1) gene product expression in breast carcinoma. Correlation with Ki-67 growth fraction and biopathological profile

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## Abstract

**Aims**—To investigate the expression of retinoblastoma protein (pRb) in invasive breast tumours and compare its expression with the major biopathological prognostic indicators to identify more aggressive subgroups.

**Material**—Archival paraffin embedded tissues from 153 consecutive primary breast carcinomas.

**Methods**—pRb, Ki-67, and oestrogen receptor/progesterone receptor proteins were identified by immunohistochemistry and score values were recorded by image cytometric analysis; p53 and EGFr expression was also evaluated.

**Results**—pRb scores correlated strongly with proliferation activity as determined by Ki-67 staining. Positive relations were also observed between pRb scores, tumour size, nuclear and histological grade, and oestrogen receptor/progesterone receptor content, while abnormal p53 accumulation was not associated with pRb expression. Among the high proliferating carcinomas it was possible to identify 13 cases with loss of pRb expression.

**Conclusions**—pRb expression paralleled proliferative activity in the majority of breast carcinomas examined, suggesting that in these cases the protein behaves normally in regulating the cell cycle. Conversely in cases with loss of pRb immunostaining, the combined expression of specific highly aggressive factors (EGFr and p53 expression, oestrogen receptor/progesterone receptor negative status, and high K67) seems to characterise a more aggressive phenotype showing growth advantage and cellular "progression" rather than significant nodal involvement.

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Keywords: pRb; retinoblastoma; breast cancer; immunohistochemistry

Mutational inactivation of the retinoblastoma (RB1) gene is considered to be of central importance in the pathogenesis of many human malignant neoplasms. The negative regulator function now attributed to retinoblastoma protein (pRb) in the control of G1/S phase progression has renewed interest in its key role in tumour growth control.<sup>1</sup> It is known that pRb influences the activity of E2Fs and other transcriptional regulators by a direct protein to protein interaction<sup>2</sup>; pRb molecules, sequentially phosphorylated by cyclin depend-

ent kinases (cdk4/cdk6 and cdk2), release E2F factors, allowing the transcription functions of pRb to initiate the DNA duplication machinery and G1/S transition.<sup>1-3</sup> Over the past decade it has become clear that the genomic or epigenetic loss of pRb function leads to uncontrolled cell growth and apoptosis.<sup>4-5</sup> This is not only an initiating event in tumorigenesis, but also a step associated with malignant progression and aggressive outcome.<sup>1-6-9</sup>

Most studies investigating pRb in human malignancies have been conducted with the aim of demonstrating loss of heterozygosity or the presence of mutations at the RB1 gene locus. As well as in retinoblastoma,<sup>10</sup> RB1 structural alterations have been detected in several mesenchymal and epithelial malignancies,<sup>11-15</sup> including breast cancer.<sup>16-19</sup>

Surprisingly, allelic loss of one RB1 gene and alterations in the second alleles have not been shown in several breast cancers not expressing pRb, which suggests that there may be pRb inactivating mechanisms other than the unmasking of a recessive mutation.<sup>20</sup> However, the presence of a well defined gene product easily detectable by immunohistochemistry, the finding that pRb inactivation may also occur through the formation of complexes with viral proteins, and the proven value of the immunohistochemical assay independent of heterozygosity analysis,<sup>6-9, 21-23</sup> have aroused interest in the study of the presence and distribution of pRb in neoplastic cells. Immunohistochemical studies on pRb expression in large tumour series are limited and often use incompatible methodology.<sup>6-9, 19, 24-25</sup> There are still fewer investigations comparing pRb immunostaining with the clinical behaviour of breast carcinoma and with the main prognostic indices used in breast cancer.<sup>19, 24-27</sup>

Our aims in this study were to determine the extent to which the presence of pRb immunoreactivity correlates with cell proliferation in breast carcinoma, and to evaluate whether an integrated approach comparing pRb presence with the major biopathological prognostic indicators may help in identifying aggressive subtypes.

## Methods

We assessed 153 invasive breast carcinomas. The tumours were staged and histologically classified according to the UICC-TNM and WHO criteria, respectively. Ductal not otherwise specified (NOS) invasive carcinoma was histologically graded (G).<sup>28</sup> Irrespective of the histopathological diagnosis, tumours were typed by nuclear grading (NG) as follows: well

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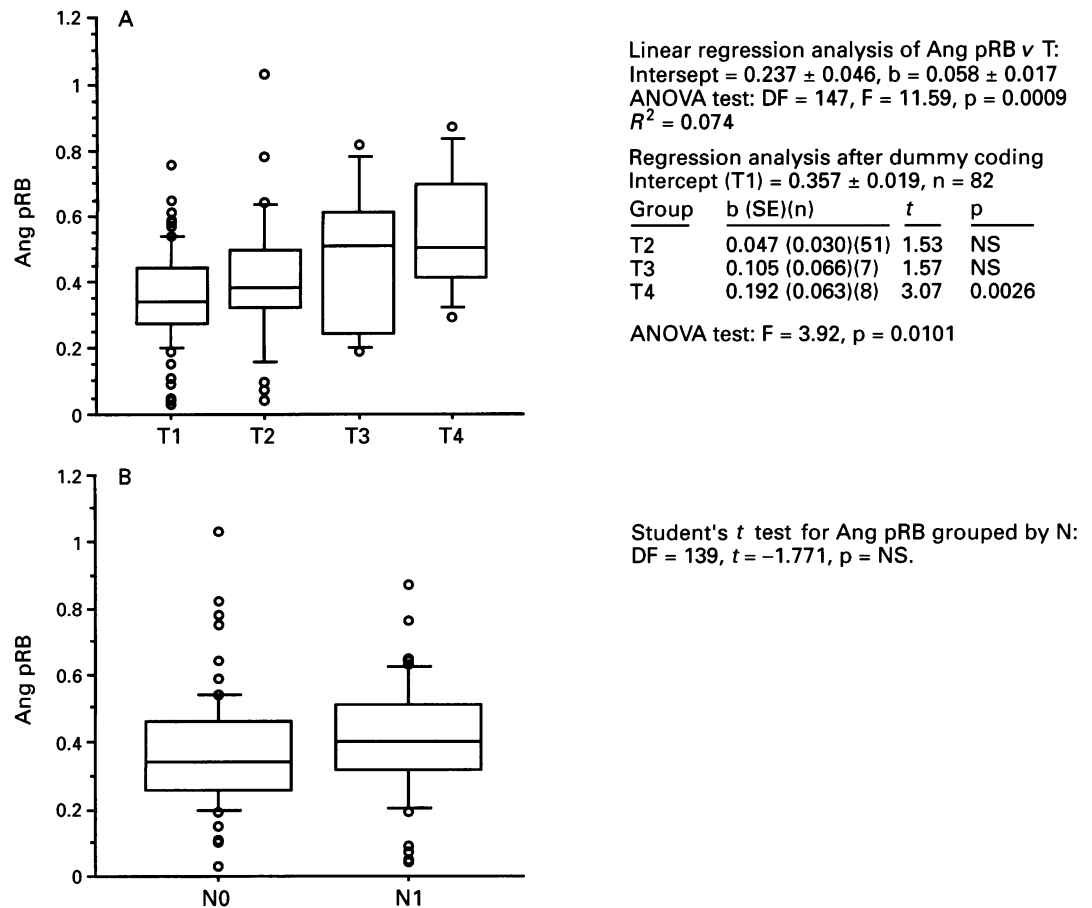


Figure 1 (A) Box plot graphic display (left), and linear regression analysis (right) for angular pRb (Ang pRb) values grouped by tumour size (T). (B) Box plot graphic display (left), and linear regression analysis (right) for angular pRb (Ang pRb) values grouped by N status. The three horizontal bars in the box for top to bottom are the 75th, 50th (median), and 25th centiles. The upper line ends at the 90th centile and the lower line at the 10th centile. Outliers are represented by circles.

differentiated (NG1), intermediate (NG2), poorly differentiated (NG3). Axillary node involvement was recorded (N0/N+) using a three point scale: N0, no lymph node metastases; N+ 1–3, one to three positive nodes; N+ > 3, more than three lymph node metastases.

#### IMMUNOHISTOCHEMISTRY

Serial sections from formalin fixed, paraffin embedded tissue blocks were collected on 3-aminopropyltriethoxy-silane treated slides (Sigma), dried overnight at 37°C, and processed for immunohistology according to a slightly modified SABC technique.<sup>29</sup> Microwave pretreatment was performed with a citrate buffer solution (pH 6.0) when using anti-oestrogen receptor and anti-progesterone receptor monoclonal antibodies (clones 1D5 and 1A6, respectively; BioGenex Laboratories), anti-p53 (clone BP53-12.1; BioGenex Laboratories), and the polyclonal antiserum anti-Ki67 (Dakopatts a/s); or with an EDTA buffer solution (pH 8.0) when using anti-pRb monoclonal antibody (clone G3-245, BioGenex Laboratories). This antibody was developed at PharMingen (Torreyana, San Diego, USA) and then widely characterised.<sup>30</sup> Finally, we performed enzyme digestion with 0.05% protease XIV (Sigma) in 0.1 M phosphate buffered saline solution (pH 7.4) at 37°C for five minutes before application of anti-EGFr monoclonal antibody (clone 31G7; Zymed Laboratories).

Nuclear and cell membrane immunostaining for p53 and EGFr, respectively, was scored independently by two observers (CC and DS) using a three point ordinal scale as follows: 0 = absence of labelled neoplastic cells; 1 = less than 25% of neoplastic cells immunopositive; 2 = more than 25% of neoplastic cells positive.

#### IMAGE CYTOMETRY

Nuclear immunostaining of pRb, oestrogen receptor, progesterone receptor, and Ki-67 was quantified by image cytometry with "Cytometrica" software (C & V, Bologna, Italy) as detailed previously.<sup>31</sup> The labelling index was expressed as the percentage of labelled over total neoplastic nuclear area (%LLa) in the section. With an optimally contrasted image, the error introduced in the final measurement by the segmentation procedure used is below 5%.<sup>32 33</sup>

#### STATISTICAL ANALYSIS

Labelling indices of pRb and Ki-67 were subjected to angular transformation (arcsin proportion) to stabilise variance of binominal proportions, and their relation was evaluated by linear regression.<sup>34</sup> Labelling indices of oestrogen receptor (OR) and progesterone receptor (PGR) were pooled ( $0.5OR + 0.5PGR$ ) to a single variable, OR/PGR, which was categorised into three groups: low, mid, and high, including respectively the lowest 33% of the

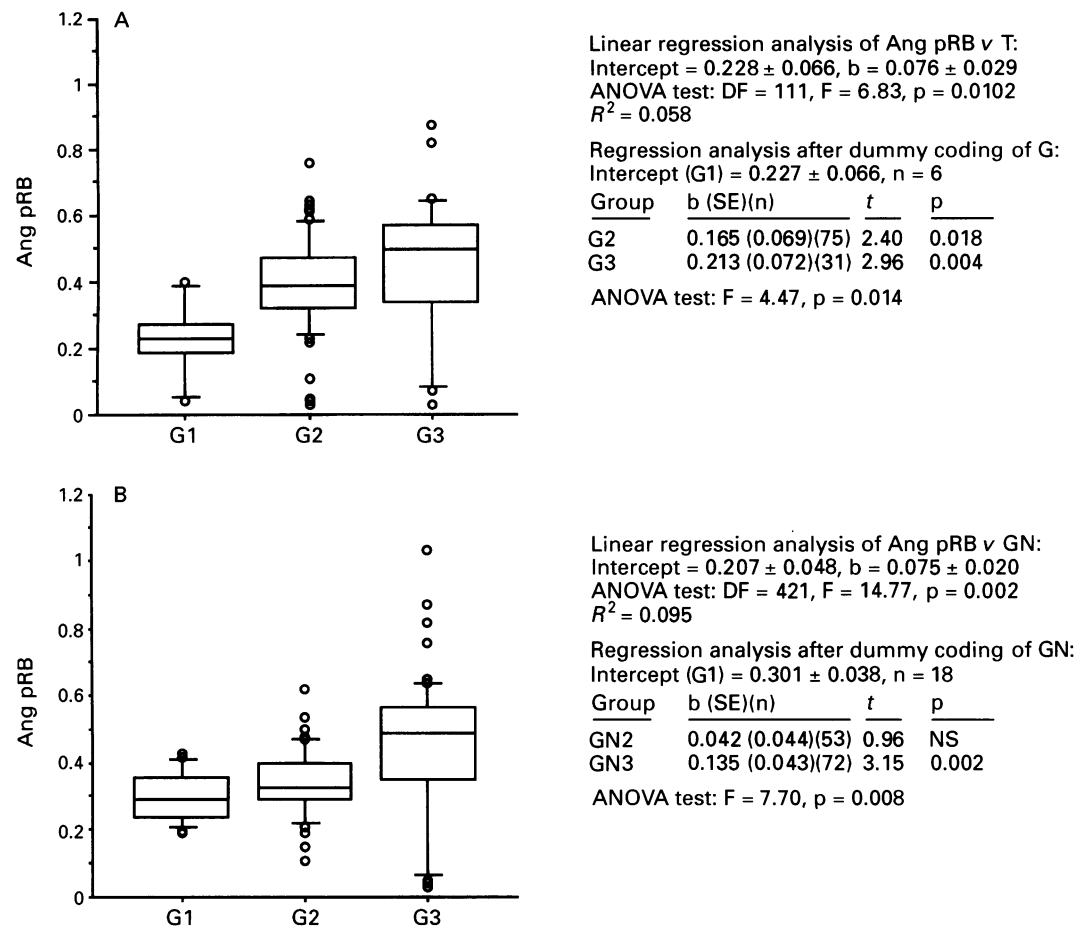


Figure 2 (A) Box plot graphic display (left), and linear regression analysis (right) for angular pRb (Ang pRb) values grouped by histological grade (G). (B) Box plot graphic display (left), and linear regression analysis (right) for angular pRb (Ang pRb) values grouped by nuclear grade (NG). For explanations about box plots, see fig 1.

values, the intermediate values, and the highest 33% of the values.<sup>35</sup>

The association of pRb with the clinicopathological variables (T, G, and NG) was quantified by analysis of variance (ANOVA). Since the groups were heavily unbalanced, ANOVA was performed using linear regression analysis after dummy coding of explanatory variables which did not depend upon equal numbers for their interpretation.<sup>36</sup> The lowest score of each clinicopathological variable (T1, G1, and NG1) was coded "0" on all sets of dummy codes and taken as the reference group in the analysis. Thus the intercept in the regression equation is the mean of the reference group, and each regression coefficient is the mean difference between the corresponding group and the reference group. The association of pRb with nodal status was examined by Student's *t* test because the two groups were balanced and—following angular transformation of pRb values—of similar variance.

Statistical calculations were performed using software from CSS Statistica (Statsoft) and Statistix (Analytical Software).

## Results

### HISTOPATHOLOGY

Of the 153 cases, 128 were diagnosed as ductal NOS invasive carcinoma, 13 as lobular, seven as mucoid, three as medullary, and two as sarcomatoid carcinomas. Eighty two of 148 cases (55.4%) were classified as pT1, 51 (34.5%) as

pT2, seven (4.7%) as pT3, and eight (5.4%) as pT4; five cases were of uncertain extent. Lymph node metastases (N+) were present in 70 cases (49.6%). Histological grading was performed in 112 ductal NOS carcinomas: six cases (5.3%) were G1, 75 (67.0%) were G2, and 31 (27.7%) were G3. Nuclear grading was evaluated in 143 cases: 18 cases (12.6%) were GN1, 53 (37.1%) were GN2, and 72 (50.3%) were GN3.

### ASSOCIATION OF pRb LABELLING WITH CLINICOPATHOLOGICAL PROGNOSTIC INDICATORS AND Ki-67

The labelling values for pRb ranged from 0% to 73% LIa, with a mean (SD) of 16.2 (12) and a median of 14. Ki-67 values ranged from 3.1% to 82.3% LIa with a mean of 26.3 (18) and a median of 22.

A positive trend was found between the pRb values T (fig 1A) and G (fig 2A). When nuclear grade (NG) was considered, NG3 tumours had a higher degree of pRb labelling than NG2 and NG1 cases; the labelling values for the latter were not significantly different (fig 2B). No difference in pRb labelling was found between N0 and N+ patients (fig 1B).

Like Ki-67 (fig 3A), pRb was always located in the nucleus (fig 3B). A strong correlation was found between pRb and Ki-67 labelling values in the different tumours (fig 4A). Moreover, pRb distribution overlapped Ki-67 immunolabelling in serial sections (fig 3A and 3B). In the scatter plot of fig 4, it appears that

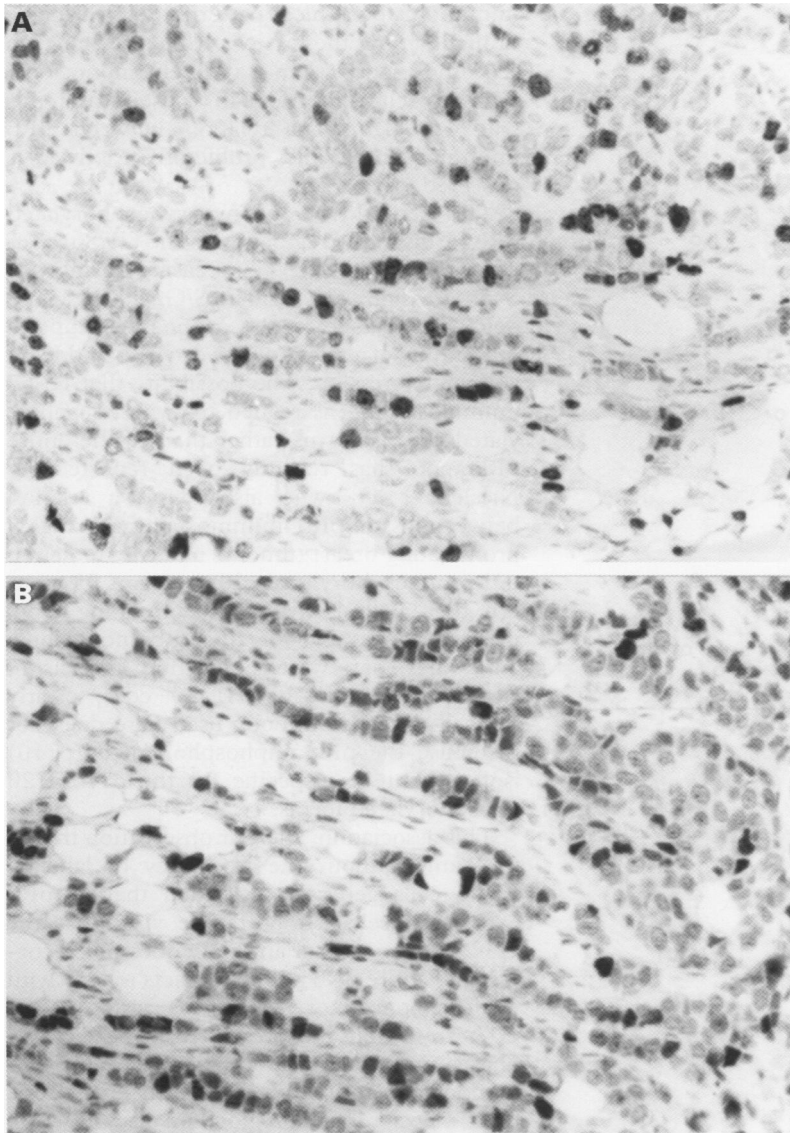


Figure 3 Serial sections of infiltrating ductal carcinoma of the breast immunostained with (A) an anti-Ki-67 polyclonal antiserum, and (B) anti-pRb monoclonal antibody G3-245. pRb immunostained nuclei overlapped the Ki-67 labeled neoplastic population. (Immunoperoxidase SABC method,  $\times 345$ .)

most of pRb and Ki-67 values were positioned close to the hypothetical line of perfect positive relation, with pRb generally accumulating below the line, indicating a lower pRb labelling compared to Ki-67 in the same tumour. Starting at a Ki-67 value of about 25%LIa, however, several tumours showed a pRb content much lower than the corresponding Ki-67 value. In at least 13 tumours with high proliferative activity, neoplastic cells were devoid of pRb. Among this latter group, six cases were T1, five were T2, and two were T3. The majority of pRb negative tumours were G2 (3/11) or G3 (8/11), while all tumours were NG3. If the data are split into two subsets at a Ki-67 labelling of 25%, where the first pRb negative tumour appeared (fig 4), it can be seen that pRb expression paralleled Ki-67 expression in tumours with low proliferative activity ( $r = 0.85$ ,  $p < 0.0001$ ), at the bottom left of fig 4. In the subset of tumours with high proliferative activity at the centre right of the same figure, there was no correlation ( $r = 0.07$ ) because tumours expressing or not expressing

pRb were randomly scattered along the whole range of Ki-67 values.

Although a bivariate relation between the extent of pRb labelling and N status was absent (fig 1B), if nodal status was re-evaluated by considering the two regression lines of pRb plotted as a function of Ki-67 low and high labelling values (fig 5), and with patients stratified by number of metastatic nodes, it was nevertheless evident that all tumours with massive lymph node involvement (nodes  $> 3$ ) were expressing pRb, irrespective of proliferative activity. Conversely, all cases without or with very low pRb labelling of cells showed a lesser metastatic potential (nodes = 0 or  $\leq 3$ ).

#### ASSOCIATION OF pRb LABELLING WITH OESTROGEN RECEPTOR/PROGESTERONE RECEPTOR STATUS, p53 IMMUNOSTAINING, AND EGFR EXPRESSION

Oestrogen receptor and progesterone receptor values ranged from 0 to 98.9% (mean (SD) 46.0(33.9)%) and from 0 to 97.0% (19.2(23.7)%), respectively. The correlation between pRb and oestrogen receptor/progesterone receptor status is summarised in fig 6B. Cases associated with low Ki-67 values mainly showed high or intermediate oestrogen receptor/progesterone receptor expression. The great majority of pRb negative tumours and most of the highly proliferative tumours were devoid of, or expressed low levels of, oestrogen receptor/progesterone receptor.

Immunoreactive p53 nuclear protein accumulation was observed in 52 cases (34%): 28 (18.3%) scored highly positive and 24 (15.7%) had intermediate scores; the highly positive p53 cases started to appear at a 17% Ki-67 Lia. Abnormal p53 accumulation was not associated with pRb expression, being present in eight (61%) of the 13 cases devoid of pRb. Because of the strong association between p53 accumulation and proliferative activity,<sup>37</sup> problems of colinearity did not allow us to make any further exploration of the relations with pRb in highly proliferating tumours.

Finally, the correlation between pRb and EGFr scores is shown in the scattergram in fig 6A. EGFr expression was mostly found in tumours with high proliferative activity without any apparent relation with pRb expression. Nevertheless, 11 of the 13 cases (84.6%) with high proliferative activity and low or absent pRb expression had an appreciable EGFr immunopositive neoplastic population.

#### Discussion

We found that the extent of pRb expression in primary breast carcinoma paralleled proliferative activity, as determined by Ki-67 labelling. This was particularly the case for tumours with low proliferative activity ( $r = 0.85$ ,  $p < 0.0001$ ). However, a consistent fraction of highly proliferative tumours (13 cases) had negligible pRb expression.

A similar correlation in breast cancer has not been reported in previous studies, which have mainly emphasised the association between abnormal pRb expression, or loss of pRb expression, and proliferative activity in tumour cells.<sup>9 19 24 26 38</sup> Loss of expression has generally

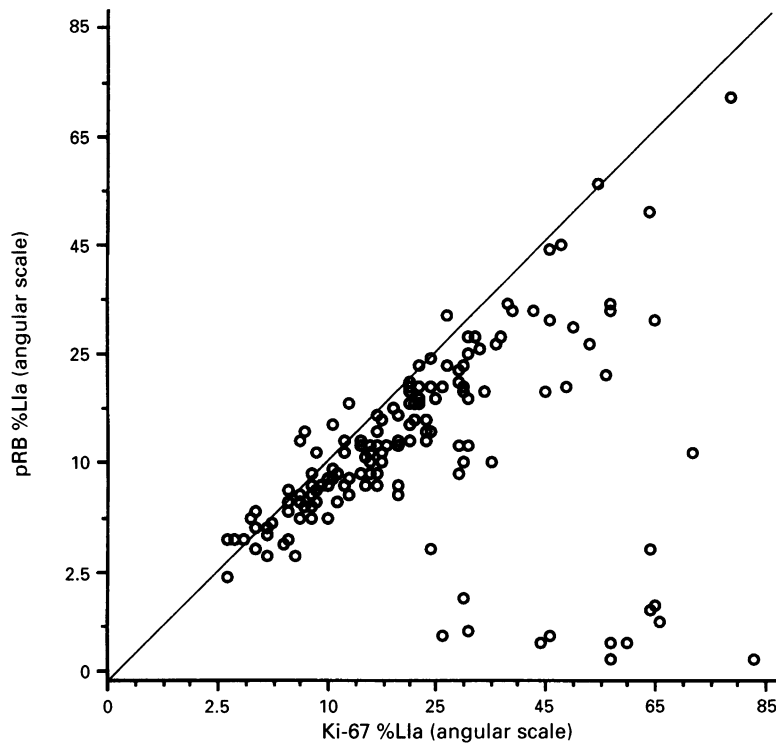


Figure 4 Scatter plot of pRB and Ki-67 %Lia values (angular scale). Diagonal line from bottom left to top right represented the hypothetical line of the perfect positive relation between the two variables.

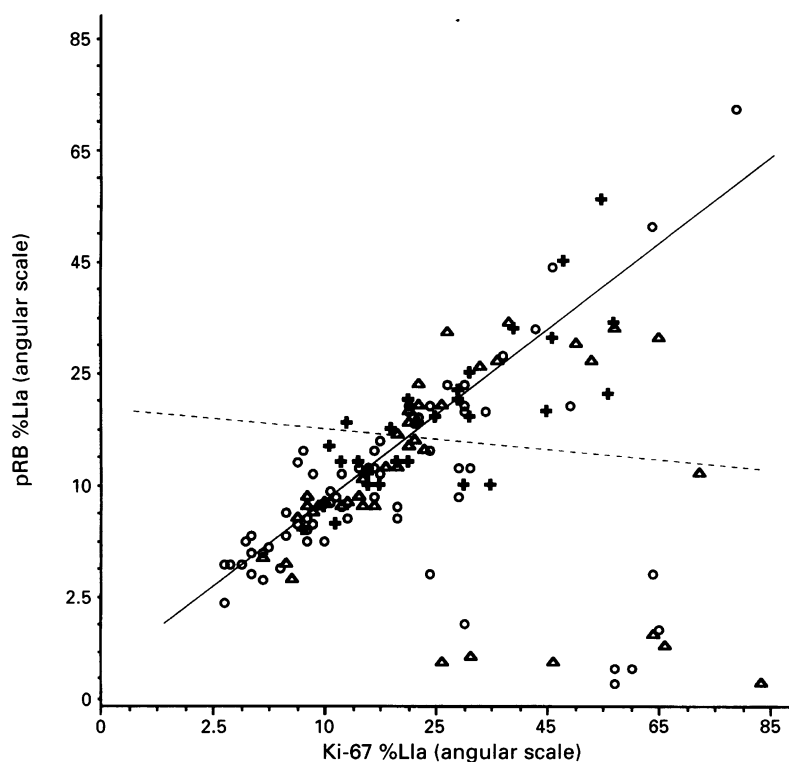


Figure 5 Same graphic scatterplot as in fig 4. The continuous line was obtained by regression of pRB v Ki 67 values lower than 25% Lia, the dotted line by regression of pRB v Ki 67 values higher than 25% Lia. Regression was calculated on angular transformed pRB and Ki67 variables. Cases were labelled according the number of metastatic lymph nodes:  $\circ$  = no metastatic involvement;  $\triangle$  = from one to three metastatic lymph nodes;  $+$  = more than three metastatic lymph nodes.

been identified using specific monoclonal antibodies such as clone 84-B3.1 (Novocastra Laboratories), which give positive results in normal and neoplastic populations without apparently discriminating between them. This

immunohistochemical pattern is only capable of highlighting the loss of pRB expression. On the other hand, when a different monoclonal antibody (clone PGM3-245) is used—as in this study—a significant relation between pRB immunostaining and proliferative activity is observed.<sup>6-8</sup>

The central role of hyperphosphorylated pRB forms in the regulation of the G1/S transition of the cell cycle<sup>1,3</sup>—increasing during G1/S progression, constant in S/G2 phases, and dephosphorylated in the M phase—supports our finding of a relation between pRB and Ki67 antigen expression. The possibility that Ki-67 immunostaining is related to hyperphosphorylated pRB isoforms rather than to the entire pRB pool has recently been reported by Nieslen *et al*,<sup>39</sup> who also found a relation between loss of pRB immunostaining in highly proliferating breast tumours and accumulation of hypophosphorylated pRB isoforms by western blot analysis. More recently Ping Dou, using a wide range of anti-pRB monoclonal antibodies in a western blotting analysis of exponentially growing HL-60 cells, clearly showed that the antibody used here does not recognise the pRB unphosphorylated (p110) isoform but it is specific for the p115/p120 phosphorylated forms.<sup>40</sup>

This association, and our observation that in the same tumour the frequency of labelled Ki-67 cells generally exceeded that of pRB expressing cells, supports the view that the G3-245 monoclonal antibody does not recognise the entire pRB pool but reacts mainly with wild-type phosphorylated forms arising during G1/S transition and preserved in the S/G2/M phases until dephosphorylation. The G3-245 monoclonal antibody reacts with an epitope that maps to the region of aa 300-380, corresponding approximately to exons 9-12. This region borders the “pocket” domain (aa 378-869) involved in E2F interaction and is characterised by the presence of the four most critical cysteine residues phosphorylated during E2F release. It is therefore possible that phosphorylation, inducing conformational changes of the protein, unmasks sites recognised by the monoclonal antibody used here.

Whatever the unmasking mechanism, pRB expression paralleled proliferative activity in most of the breast carcinomas examined, suggesting that in these cases the protein behaves normally in regulating cell proliferation and may also be considered a potential growth marker. A relation between pRB expression and proliferative activity has already been reported in reactive lymphoid tissue, and stimulated lymphocytes in the maturing compartment of several normal tissues as well as in some neoplasms such as lymphomas and hepatocellular carcinomas.<sup>6-8, 41</sup>

Our results showed a positive trend between pRB values and tumour size, with T3 and T4 cases having the highest content (fig 1A). A similar positive association was also found for histopathological grade (G) (fig 2A) and nuclear grade (NG) (fig 2B). Thus a high pRB content was associated with histologically poorly differentiated tumours, a finding which

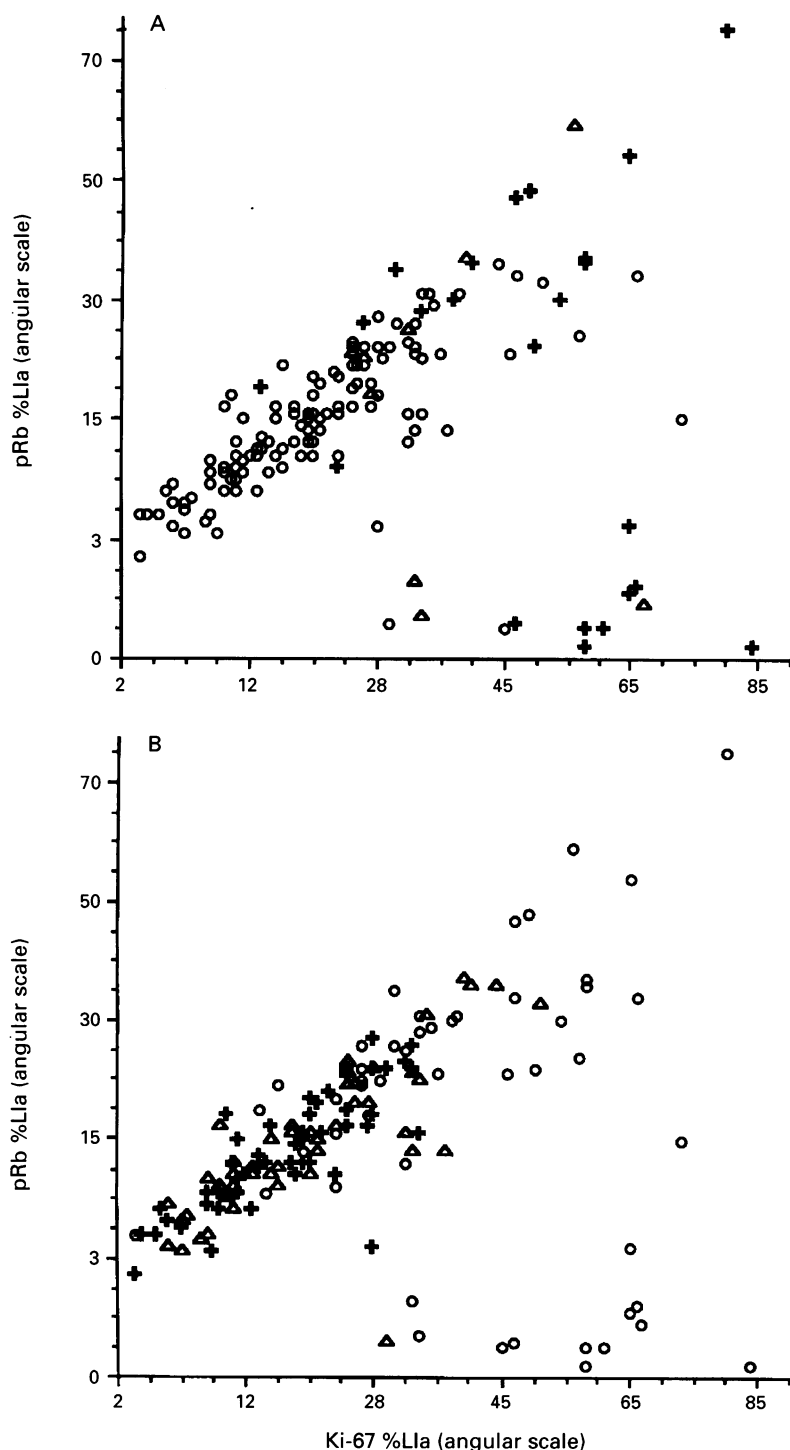


Figure 6 Same graphic scatterplot as in fig 4. In (A) cases were labelled according to EGFR cellular immunostaining:  $\circ$  = absent;  $\Delta$  = less than 25% neoplastic immunopositive cells;  $+$  = more than 25% neoplastic immunopositive cells. In (B), cases were labelled according to categorised oestrogen receptor/progesterone receptor content as follows:  $\circ$  = absent or low;  $\Delta$  = mid;  $+$  = high.

reinforces the observation that pRb expression parallels proliferation and is usually greatly increased in aggressive breast carcinomas. It is therefore probable that pRb expression is normally regulated in these more aggressive breast carcinomas. On the other hand, among these highly proliferative tumours we observed another group of cases without pRb expression in which the correlation between proliferative activity—as determined by Ki67 immunostain-

ing and pRb—was lost. These neoplasms showed loss of oestrogen receptor/progesterone receptor proteins, EGFR overexpression (84.6%), and to a lesser extent altered p53 expression (61%). While loss of Rb function has been shown to be a critical initiating factor in the development of retinoblastoma,<sup>10</sup> in our cases it is equally possible that some tumours possess this pRb phenotype from the start, and that in others the loss of pRb may have been a later event. The pRb expression was observed in both slow and fast growing tumours and it is plausible that in rapidly growing tumours the rate of pRb mutation increases and consequently tumours with pRb loss may emerge. In our study no apparent relation between N status and pRb expression was observed, suggesting that cell cycle deregulation (pRb loss) may not be directly related to metastasis, even if the existence of other unknown, indirect, mechanisms cannot be excluded. The EGFR positivity in 84.6% of our pRb negative cases should reinforce this hypothesis, in view of the fact that its expression was not related to N status in the majority of studies.<sup>42</sup> Thus the absence of any relation between pRb loss and N status may be explained by assuming that this oncogenic factor enhances cell division rather than cell motility, which in breast cancer seems to be mediated mainly by other factors such as ErbB-2 expression.<sup>43-44</sup>

There is recent evidence that in breast carcinoma an “interconverted phenotype” may be associated with some markers of poor prognosis and adverse outcome, such as tumour size, nodal status, high proliferative index, aneuploidy, vimentin expression, absence of oestrogen receptor/progesterone receptor, and EGFR positivity.<sup>45-46</sup> Thus the particular combination of markers (high nuclear grade, EGFR expression, absence of oestrogen receptor/progesterone receptor, high proliferative status, and p53 positivity) found in our pRb negative cases might identify a subset of neoplasias with an aggressive biological phenotype characterised mainly by growth advantage and cellular “progression”—an observation which appears to be in line with the recently recognised role of pRb in cell cycle withdrawal during differentiation.<sup>47</sup>

In conclusion, pRb immunolabelling strongly paralleled the Ki-67 proliferative fraction, showing that this nuclear protein is a potential growth marker in breast carcinoma. Because hyperphosphorylated pRb forms are closely associated with G1/S progression, this could provide additional in situ information on proliferative growth and cell cycle progression of neoplastic cells.

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