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Relationship between the RB1 mRNA level and the expression of phosphorylated RB protein in human breast cancers: their relevance in cell proliferation activity and patient clinical outcome

M. Derenzini[1], L. Montanaro[1], M. Vici[1], S. Barbieri[1], C. Ceccarelli[2], D. Santini[3], M. Taffurelli[4], G.N. Martinelli[3] and D. Treré[1]

[1]Department of Experimental Pathology, Unit of Clinical Pathology, University of Bologna, Bologna, Italy,

[2]Centre for Applied Biomedical Research, S. Orsola–Malpighi Hospital, Italy and [3]Institute of Surgical Pathology, S. Orsola–Malpighi Hospital, Italy and [4] First Surgical Clinic, Breast Cancer Surgical Unit, S. Orsola–Malpighi Hospital, Italy

Summary. The aim of the present study was to ascertain the relationship between the level of RB1 mRNA and the expression of phosphorylated RB protein and the relevance of these two parameters in cancer cell proliferation and clinical outcome in human breast cancer. Sixty-eight primary human breast cancers were considered. The amount of RB1 mRNA was evaluated by quantitative RT-PCR analysis. The level of RB phosphorylation was immunohistochemical defined by measuring the phosphorylated (pp) RB labelling index (LI). Cell proliferation rate was measured by calculating the Ki67 LI. No relation was found between the RB1 mRNA level and the ppRB LI (p=0.565). Both RB1 mRNA value and ppRB LI were related (in an inverse and direct manner, respectively) to Ki67 LI. RB1 mRNA expression was more strictly associated with KI67 LI (p=0.001) than the ppRB LI (p=0.013). Regarding the patient clinical outcome, the separately considered RB parameters did not reach the prognostic significance. However, patients with low RB1 mRNA quantity and patients with high ppRB LI, taken together, had a significantly shorter disease free and overall survival than the group comprehending patients with high RB1 mRNA value and low ppRB LI, and this despite the low number of patients considered. Our results demonstrated that the ppRB LI was independent of the RB1 mRNA level; that both RB parameters are related to the cell proliferation rate and, if collectively considered, have a high informative value on breast tumour prognosis.

Offprint requests to: Prof. Massimo Derenzini, Department of Experimental Pathology, Unit of Clinical Pathology, University of Bologna Via S. Giacomo 14, Bologna 40126, Italy. e-mail: massimo.derenzini@unibo.it

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Introduction

In mammalian cells the progression through the cell cycle is tightly controlled by a set of regulatory devices operating at the end of the G1 phase, at the so called restriction point which defines the limit beyond which the cell is committed to divide independently of growth factor signalling (Pardee, 1989). The retinoblastoma (RB) tumour suppressor protein, encoded by the RB1 gene, controls the passage throughout the restriction point by interacting with the family of transcription regulators termed E2Fs (Sherr and McCormick, 2002). The E2Fs regulate the expression of those genes whose products are necessary for the S phase progression (Harbour and Dean, 2000). In its active hypo-phosphorylated form, RB is bound to E2Fs and prevents them to activate the E2Fs target genes, whereas, in the hyper-phosphorylated form, RB no longer binds to E2Fs which are let free to activate the target genes. Phosphorylation of RB is triggered in the early G1 phase by the cyclin D-cyclin-dependent protein kinase (CDK)-4 and -6 complexes and is completed, at the end of G1 phase, by cyclin E-CDK-2 complexes. The activities of the CDKs are in turn constrained by the CDK inhibitors (CKIs): CDK-4 and CDK-6 are inhibited mainly by p16INK4a, whereas the CDK-2 is negatively regulated by p21Cip1 and p27 (Sherr and Roberts, 1999). The components of the regulatory machinery that controls G1/S phase transition behave as tumour suppressors or proto-oncogenes and are frequently altered in cancer

cells. RB1 mutation or deletion, INK4a mutation, deletion or gene silencing and cyclin D1 or CDK4 over-expression characterise many human cancers (Sherr and McCormick, 2002). These changes, causing either RB1 loss or RB hyper-phosphorylation, render out of order the major control mechanism of the G1/S phase check point. The RB status has been deeply investigated in human tumour pathology in order to clarify its role in tumour biology and its relevance in both tumour progression and patient clinical outcome. In breast cancer, the expression of RB has been evaluated either by reverse transcriptase-polymerase chain reaction analysis of RB1 mRNA (Bieche and Lidereau, 2000) or, much more frequently, by immunocytochemical analysis of RB protein on histological sections (Anderson et al., 1996; Jares et al., 1997; Wakasugi et al., 1997; Ceccarelli et al., 1998; Gillett et al., 1999; Nielsen et al., 1999). In both these studies breast tumours were only defined as RB positive or negative on the basis of either normal or under-expressed RB1 or the presence or absence of RB immuno-stained cells. Moreover, in immunohistochemical investigations to detect RB different anti-RB antibodies were used, which, in some cases, have been later shown to reveal the phosphorylated form of RB (Jares et al., 1997; Ceccarelli et al., 1998; Gillet et al., 1998). Regarding the relation with the cell proliferation rate, these studies indicated that either tumours with under-expressed RB1 or RB-negative tumours were associated with higher proliferative activity than RB1-normally-expressing or RB-positive tumours. As far as the relationship between RB phosphorylation and cell proliferation is concerned, contradictory results have been produced (Ceccarelli et al., 1999; Loden et al., 1999). Furthermore, no clear evidence has been produced on the relevance of RB1 mRNA value and phosphorylated RB protein expression and the clinical behaviour of breast cancer (Berns et al., 1995; Anderson et al., 1996; Wakasugi et al., 1997; Bieche and Lidereau, 2000).

The aim of the present paper was first to ascertain whether a relationship exists between the level of RB1 mRNA and the expression of phosphorylated RB protein and second to define the importance of these two parameters, taken together, in cell proliferation and prognosis in human primary breast cancers. For this purpose, we measured the level of RB1 mRNA by quantitative RT-PCR analysis and the expression of phosphorylated RB protein by immunohistochemistry. The levels of RB1-mRNA and the expression of RB phosphorylated protein were related to cell proliferation rate, evaluated by measuring the Ki67 labelling index, and to the pathological and clinical characteristics of the breast cancers considered.

Materials and methods

Patients

A total of 68 carcinomas of the breast were studied. Cases were selected from a series of consecutive patients who underwent surgical resection for primary infiltrating carcinomas of the breast at the Surgical Department of the University of Bologna between 1994 and 1995 on the only basis of frozen tissue availability. Patients' age ranged from 28 to 87 years with an average (± SD) of 60.9 (± 15.1) years (median value: 62 years). Tumours were histologically classified according to the World Health Organisation (WHO) criteria. Invasive ductal carcinomas were histologically graded (G) following Elston and Ellis's method (1991). The tumours were also typed by nuclear grading (NG) as follows: mild (NG1), moderate (NG2), and severe (NG3) nuclear atypia. Tumour size was evaluated in freshly obtained tissue, before formalin fixation, and coded according to the UICC pT recommendations. Axillary node status was assessed by pathological staging after axillary node dissection. Due to patient age, axillary dissection was not performed in 3 cases. Axillary lymph node metastases were reported as absent (N0) or present (N+). Table I reports the histological diagnosis and pT, G, NG and N distribution of all cases.

Quantitative analysis of Rb1 mRNA expression

Total RNA was extracted from frozen samples using Trizol reagent (Invitrogen, Darmstadt, Germany). For each sample, 10 mg of total RNA was reverse transcribed using High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA), following manufacturer's instructions. The obtained cDNAs were diluted 1:10 and 5 ml of the diluted cDNA was used for each single quantitative determination. The cDNA was subjected to real-time PCR analysis using the Gene Amp 7000 Sequence Detection Systems (Applied Biosystems). The analysis was performed using the TaqMan assay: for each single reaction, in a total volume of 25 µl, we used the TaqMan PCR Master Mix (Applied Biosystems) containing Ampli Taq Gold DNA polymerase with the following cycling conditions: 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 sec, and 60°C for 1 min. For each sample three replicates were analysed. The relative amounts of RB1 mRNA expression was calculated using the expression of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ß-glucuronidase (GUS) as endogenous controls (TaqMan gene expression assays, Applied Biosystems). Specific sets of primers and fluorogenic probes for target mRNAs were purchased from Applied Biosystems. Final results, expressed as N-fold differences in target gene expression relative to both the endogenous control gene expression and the calibrator, were determined as follows: N target = $2^{-(\Delta Ct\ sample - \Delta Ct\ calibrator)}$ where ΔCt values of the sample and calibrator were determined by subtracting the Ct value of the endogenous control gene from the Ct value of the target gene. In each single determination the value obtained using a cDNA preparation from the RB proficient U2OS cell line was used as a calibrator.

Immunohistochemical assessment

From each case, one block of formalin-fixed and paraffin-embedded tissue was selected, including a representative tumour area. Four µm – thin serial sections were cut, collected on 3-ethoxy-aminoethyl-silane treated slides, and allowed to dry overnight at 37°C. Sections were then processed for immunohistochemistry according to SABC (Stretavidin-Biotin-Peroxidase Complex) protocol combined with a microwave-based antigen retrieval pre-treatment in citrate buffer solution (pH 6.0), and subsequently highlighted using a peroxidase/DAB enzymatic reaction. The following monoclonal antibodies (MoAbs) were used: anti-RB (clone G3-245, which specifically recognises the phosphorylated form of RB protein), anti-Ki67 (clone MIB-1), anti-oestrogen receptor (anti-ER; clone 1D5), anti-progesterone receptor (anti-PGR; clone 1A6), all from BioGenex Laboratories, San Ramon, CA, USA). The immunostaining reactions were semi-quantitatively assessed using the Cytometrica program (C & V, Bologna, Italy), as previously detailed (Faccioli et al., 1996), and expressed as the percentage of labelled nuclear area over the total neoplastic nuclear area in the section (labelling index: LI). Previous studies have found that the error induced in the final measurement by the segmentation procedure was < 5% (Caulet et al., 1991). For each case, at least 2000 cells were evaluated.

To check the specificity of the MoAb clone G3-245 versus the phosphorylated form of RB protein we used a human osteosarcoma cell line (U2-OS cells), normally expressing RB, characterised by a doubling time of 24 hours. The cells were cultured with Dulbecco's modified Eagle's medium supplemented with 20% foetal bovine serum and synchronised by adding Nocodazole (0.6mg/ml) for 24 hours. Mitotic cells were collected by mechanical shake-off from the culture flask and washed with normal medium. Collected mitotic cells were then seeded on 22x22mm glass coverslips in six well plates. Six and 18 hours after seeding the cells were washed in PBS and fixed and permeabilised for 4 min with 2% paraformaldehyde added with 1% Triton X-100 diluted in PBS. Cells were treated with 1.5% H_2O_2 for 5 min in the dark, in order to suppress endogenous peroxidase activity. The cells were incubated with the anti-Phospho-Rb antibodies over night at 4°C in a humidified chamber. The cells were washed in PBS and incubated at first with a biotinylated secondary antibody for 30 min, and then with the streptavidin-peroxidase conjugate for 25 min. The streptavidin-peroxidase complex was visualised using diaminobenzydine. Cells were finally dehydrated and mounted in a synthetic medium on microscope slides.

Statistical analysis

Correlation between continuous variables has been analysed using the Spearman rank correlation test. Differences between categorical variables were analysed using the Mann-Whitney tests. Disease free survival (DFS) and overall survival (OS) were evaluated by the Kaplan and Meier method, and differences in survival curves were assessed by the Log Rank test (Altman et al., 1995). Univariate and multivariate DFS and OS analyses were performed according to the Cox proportional hazards model (Cox, 1972). Statistical evaluations were performed using the SPSS program package (Statistical Package for Social Science, SPSS Inc., Chicago, IL, USA). Values for p less than 0.05 were regarded as statistically significant.

Results

Relationship among RB1 mRNA level, expression of phosphorylated RB protein and cell proliferation

We have evaluated the level of RB1 by quantitative real-time RT-PCR. The level of RB mRNA was standardised to the value of GAPDH and GUS house keeping genes. The use of these two house keeping

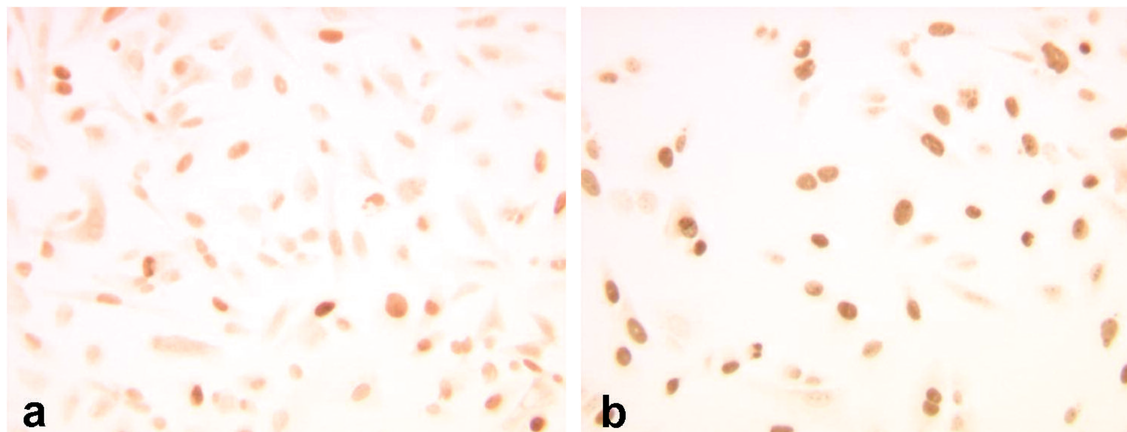

Fig. 1. Synchronised U2-OS cells, 6 **(a)** and 18 **(b)** hours after the end of the synchronisation procedure, immunostained for the phosphorylated form of RB protein. Note the higher percentage of intensely stained nuclei in **(b)** than in **(a)**. x 400

genes gave rise to very similar results; the data reported were obtained using the GAPDH. RB1 level, measured in the U2-OS cell line, was used as calibrator. In our series of breast cancers the RB1 mRNA values, expressed in arbitrary units (a.u.), ranged from 0.62 to 17.92 with a mean (± SD) value of 5.61 (± 3.67). The phosphorylated (pp) status of RB has been evaluated by measuring the percentage of cell nuclei stained by anti-RB MoAbs which specifically recognise the phosphorylated form of RB (ppRB labelling index). To control the specificity of the immuno-staining reaction we evaluated the stainability of phosphorylated protein in synchronised U2-OS cells during cell cycle phases. There is evidence that phosphorylation of RB occurs during G1 phase, is greatly accelerated at the end of G1, and the highly phosphorylated state of RB persists until the exit from mitosis (Adams, 2001). Accordingly, we observed that a progressive increase of the percentage of intensely stained nuclei occurred in synchronised cells from the beginning of the G1 phase, 6 after the end of the synchronisation procedure, to the S phase, 18 after the end of the synchronisation procedure (compare Fig. 1a with Fig. 1b). Since the level of RB protein expression is constant during the cell cycle phases (Classon and Harlow, 2002), variations in the intensity of the staining reaction using antibodies versus the phosphorylated form of RB indicate quantitative changes in RB protein phosphorylation. Therefore, in this study we considered the intensely stained nuclei as those containing RB protein in a highly phosphorylated state. The ppRB LI ranged from 0 to 53.4, with a mean value of 18.15 (± 12.31). For the evaluation of the cell proliferation rate we measured the percentage of cancer cells expressing the Ki67 antigen, which was immunohistochemically detected using the MIB-1monoclonal antibody. The Ki67 antigen, being expressed in cycling cells from G1 to M phase, is in fact the most reliable marker of cell proliferation (Scholzen and Gerdes, 2000). In our series the Ki67 LI ranged from 9.5 % to 79.7 % with a mean value of 33.75 % (± 19.04).

No correlation was found between the quantitative level of RB1 mRNA and ppRB LI (r=0.077; p=0.565). On the other hand, both RB parameters were linearly related to cell proliferation. In fact, a strong, inverse

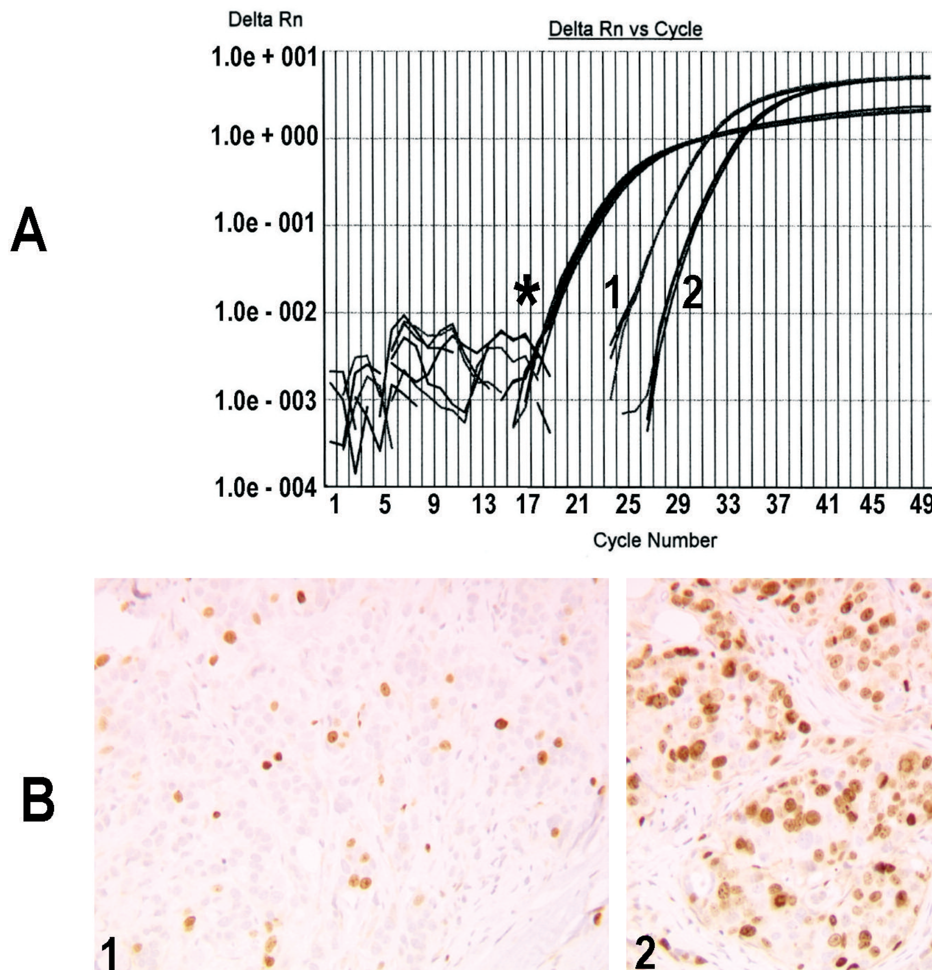

Fig. 2.A. Representative real-time RT-PCR triplicate amplification plots from two infiltrating ductal carcinomas characterised by high (1) and low (2) RB1 mRNA expression (9.57 and 2.20 a.u, respectively). The two samples displayed similar amplification plots for the endogenous control mRNA (GAPDH; *). **B:** Ki67/MIB1 staining of the same samples showing the labelling index of 13.6% (1) and 66.6% (2), respectively. x 250

relationship was observed between the amount of RB1 mRNA and Ki67 LI (r=-0.424; p= 0.001), while a direct relationship existed between ppRB LI and Ki67 LI (r=0.324; p= 0.013). These relationships are illustrated in Figs. 2 and 3. In Fig. 2, two infiltrating ductal carcinomas are shown: they are characterised by low and high value of RB1 mRNA, whereas the values of ppRB LI are quite similar. In the carcinoma with low RB1 level, the Ki67 LI is very high. Conversely, the carcinoma characterised by high RB1 mRNA value exhibits very low Ki67 LI. The infiltrating ductal carcinomas shown in Figure 3 are characterised by quite similar values of RB1 mRNA level but different ppRB LI values. In the carcinoma with a high ppRB LI value, the Ki67 LI is also elevated, whereas in the carcinoma with a low ppRB LI value, the Ki67 LI is low.

RB1 mRNA level and phosphorylated RB protein expression in relation to breast cancer anatomo-clinical variables

First we have evaluated the association among the level of RB1 mRNA and the ppRB LI, from one side, with the parameters used for defining tumour differentiation and progression, from the other side. Regarding RB1 mRNA level, we found that it was significantly higher in cancers with a more differentiated

Table 1. Histopathological characteristics of all cases.

Histological diagnosis:	n (%)
ductal carcinomas	60 (88.2)
lobular carcinomas	8 (11.8)
Tumour size:	n (%)
T1	24 (35.3)
T2	33 (48.5)
T3	6 (8.8)
T4	5 (7.4)
Histological grade:	n (%)
(only for ductal carcinomas: n=60)	
G1	3 (5)
G2	28 (46.7)
G3	29 (48.3)
Nuclear grade:	n (%)
NG1	5 (7.4)
NG2	23 (33.8)
NG3	40 (58.8)
N-status:	n (%)
N (0)	27 (41.5)
N (+)	38 (58.5)
ER-status (LIa):	n (%)
< 10%	26 (38.2)
≥ 10%	42 (61.8)
PGR-status (LIa):	n (%)
< 10%	43 (63.2)
≥ 10%	25 (36.8)

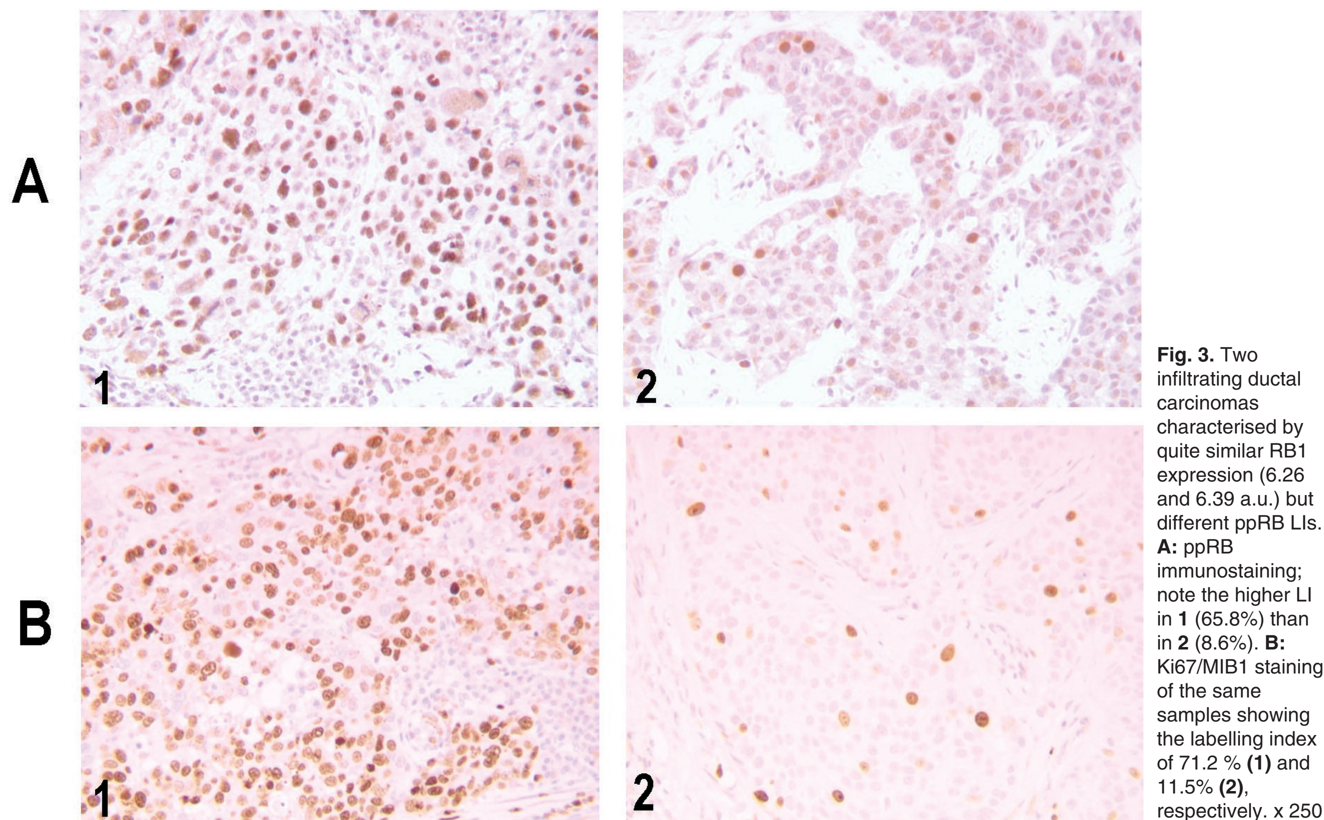

Fig. 3. Two infiltrating ductal carcinomas characterised by quite similar RB1 expression (6.26 and 6.39 a.u.) but different ppRB LIs. **A:** ppRB immunostaining; note the higher LI in **1** (65.8%) than in **2** (8.6%). **B:** Ki67/MIB1 staining of the same samples showing the labelling index of 71.2 % **(1)** and 11.5% **(2)**, respectively. x 250

phenotype, as defined by low histological and nuclear grade and by high ER expression (Table 2). On the other hand, no relation was found between RB1 mRNA level and the tumour progression parameters. In fact, RB1 mRNA levels were not related to either tumour size or lymph node involvement (Table 2). Regarding the phosphorylated status of RB protein, no association was observed with the above considered parameters, with the exception of the nuclear grade of tumours: the higher the nuclear grade, the greater the ppRB LI, with a p value very close to statistical significance (p=0.055) (Table 2).

Considering the fact that both RB1 mRNA level and ppRB LI were found to be associated with the Ki67 LI, which is related to the clinical outcome of patients with breast cancer (Treré et al., 2006), we also evaluated the prognostic relevance of the RB1 mRNA level and the expression of RB protein phosphorylation in the present series of breast cancers. Only patients with infiltrating ductal carcinomas (n=60) were considered. The mean follow-up time of this subgroup of patients was 84 months (range 4-115 months). For survival analysis, each variable was dichotomised. As far as the RB1 mRNA level was concerned, patients were divided into two groups using the cut-off generating the highest predictive value (2.5 a.u.). Regarding the ppRB LI variable, the cut-off of 25% was used according to a previous study carried out on a large cohort of breast cancer patients (Derenzini et al., 2004). Separately evaluated, neither RB1 nor ppRB variables reached the significant value of p <0.05 (Table 3). This was very likely due to the small number of patients considered. However, when cases with RB1 expression values < 2.5 a.u. were considered together with cases showing a ppRB LI > 25% and then compared with the group with RB1 mRNA values > 2.5 a.u. and ppRB LI < 25%, a highly significant difference was observed both in DFS and OS analysis (Figures 4 and 5, respectively), despite the very low number of patients considered. Among the other well established prognostic variables considered in the univariate Cox proportional hazards model, only the Ki67 LI was significantly associated with DFS and OS

Table 2. Comparison of RB1 and ppRB mean values in subgroups of patients identified by histopathological characteristics.

variable:	RB1 *		ppRB LI **	
	mean±SD	p value	mean±SD	p value
lymph-node status:				
negative	5.94±2.89	= 0.382	15.85±14.72	= 0.172
positive	6.20±4.05		19.45±10.25	
tumour size:				
T1	6.66±4.09	= 0.193	15.86±10.60	= 0.558
T2, T3, T4	5.47±3.61		18.79±12.81	
histological grade:				
G1, G2	6.94±3.97	= 0.015	19.03±11.13	= 0.651
G3	4.68±3.24		17.12±13.55	
nuclear grade:				
NG1, NG2	7.73±3.97	= 0.001	14.42±6.71	= 0.055
NG3	4.61±3.02		20.61±14.46	
ER-status (LI)				
< 10%	4.10±2.62	= 0.008	20.09±16.64	= 0.467
≥ 10%	6.86±4.02		17.05±9.09	
PGR-status (LI)				
< 10%	5.62±3.72	= 0.439	19.39±13.98	= 0.437
≥ 10%	6.38±3.99		15.97±8.50	

*: evaluated by quantitative real time RT-PCR; **: evaluated by immunohistochemical analysis

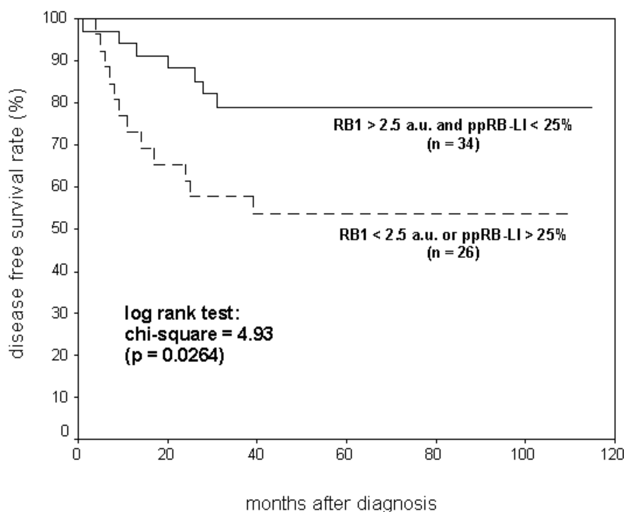

Fig. 4. Disease free survival curves (Kaplan-Meier estimates) with respect to RB status.

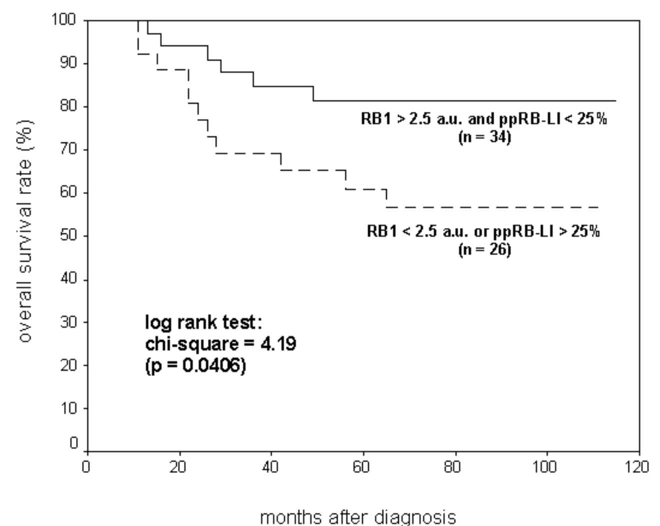

Fig. 5. Overall survival curves (Kaplan-Meier estimates) with respect to RB status.

(Table 3).

When multivariate analysis was carried out including all prognostic parameters considered in the study, no variable resulted prognostically independent (data not shown). This was probably due to the fact that a significant association was present between the RB variables and other parameters including histological/ nuclear grade, ER/PGR-status, and Ki67 LI. Therefore, a multivariate analysis was performed considering only parameters not significantly correlated to each other,

Table 3. Cox proportional hazards analysis of prognostic factors for DFS and OS (univariate analysis).

	DFS		OS	
factor	hazard ratio (95% CI)	p-value	hazard ratio (95% CI)	p-value
N-status				
N0	1.00	= 0.1236	1.00	= 0.2011
N+	2.25 (0.80 – 6.32)		1.97 (0.69 – 5.61)	
tumor size				
pT1	1.00	= 0.3469	1.00	= 0.2605
pT2, pT3, pT4	1.63 (0.58 – 4.53)		1.90 (0.62 – 5.85)	
nuclear grade				
NG1, NG2	1.00	= 0.1858	1.00	= 0.1809
NG3	1.99 (0.71 – 5.53)		2.14 (0.70 – 6.59)	
ER-status (LI)				
≥ 10%	1.00	= 0.2903	1.00	= 0.2027
< 10%	1.63 (0.65 – 4.06)		1.85 (0.71 – 4.81)	
PGR-status (LI)				
≥ 10%	1.00	= 0.2393	1.00	= 0.1915
< 10%	1.84 (0.66 – 5.12)		2.11 (0.68 – 6.48)	
RB1 (a.u.)				
< 2.5	1.00	= 0.2182	1.00	= 0.2064
≥ 2.5	1.83 (0.69 – 4.84)		1.90 (0.70 – 5.14)	
ppRB LI				
< 25%	1.00	= 0.1003	1.00	= 0.2134
≥ 25%	2.27 (0.85 – 6.07)		1.95 (0.67 – 5.63)	
RB1 and ppRB LI				
class 1 (*)	1.00	= 0.0227	1.00	= 0.0457
class 2 (**)	2.88 (1.15 – 7.19)		2.67 (1.01 – 7.04)	
Ki67 LI				
< 20%	1.00	= 0.0326	1.00	= 0.0421
≥ 20%	2.76 (1.08 – 7.04)		2.80 (1.03 – 7.59)	

(*) class 1: patients with RB1 > 2.5 a.u. and patients with ppRB LI < 25%; (**) class 2: patients with RB1 < 2.5 a.u. or patients with ppRB LI > 25%.

Table 4. Cox proportional hazards analysis of N, pT and RB status for DFS and OS: multivariate analysis.

	DFS		OS	
factor	hazard ratio (95% CI)	p-value	hazard ratio (95% CI)	p-value
N-status				
N0	1.00	= 0.1142	1.00	= 0.2563
N+	2.32 (0.81 – 6.64)		1.85 (0.63 – 5.36)	
tumor size				
pT1	1.00	= 0.8504	1.00	= 0.5243
pT2, pT3, pT4	1.11 (0.37– 3.30)		1.46 (0.45 – 4.76)	
RB1 and ppRB LI				
class 1 (*)	1.00	= 0.0316	1.00	= 0.1104
class 2 (**)	2.96 (1.10 – 7.96)		2.25 (0.83 – 6.12)	

(*) class 1: patients with RB1 > 2.5 a.u. and patients with ppRB LI < 25%; (**) class 2: patients with RB1 < 2.5 a.u. or patients with ppRB LI > 25%

including N, pT and RB status. As reported in Table 4, the RB parameter come out as the only independent variable in DFS analysis.

Discussion

Changes of the various components of the cyclin/cyclin-dependent kinase/ p16INK4a/retinoblastoma protein pathway occur in all human cancer. Particular genetic abnormalities of the RB pathway exhibit certain tissue specificity. As far as breast cancer is concerned these are represented by over-expression of cyclin D1, D3 and E1, and reduced expression of p27 Kip1 and p16INK4a (Sutherland and Musgrove, 2004). All these changes, each of which is present in 30 to 45% of primary tumours, lead to an uncontrolled RB protein phosphorylation which may be responsible for tumourigenesis and tumour progression. Loss of RB protein expression is much lesser frequent, occurring in about 6% of breast cancers (Geradts and Wilson, 1996). In the present study we have analysed both the expression of the phosphorylated form of RB protein and the level of RB1 mRNA in a series of human breast cancer in order to ascertain the relevance of these two parameters in cancer cell proliferation and clinical outcome. First, we noticed that the quantitative level of RB1 mRNA was not related to the expression of phosphorylated RB protein. Since it has been demonstrated that the levels of the RB1 mRNA correlate with the expression of total RB protein (Kobayashi et al., 1998; Xing et al., 1999; Bieche and Lidereau, 2000; Roesch et al., 2000; Semczuk et al., 2002), our results reasonably imply that no relationship exists between the quantitative levels of the total and the phosphorylated form of RB protein in breast cancer cells. Regarding the relationship with cell proliferation activity, we found that the quantity of RB1 mRNA was inversely linearly related to Ki67 LI, whereas the ppRB LI was directly linearly related to this parameter. The fact that the level of RB1 mRNA and RB protein phosphorylation are parameters which are related to cell proliferation in an inverse manner can be explained by considering the mechanism by which RB protein controls the cell cycle progression. Indeed, the hypo-phosphorylated form of RB protein hinders the cell to transit from G1 to S phase, whereas the hyper-phosphorylated form allows the cell to pass to S phase. Interestingly, the relevance of the level of RB1 mRNA in cell proliferation appeared to be higher than that of the ppRB LI ($p=0.001$ vs. $p=0.013$).

In the present study we have also investigated the relationship between RB1 level and phosphorylated RB expression and the anatomo-clinical characteristics of the breast cancers examined. In this context, we found that the level of RB1 mRNA was significantly associated with the tumour differentiation degree. In fact, cancers with high RB1 level exhibited both low histological and nuclear grade, whereas those with reduced RB1 level were characterised by high histological and nuclear grade, with significant differences between the values of the two groups ($p=0.015$ and $p<0.001$, respectively). Furthermore, the higher the RB1 mRNA value, the higher the expression of oestrogen receptor ($p=0.008$). On the contrary, the expression of the phosphorylated RB protein appeared to be very weakly linked to tumour differentiation (nuclear grade) ($p=0.055$). Regarding the relationship between the quantitative expression of RB1 mRNA and tumour progression parameters, our results showed that RB1 mRNA values were not associated either with tumour size or lymph node status, thus suggesting that the different levels of RB1 mRNA were a constitutive characteristic of a given tumour and not the consequence of changes occurring during the tumour progression. The same was true of the expression of RB phosphorylated protein.

There is evidence that RB mutations and altered expression of its encoded product appear to be of clinical significance, often correlating with prognosis in many type of cancers (Cordon-Cardo, 1995). Regarding the breast tumours, despite the observation that molecular defects of tumour suppressors controlling the G1-S phase transition predict the clinical outcome in breast cancers (Nielsen et al., 1999), both the immunohistochemical assessment of the expression of RB protein and the quantity of RB1 mRNA evaluated by reverse transcriptase-polymerase chain reaction (Berns et al., 1995; Anderson et al., 1996; Wakasugi et al., 1997; Bieche and Lidereau, 2000) were shown not to be significantly related to prognosis. Indeed, in the present study we observed that neither RB1 mRNA level nor the expression of phosphorylated RB protein reached a prognostic significance, if separately considered. On the other hand, our data, indicating that RB1 mRNA level and expression of phosphorylated RB protein are independently related to cell proliferation, suggested that these two parameters should be considered together, and not separately, in order to obtain information on the importance of RB alterations as prognostic factor in human cancer. Accordingly, in the present investigation we found that if patients with low levels of RB1 mRNA were considered together with patients with hyper-phosphorylated RB protein, this group was characterised by a significantly lower disease free ($p=0.0264$) and overall survival ($p=0.0406$) rate than that comprehending patients with high RB mRNA level and hypo-phosphorylated RB protein. This, despite the small number ($n=60$) of patients considered.

In conclusion, in the present study we demonstrated that the level of RB1 mRNA was highly variable in human primitive breast cancers and not related to the expression of phosphorylated RB protein. Total RB expression and RB protein phosphorylation should be considered together, and not separately, to obtain valuable information on breast cancer prognosis.

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