Pergamon

European Journal of Cancer Vol. 31A, No. 3, pp. 329–333, 1995

0959-8049/95 $9.50+0.00

0959-8049(94)00463-3

Expression of Retinoblastoma Gene Protein (Rb) in Breast Cancer as Related to Established Prognostic Factors and Survival

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The expression of retinoblastoma gene product (Rb protein) was studied by immunohistochemical analysis of 205 cases of breast cancer. Rb protein was invariably expressed in non-neoblastic breast epithelium, in dysplastic and hyperblastic lesions adjacent to tumours, and none of the breast tumours was totally negative for Rb protein. According to the scoring system used, the expression of Rb protein was abnormal in 36.6% of cases. Abnormal expression of Rb protein was significantly related to grade ($P < 0.00004$), type ($P = 0.0183$), margin formation ($P = 0.0116$), DNA ploidy ($P < 0.0002$) and nuclear pleomorphism ($P < 0.0001$). Abnormal expression of Rb protein was related to high S phase fraction ($P = 0.004$), high mitotic index ($P < 0.001$) and high morphometric nuclear factor values ($P < 0.01$). The expression of Rb protein had no prognostic value in univariate or multivariate analysis. The results show that the tumour suppressor gene Rb participates in the growth regulation of breast cancer cells *in vivo*, but immunohistochemical assessment of the expression of Rb protein has no prognostic significance in clinical breast cancer over already established prognostic factors.

Key words: Rb gene protein, breast cancer cells, prognosis, immunohistochemistry
Eur J Cancer, Vol. 31A, No. 3, pp. 329–333, 1995

INTRODUCTION

BREAST CANCER is the commonest malignancy in females and still fatal in approximately 30% of cases. The development of breast neoplasia is influenced by genetic factors, hormonal factors and other growth regulating stimuli including growth factor and sex steroid receptor expression [1–6]. A small proportion of breast cancers are related to hereditary alterations in tumour suppressor gene *p53* [6], which normally regulates cell proliferation [7, 8] and programmed cell death [9, 10]. In breast cancer, the importance of the expression of p53 oncoprotein has been analysed in several reports [2, 3], while the role of the retinoblastoma gene protein expression has been more rarely assessed [3, 11]. The retinoblastoma gene is a prototype of tumour suppressor genes which controls the cell cycle at the G1 phase [12, 13]. The Rb gene product, pp110Rb, is a nuclear phosphoprotein [13] with DNA binding properties [13], and it is cyclically phosphorylated and dephosphorylated during the cell cycle, playing a significant role in regulation [12–14]. The present analysis was designed to assess the expression of retinoblastoma gene protein (Rb) in breast cancer and to relate the results of immunohistochemical analysis to standard clinical prognostic factors, histological differentiation, proliferation rate of cancer cells and sex steroid receptor expression. A survival analysis was also performed in which the prognostic potential of Rb expression was compared with established prognostic factors in a multivariate analysis of a cohort of 205 cases of breast cancer patients followed up for more than 8 years in one university clinic.

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Revised 6 Sep. 1994; accepted 28 Sep. 1994.

MATERIALS AND METHODS

The present series consists of 205 women selected from the original cohort of 688 patients, recently examined for various prognostic factors at the University of Kuopio [15]. The original cohort was treated and followed up between 1968 and 1990, while the patients in this study were treated and followed up between 1978 and 1990. The patients in this study were almost consecutive, since only cases with insufficient biopsy specimens for immunohistochemistry were excluded. Axillary lymph node status was determined by histological examination in 196 of 205 cases (96%), and by clinical judgement in 9 cases. Tumour size was recorded as the maximum tumour diameter in a fresh mastectomy specimen. The follow-up was conducted at 3-month intervals during the first year, at 6-month intervals during the next 2 years and annually thereafter. Metastases were detected by routine chest radiographs, bone radiographs, ultrasonography and laboratory tests reflecting bone and liver metabolism. All other pertinent clinical data are summarised in Table 1.

Histological methods

The operative specimens from the primary tumours were fixed in buffered formalin (pH 7.0), embedded in paraffin,

Table 1. *Patients characteristics* (n = 205)

Age (years), mean (S.E.)	60.9 (1.0)
Range	27–92
Follow-up (years), mean (S.E.)	8.4 (0.1)
Range	5–12
Tumour diameter (cm), mean (S.E.)	3.4 (0.1)
Range	1.0–12.0
Axillary lymph node negative positive*	97/99
Metastasis at diagnosis	9
Recurrence	
Yes	78
No	127
Died of breast cancer/other	79/18
Primary treatment	
Modified mastectomy alone	92
Lumpectomy	2
Mastectomy and radiation therapy	52
Mastectomy and adjuvant chemotherapy	8
Mastectomy and radiation and systemic adjuvant therapy	51

* Histological examination.

sectioned at 5 μm and stained with haematoxylin and eosin. Histological grading of tumours was completed using a grading system already described [16], and the typing of all tumours was completed according to WHO standards [17]. Mitotic figures were counted using a dual-headed microscope by two observers with an objective magnification of ×40 (field diameter 490 μm) [15]. The volume-corrected index (M/V) method was used which expresses the number of mitotic figures/mm^2 of neoplastic epithelium in the section [15]. The density of tumour infiltrating lymphocytes (TIL) was scored into three categories as previously described [18]. The categories of TIL were (a) absent or weak (TIL 1); (b) moderate (TIL 2); and (c) dense (TIL 3). All the tumours were invasive and the special histological features were assessed as detailed in our previous report [15] and in this analysis: the proportion of intraductal growth (none, some, principal), and the degree of tubule formation (slight/none, moderate, extensive), margin formation (none, questionable, definite) and the degree of nuclear pleomorphism (some, moderate, severe) were also used.

Flow cytometry, nuclear morphometry and steroid receptor assay

Flow cytometry and nuclear morphometry were completed as described previously [19]. DNA index was available in 175 of 205 cases (85%) and the S-phase fraction could be analysed in 120 or 205 of cases (58%). Tumours with a DNA index value ≤1.05 were considered diploid, and tumours with a DNA index >1.05 were aneuploid. Morphometric nuclear factors were determined by interactive measurement of cancer cell nuclei by using IBAS 1&2 image analyser system. In this analysis, mean nuclear area (NA), SD of nuclear area (SDNA), mean nuclear perimetry (PE), mean longest nuclear axis (Dmax) and mean shortest nuclear axis (Dmin) were used. The sex steroid receptors (ER, PR) were assayed biochemically using a charcoal-dextran assay as described earlier [20], and the cut-off level of receptor positivity was 10 fmol/g cytosol protein. The sex steroid receptor content was available in 172 of 205 cases (84%).

Immunohistochemistry

For immunohistochemical demonstration of Rb protein, 5-μm sections from the primary BCs were deparaffinised, rehydrated, washed for 5 min with PBS and treated with 0.5% pepsin in 0.1% HCl for 60 min at 37°C. Endogenous peroxide was blocked by 3% hydrogen peroxide for 5 min followed by washing for 5 min with PBS. The tissue sections were incubated overnight (24 h) with the monoclonal anti-Rb-protein antibody (NCL-RB1); Novocastra Laboratories Ltd, Newcastle upon Tyne, U.K., diluted 1:40 in PBS. Several dilutions of the antibody and several enzymatic pretreatment conditions were tested to avoid background staining, and to find optimal nuclear staining before the entire series was processed. Sections were washed twice for 5 min with PBS, incubated for 20 min with biotinylated secondary antibody (Vector, California, U.S.A.) diluted 1:200 in PBS. Slides were washed twice in PBS for 10 min and incubated for 20 min in preformed avidin-biotinylated peroxidase complex (ABC, Vectastain Elite kit, Vector, California, U.S.A.). Sections were washed twice for 5 min with PBS, developed with diaminobenzidine tetrahydrochloride substrate (Sigma, U.K.), lightly counterstained with Mayer's haematoxylin, dehydrated, cleared and mounted.

Scoring of Rb protein expression

The fraction of nuclei staining positive for Rb protein was scored for the entire section by using objective magnification 40× (field diameter 490 μm). The fraction of nuclei (%) positive for Rb protein was obtained by averaging the fraction of positive cancer cell nuclei in ten random fields (corresponding to approximately 1 mm^2 in the section). As a second parameter, the intensity and heterogeneity of nuclear staining were defined by three categories: tumours with a uniform, homogeneous nuclear staining similar to that of normal breast epithelium were scored as normal Rb expression, "2" (Figures 1a, b); completely Rb negative tumours were scored as "0"; and tumours with heterogeneous abnormal nuclear expression of Rb protein were scored as "1" (Figure 1c). In these tumours, Rb protein was expressed as granular positivity not covering the entire nuclear surface and the staining intensity was clearly weaker than in tumours that expressed Rb protein normally. A breast cancer biopsy specimen (consecutive sections) showing intense uniform positivity for Rb protein was used as a positive control, and it was positive for Rb protein in all of the experiments. A negative control was always negative for Rb protein (the same biopsy specimen as positive control processed without primary antibody). For statistical analysis of Rb expression, the tumours were further categorised into two groups: tumours which expressed Rb protein in over 90% of cells with strong staining (i.e. normal) were considered normal expressors of Rb protein; and tumours which expressed Rb protein in less than 90% of cells or were weakly stained were considered abnormal expressors of Rb protein. This classification is based on the observations of previous studies [21] which suggest that point mutations in the Rb gene may result in low staining intensity in immunohistochemistry. Deletions and corresponding genomic changes usually result in low fraction and/or low staining intensity of cancer cells [21].

Statistical analysis

In basic statistical calculations, the SPSS/PC+ program was used in an IBM computer and the statistical tests used are indicated in the results when appropriate. Univariate survival analysis (logrank analysis, SPSS-X) was based on the life-table method with the statistics of Lee and Desu [22]. Several group limits were tested for the expression of Rb protein, and the limit showing the highest discriminatory power between the groups

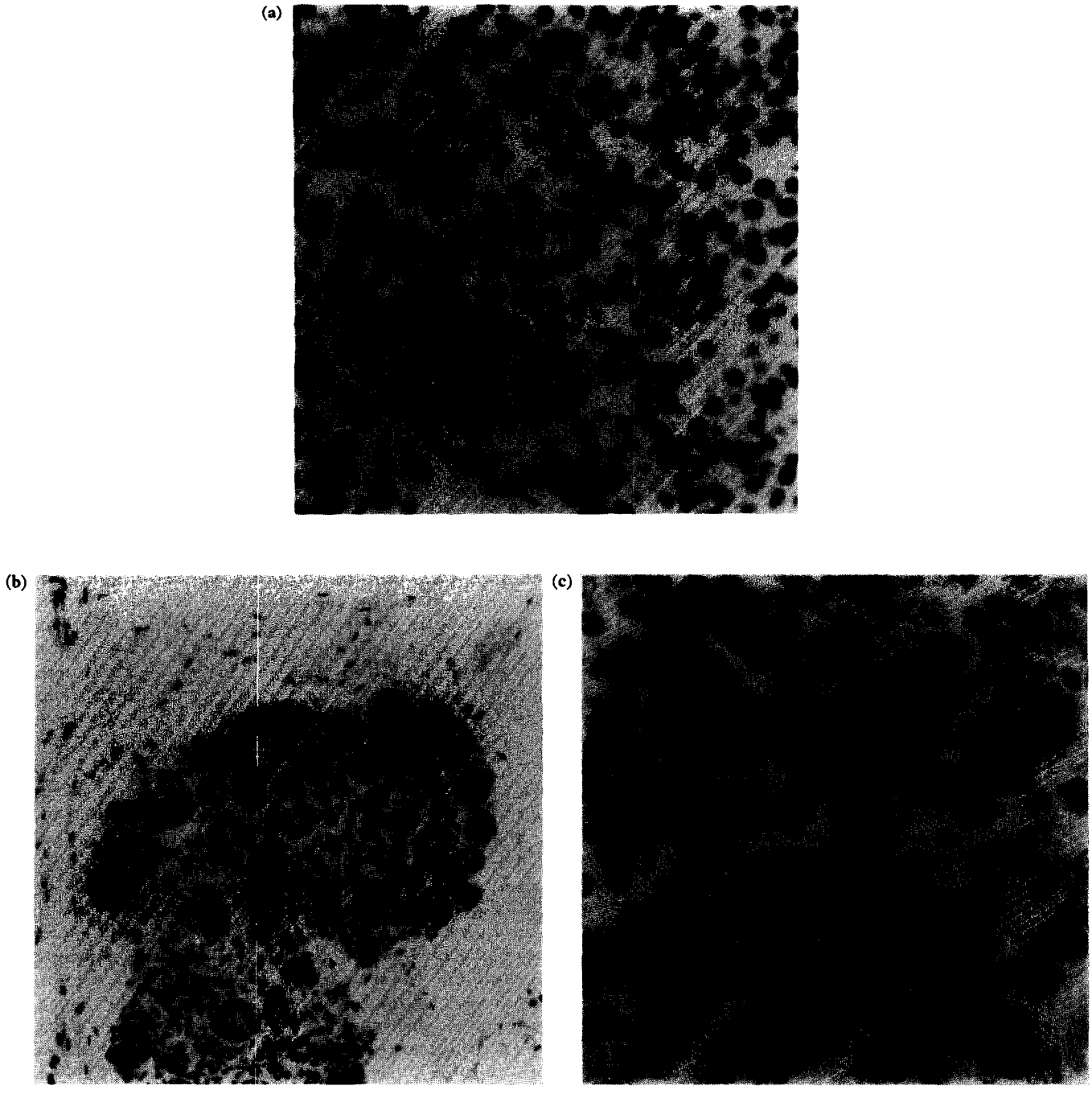

Figure 1. (a) **Expression of Rb protein in cancer cell nuclei in an invasive ductal breast carcinoma. The expression was scored as normal "2" in this section. Magnification × 200.** (b) **Normal breast epithelium adjacent to tumours exhibited strong uniform (normal) staining for Rb protein. Magnification × 50.** (c) **Altered expression "1" of Rb protein in an invasive breast carcinoma. Note weak staining of the nuclei, granular positivity, and there are also completely Rb protein negative nuclei. Magnification × 200.**

was selected. Multivariate survival analysis [23] was performed with the BMDP (2L) programme package in a stepwise manner, and continuous variables were used as absolute numbers in this analysis. The enter limit was $P < 0.1$ and the remove limit was $P > 0.15$. Multivariate analysis included patient age, menopausal status and the year of treatment to control their confounding effect. Multivariate analyses included only cases with histologically confirmed axillary lymph node status. When the recurrence-free survival was analysed, the cases ($n = 9$) with distant metastasis at diagnosis were not included in the analyses.

RESULTS

Rb protein was invariably expressed in normal breast epithelium adjacent to tumours (Figure 1b) ($n = 20$), and also in dysplastic and hyperplastic lesions ($n = 10$). The mean S.E. fraction of positive cancer cell nuclei was 96.2 ± 0.7% and the fraction of positive nuclei ranged between 30 and 100%. None of the tumours was totally Rb protein negative, while marked intratumour variation was present in the expression of Rb protein. Low staining intensity of cancer cell nuclei was detected in 76 of 205 cases (37%). Low staining intensity and low fraction

Table 2. The relationship between abnormal expression of Rb protein, grade, type, degree of nuclear pleomorphism, margin formation and DNA ploidy

	Expression of Rb protein		
	Abnormal	Normal	χ^2, P*
Grade 1	12	52	20.3, 0.00004
Grade 2	23	44	
Grade 3	41	33	
Ductal†	71	108	7.9, 0.0183
Other	2	20	
Nuclear pleomorphism†			
Some	1	8	32.0, <0.0001
Moderate	22	84	
Severe	49	34	
Margin formation			
No	19	13	8.9, 0.0116
Questionable	51	107	
Definite	2	6	
Diploid†	17	64	13.7, 0.0002
Aneuploid	45	49	

* Chi-square test; † not available in all cases.

of positive cells were highly significantly interrelated ($\chi^2 = 157$, $P < 0.00001$).

According to the classification system (see scoring of immunoreactivity), 75 of 205 tumours (36.6%) were abnormal expressors of Rb protein. Abnormal expression of Rb protein was not related to axillary lymph node status ($P = 0.4$), metastasis at diagnosis ($P = 0.2$), ER content ($P = 0.2$), PR content ($P = 0.07$), diameter ($P = 0.7$), tubule formation ($P = 0.7$), proportion of intraductal growth ($P = 0.3$) or density of tumour infiltrating lymphocytes ($P = 0.16$). There was a significant relationship between the abnormal expression of Rb protein, histological type, grade nuclear pleomorphism, margin formation and DNA ploidy (Table 2). The significant relationship between quantitative variables and abnormal expression of Rb protein is shown in Table 3.

In univariate survival analysis, abnormal expression of Rb protein had no prognostic value in the entire cohort ($P = 0.2$), in axillary node negative ($P = 0.13$) or in axillary node positive tumours ($P = 0.7$). Recurrence-free survival in these subcategories was independent of expression of Rb protein (for all $P > 0.1$). In multivariate survival analysis, independent prognostic factors were tumour diameter (RR = 1.02, $P < 0.001$), histological type (RR = 2.10, $P = 0.02$), ER content (RR = 0.48, $P = 0.025$) and axillary lymph node status (RR = 1.68, $P = 0.052$). Recurrence-free survival was predicted by mitotic index (RR = 1.02, $P = 0.006$) and histological type (RR = 2.08, $P = 0.031$).

Table 3. The mean (S.E.) of S-phase fraction, mitotic index and morphometric nuclear factors as related to expression pattern of Rb protein

	Expression of Rb protein		Statistics*
	Abnormal	Normal	
S-phase (%)	9.0 (0.9)	5.6 (0.5)	$t = 3.0, P = 0.004$
M/V index	28.8 (2.7)	15.0 (1.1)	$t = 4.6, P < 0.001$
NA, μm²	115.8 (9.5)	89.9 (2.5)	$t = 2.6, P = 0.010$
SDNA, μm²	32.9 (1.7)	25.1 (1.0)	$t = 3.9, P < 0.001$
PE, μm	40.4 (0.9)	37.2 (0.6)	$t = 2.9, P = 0.004$
Dmin, μm	9.0 (0.2)	8.3 (0.1)	$t = 3.2, P = 0.002$
Dmax, μm	14.4 (0.3)	13.3 (0.2)	$t = 3.2, P = 0.002$

* t-test.

DISCUSSION

Retinoblastoma gene is the prototype of tumour suppressor genes which controls cell cycle at the G1 phase [12, 13]. Deletion or inactivation of both of the alleles of Rb gene results in retinoblastoma and osteosarcomas [24] that arise in families carrying a mutated Rb gene. Rb inactivation has also been found in other tumours such as small cell carcinoma of the lung [25], breast carcinoma [1], prostate carcinoma [26] and renal adenocarcinoma [27].

The relationship between mutations in the Rb gene and altered Rb protein expression is not clear-cut since immunohistochemically detectable Rb protein may be present even in cases with a mutated Rb gene [11, 27]. In breast cancer, loss of protein expression was detected in 29% of cases of known altered gene structure [11]. The protein expression was also variable from cell to cell which may cause difficulties in interpreting the results. However, it has been established that altered Rb protein expression pattern gives a fairly good estimate of the mutation frequency in the Rb gene [21, 28]. Xu and colleagues [28] found altered Rb protein expression in 88% of tumours that displayed loss of heterozygosity (LOH) whereas 86% of tumours without LOH expressed Rb protein normally. These variable results may be related to additional regulation of Rb gene at the transcript level [29], methodological factors related to immunohistochemistry [21] or differences in tumour cell populations.

In this series, altered expression of Rb protein was found in 36.6% of cases, while none of the tumours was totally Rb negative. These results are at variance with the results by Spandidos and associates [30], who reported 42% of Rb negative tumours, and Sawan and associates [3] who reported 28% of Rb negative cases. Varley and colleagues [11] found 1% of negative cases and 15% with altered expression of Rb protein. Moreover, some results suggest that Rb is not always expressed in normal breast epithelium [30], while in this study all sections, including non-neoblastic breast epithelium adjacent to tumours, exhibited nuclear positivity for Rb protein. These differences are most probably due to the different antibodies used, differences in scoring methods and also the variable number of cases included in these studies.

Previous reports suggest a significant relationship between altered Rb expression and axillary lymph node status [3, 11], while in this study only a weak non-significant trend was found between those features. In other neoplasia [31], stage and expression of Rb are independent features which supports the present results. Spandidos and colleagues [30] found a significant relationship between tumour size and expression of Rb, while in this analysis tumour size was independent of the expression of Rb protein.

Experimental studies suggest that tumour cell morphology is independent of Rb gene status [32], while our results showed that in human breast cancer histological differentiation, nuclear morphology and expression of Rb protein are related. Again, the results are variable since some reports have failed to relate the

expression of Rb to histological features in breast cancer [3, 30]. Since DNA aneuploidy, rapid cell proliferation and poor histological differentiation were related to altered expression of Rb, the results suggest that, along with increasing genetic instability, mutations in the Rb gene become more common, which contributes to the relationship between malignant histological features and expression of Rb. Overall these results suggest that the role of the retinoblastoma gene mutations in the early phases of breast cancer development is questionable.

In this analysis, altered expression of Rb protein was clearly related to cell proliferation as measured by S-phase fraction or mitotic index. These results correspond well to the basic regulatory functions of these proteins on cancer cell proliferation [32, 33].

Our survival analysis failed to demonstrate any association between the expression of Rb protein and survival. The present results are supported by the results of Sawan and colleagues [32] who reported non-correlation between prognosis and expression of Rb. The expression of Rb was entered in a multivariate analysis of various prognostic factors, but the role of Rb protein expression on prognosis remained non-significant.

We conclude that abnormalities in the tumour suppressor gene Rb product occur in approximately 35% of breast cancers. There is a weak relationship between cell proliferation, histological differentiation and expression of Rb, while this association is not reflected in survival of patients. Accordingly, Rb protein immunohistochemistry has hardly any use in clinical pathology in defining the correct prognostic category for individual breast cancer patients.

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Acknowledgements—The technical assistance of Mrs Kaarina Hoffren and Aija Korkalainen is gratefully acknowledged.