Controversial relationship between the expression of the RB pathway components and RB protein phosphorylation in human breast cancer

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Summary. Recent data challenge the relevance of the RB pathway to cancer based on RB inactivation, at least in breast tumors. To obtain information on the actual role of the components of the RB pathway in tumor progression we decided to investigate whether their quantitative changes were associated with variations in the level of RB phosphorylation in human breast cancer. A series of 68 human primary breast carcinomas was studied. Five cases were excluded from the study due to their lack of RB expression. In the remaining 63 cases the expression of cyclin D1, cdk4, cyclin E, and INK4a mRNA was assessed by real-time RT-PCR. The level of RB phosphorylated protein (ppRB) and p27 expression was immunohistochemically analyzed by measuring the percentage of stained cells (labeling index, LI). Cell proliferation rate was measured by Ki67 LI evaluation.

The ppRB LI ranged from 5.2 to 73.8 and, as expected, was strongly related to the Ki67 LI (r=0.80; p<0.001). The expression of cyclin D1 mRNA, expressed in arbitrary units (a. u.), ranged from 1.15 to 123.0 and was inversely related to the ppRB LI (p=0.021) and Ki67 LI (p<0.001). Neither the cdk4 (range from 0.07 to 1.13 a. u.) nor the cyclin E (range from 0.13 to 9.27 a. u.) mRNA expression was significantly associated with the ppRB LI (p=0.962 and p=0.103, respectively). Cyclin E was related to Ki67 LI (p=0.022). Both INK4a mRNA (range from 0.01 to 0.60 a. u.) and p27 (LI from 0.0 to 73.1) values were inversely related to the ppRB LI (p=0.022 and p=0.014, respectively). Cyclin D1, cdk4, and cyclin E mRNA expressions were not significantly related to one another.

In human primary breast cancers, the expression levels of the factors known to facilitate the cell cycle progression by RB protein phosphorylation were not positively related to ppRB-LI. Pathological increases of cyclin D, cdk4, and cyclin E are very likely associated with other biological functions other than their well-established action on cell cycle progression.

Key words Cell cycle, RB, Breast cancer, Immunohistochemistry, Real-time RT-PCR

Introduction

A set of regulatory devices tightly controls the progression of proliferating mammalian cells through the cell cycle phases, by operating at the end of the G1 phase, at the so-called restriction point. The restriction point defines the limit beyond which the cell is committed to divide independently of growth factor signaling (Pardee, 1989). There is evidence that the passage through the restriction point is mainly controlled by the retinoblastoma (RB) tumor suppressor protein. In fact, the RB protein interacts with the family of transcription regulators termed E2Fs (Sherr and McCormick, 2002), which regulate the expression of those genes whose products are necessary for the S phase progression (Harbour and Dean, 2000). In quiescent cells RB protein binds to E2Fs and prevents the latter from activating the E2Fs target genes. In proliferating cells, RB protein undergoes progressive phosphorylation and, when hyper-phosphorylated, no longer binds to E2Fs, which are let free to activate the target genes. Therefore, the phosphorylation of RB protein is a crucial event for cell cycle progression. RB protein phosphorylation begins in the early G1 phase being carried out by the cyclin D-cyclin-dependent protein kinase (cdk)-4/6 complexes. At the end of the G1 phase the phosphorylation of the RB protein is completed by cyclin E-cdk-2 complexes. The phosphorylation of the RB protein is negatively controlled by the cdk inhibitors (CKIs): cdk-4/6 is
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inhibited mainly by p16INK4a, whereas cdk-2 is negatively regulated by p21Cip1 and p27 (Sherr and Roberts, 1999). The components of the regulatory machinery that controls phosphorylation of RB protein behave as tumor suppressors or proto-oncogenes and are frequently altered in cancer cells. Over-expression of cyclin D1, cdk4, cyclin E and INK4a mutation, deletion or gene silencing characterize many human cancers (Sherr and McCormick, 2002). These changes, by causing RB hyper-phosphorylation, are thought to prevent the major control mechanism of the G1/S phase checkpoint, thus promoting tumorigenesis and influencing tumor progression. However, as far as cyclin D is concerned, there is increasing evidence that this factor does not support the genesis and progression of breast tumors by acting on cell cycle progression in a cdk-dependent manner (Ewen and Lamb, 2004; Sutherland and Musgrove, 2004; Arnold and Papanikolau, 2005; Caldon et al., 2006). These data somewhat challenge, at least in breast tumors, the relevance of the RB pathway to cancer, based on an uncontrolled RB inactivation.

In order to obtain information on the actual role of the components of the RB pathway in tumor progression, we investigated whether their quantitative changes were associated with variations in the level of RB phosphorylation in human breast cancer. For this purpose, in the present study we compared the expression of cyclin D1, cdk4, cyclin E and p16INK4a, analyzed by real-time RT-PCR, with the level of RB protein phosphorylation analyzed by immunocytochemistry, in a series of 68 human primary breast carcinomas. The analysis of factors influencing RB protein phosphorylation was completed by the immunohistochemical evaluation of the expression of p27.

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 carcinoma of the breast at the Surgical Department of the University of Bologna between 1994 and 1995 on the sole basis of frozen tissue availability. Patients’ ages ranged from 28 to 87 years, with an average (± SD) of 60.9 (± 15.1) years (median value: 62 years). Tumors were histologically classified according to the World Health Organization (WHO) criteria and histologically graded (G) following Elston and Ellis’s method (Elston and Ellis, 1991). The tumors were also typed by nuclear grading (NG) as follows: mild (NG1), moderate (NG2), and severe (NG3) nuclear atypia. Tumor 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 (NO) or present (N+).

Quantitative analysis of CyclinD1, Cyclin E and p16INK4a mRNA expression by real-time RT-PCR

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 a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA), following the manufacturer’s instructions. The cDNA was subjected to real-time PCR analysis in a Gene Amp 7000 Sequence Detection System (Applied Biosystems) using the TaqMan approach. Cycling conditions were the following: 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 analyzed. Sets of primers and fluorogenic probes specific for Cyclin D1, Cyclin E, and p16INK4a were purchased from Applied Biosystems (Assay on Demand). The relative amounts of the studied target genes were calculated using the expression of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-glucuronidase (GUS) as an endogenous control (Applied Biosystems). The final results were determined as follows: N target = 2-(ΔCt sample-ΔCt 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 each target gene. In each single determination, cDNA preparations of the RB proficient U2OS and from SAOS-2 (for INK4a) cell lines were used as calibrators.

Immunohistochemical assessment of phosphorylated RB, p27 expression, cell proliferation rate and estrogen receptor status

From each case, one block of formalin-fixed and paraffin-embedded tissue, which included a representative tumor area, was selected. 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 an SABC (Streptavidin-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. Rb immunostaining was assessed using two different monoclonal antibodies (MoAbs): clone G3-245 (BioGenex Laboratories, San Ramon, CA, USA), which specifically recognizes the phosphorylated pRB form, and clone 1F8/Rb1 (Neomarkers, Lab Vision, Newmarket Suffolk, UK), which reacts to the hyper-phosphorylated as well as the un- or under-phosphorylated forms of the Rb protein. Sections were also immunostained using the following monoclonal antibodies: anti p27 (clone DCS72.F6), from
Neomarkers (Fremont, CA, USA); anti-Ki67 (clone MIB-1) and anti-estrogen receptor (anti-ER; clone 1D5), both from BioGenex Laboratories (San Ramon, CA, USA). The immunostaining reactions were semiquantitatively assessed using the Cytomtrica program (C & V, Bologna, Italy), as previously detailed (Faccioli et al., 1996), and expressed as the percentage of labeled nuclear area over the total neoplastic nuclear area in the section (labeling index: LI). For each case, at least 2000 cells were evaluated.

Statistical analysis

Differences among categorical variables were analyzed using the Mann-Whitney and Kruskal-Wallis tests, as appropriate. Correlation between continuous variables was analyzed using the Spearman rank correlation test. Statistical evaluations were performed by using the SPSS program package (Statistical Package for Social Science, SPSS Inc., Chicago, IL, USA). p values lower than 0.05 were regarded as statistically significant.

Results

In the present study we investigated a series of 68 human primary breast carcinomas which were preliminarily characterized for the expression of RB. RB expression was analyzed by immunohistochemical detection of RB protein by using MoAbs versus total RB. In 5 out of the 68 samples examined, no stained reaction was obtained in the cancer cells. These cases were not included in this investigation, thus obtaining a homogeneous group of patients in whom the RB protein was always expressed. The anatomo-clinical characteristics of the 63 remaining cases are shown in Table 1.

Relationship between pRB phosphorylation and cell proliferation and differentiation

The available evidence indicated that the phosphorylation level of pRB is directly related to the cell proliferation rate. To ascertain whether pRB phosphorylation was related to cell proliferation also in the present series of primary breast cancers, we evaluated the relationship between the percentage of cancer cells stained by anti-RB MoAbs (ppRB Labeling Index) and the percentage of cells stained with anti Ki67 MoAbs (Ki67/LI). The ppRB-LI ranged from 5.2 to 73.8, with a mean value of 19.22 (± 14.2) and the Ki67/LI ranged from 9.5 to 79.7, with a mean value of 31.45 (± 18.71). As shown in Fig. 1, a linear relation was observed between these two parameters in our series of breast cancers. The ppRB LI was also compared with the histological grade and the expression of estrogen receptors. No relationship was found between the histological grade and the ppRB LI (p=0.688). Regarding the expression of estrogen receptors, it is well known that positive receptor status correlates with favorable prognostic features, including a lower rate of

Table 1. Histopathological characteristics of all cases

<table>
<thead>
<tr>
<th>Histological diagnosis:</th>
<th>n (%)</th>
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<tbody>
<tr>
<td>ductal carcinomas</td>
<td>55 (87.3)</td>
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<tr>
<td>lobular carcinomas</td>
<td>8 (12.7)</td>
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<table>
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<tr>
<th>Tumour size:</th>
<th>n (%)</th>
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<tbody>
<tr>
<td>T1</td>
<td>21 (33.4)</td>
</tr>
<tr>
<td>T2</td>
<td>32 (50.8)</td>
</tr>
<tr>
<td>T3</td>
<td>5 (7.9)</td>
</tr>
<tr>
<td>T4</td>
<td>5 (7.9)</td>
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<tr>
<th>Histological grade:</th>
<th>n (%)</th>
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<tbody>
<tr>
<td>G1</td>
<td>4 (6.3)</td>
</tr>
<tr>
<td>G2</td>
<td>30 (47.7)</td>
</tr>
<tr>
<td>G3</td>
<td>29 (46.0)</td>
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</table>

<table>
<thead>
<tr>
<th>Nuclear grade:</th>
<th>n (%)</th>
</tr>
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<tbody>
<tr>
<td>NG1</td>
<td>5 (7.9)</td>
</tr>
<tr>
<td>NG2</td>
<td>22 (34.9)</td>
</tr>
<tr>
<td>NG3</td>
<td>36 (57.2)</td>
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</table>

<table>
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<tr>
<th>N-status:</th>
<th>n (%)</th>
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</thead>
<tbody>
<tr>
<td>N (0)</td>
<td>23 (38.3)</td>
</tr>
<tr>
<td>N (+)</td>
<td>37 (61.7)</td>
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<tr>
<th>ER-status (Lla):</th>
<th>n (%)</th>
</tr>
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<tr>
<td>&lt; 10%</td>
<td>22 (34.9)</td>
</tr>
<tr>
<td>≥ 10%</td>
<td>41 (65.1)</td>
</tr>
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</table>

Fig. 1. Relationship between ppRB LI and Ki67 LI. The two parameters are significantly correlated. The greater the number of proliferating cells, the greater the number of cells with hyper-phosphorylated RB protein.
cell proliferation and histological evidence of tumor differentiation. The percentage of ER-positive cells ranged from 0.0 to 100.0, with a mean value of 51.90 (± 41.80). As expected, we found an inverse relationship between the ER positivity and the ppRB LI (Table 2). Finally, no relationship was found between ppRB LI and lymph node status (p=0.912).

Relationship between the expression of cyclin D, cdk4, and cyclin E mRNA and ppRB-LI

The expression of cyclin D1, cyclin E, and cdk4 mRNA was measured by quantitative real-time RT-PCR. The quantitative analysis of the expression of each gene was carried out separately and the expression level of each type of mRNA was standardized to the expression level of the GAPDH housekeeping gene. In each gene analyzed the expression level of the same gene, measured in the U2-OS human cancer cell line, was used as calibrator. The cyclin D1 mRNA values, expressed in arbitrary units (a.u.), ranged from 1.15 to 123.0, with a mean value of 35.41 (± 32.07); cdk4 values ranged from 0.07 to 1.13, with a mean value of 0.34 (± 0.24), and cyclin E values ranged from 0.13 to 9.27, with a mean

Fig. 2.A. Representative real-time RT-PCR triplicate amplification plots from two infiltrating ductal carcinomas characterized by high (1) and low (2) cyclin D mRNA expression (67.81 and 10.08 a.u., respectively). The two samples displayed similar amplification plots for the endogenous control mRNA (GAPDH; *). B. The same samples specifically immunostained for the phosphorylated form of RB protein, showing the labeling index of 13.6% (1) and 62.6% (2), respectively. x 250. C. The same samples specifically immunostained for the Ki67 antigen, showing the labeling index of 14.3% (1) and 75.1% (2), respectively. x 250.
value of 0.89 (± 1.43).

As shown in Table 2, we found that the expression of cyclin D1 appeared to be inversely related to both ppRB LI and Ki67 LI. This relationship is also seen in Figure 2, which shows two infiltrating ductal carcinomas characterized by a high and low value of cyclin D1 mRNA expression. In the case with high cyclin D1 expression, the ppRB LI and the Ki67 LI were very low. Conversely, the case characterized by low cyclin D1 expression exhibited very high ppRB LI and Ki67 LI. As has been reported in previous investigations (Hui et al., 1996; Jares et al., 1997), in this series also, Cyclin D expression is strongly correlated with the ER level (Table 2).

As for the expression of cdk4 mRNA, no relationship was found between its level and the ppRB LI. The same was true for the expression of cyclin E mRNA. Interestingly, cyclin D1, cyclin E, and cdk4 expressions were not related to one another (Table 2).

**Relationship between the expression of cyclin-dependent kinase inhibitors and ppRB-LI**

As far as the expression of the inhibitors of pRB phosphorylation was concerned, the quantity of INK4a mRNA ranged from 0.01 to 0.60 a. u., with a mean value of 0.17 (± 0.14). As expected, the level of this inhibitor was inversely related to the ppRBL1 (Table 2). For the study of the expression of p27, the other major inhibitor of cell cycle progression, we conducted an immunohistochemical analysis on the quantitative distribution of the protein rather than a RT-PCR analysis of the relative mRNA; this was because post-translational modifications of p27 appear to regulate p27 levels in human tumors through ubiquitin-mediated degradation and proteolytic processing during the G1. Furthermore, in certain breast tumors, changes of the proteasome-mediated degradation pathways have been involved in the loss of p27 protein expression. The percentage of stained cells with anti-p27 MoAbs ranged from 0.0 to 73.1, with a mean value of 16.65 (± 21.09). The p27LI was inversely related to the ppRBL1 (Table 2).

### Discussion

In the present study we investigated the relationship between the profiles of the major constituents involved in the control of cell cycle progression and RB protein phosphorylation in human breast cancers. For this purpose, we compared the expression of cyclin D1, cyclin E, cdk4, p16INK4a, and p27 to the ppRB LI. We found that no expression of a factor that is known to promote pRB phosphorylation (i.e. cyclin D1, cdk4, and cyclin E) was positively associated with the ppRB LI; furthermore, there was no relationship among them.

Cyclin D1 is one of the most commonly over-expressed oncogenes in breast tumors and up to 50% of primary breast cancers are characterized by cyclin D1 over-expression at the mRNA and protein level (Caldon et al., 2006). Formation of cyclin D-cdk4/6 complexes leads to pRB phosphorylation during the G1 phase. In addition, the cyclin D-cdk complexes titrate p27kip1 and p21cip1 cell cycle inhibitors from cyclin E-cdk2 complexes, thus increasing the pRB phosphorylation rate. The CDK4 gene is amplified and the protein is consequently overexpressed in a significant fraction of human breast cancers (Yu et al., 2006), while a continuous presence of CDK4-associated kinase activity is required to maintain breast tumorigenesis in ErbB-2-driven mammary tumors (An et al., 1999). The overexpression of CDK-4 kinase is significantly associated with the cell proliferation rate (Coqueret,
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Therefore, according to the expected theoretical effect of cyclin D-CDK4 complexes on cell cycle progression, the phosphorylation rate of RB protein should be positively related to the expression of cyclin D and/or CDK4 kinase. By showing an inverse relationship between the level of RB protein phosphorylation and the expression of cyclin D1 and no association with CDK4 expression, the present results might appear not to be consistent with the biochemical evidence that indicates that the phosphorylation of RB protein is mainly due to the cyclin D1 and cyclin-dependent kinase availability (Sherr and Roberts, 1999). However, there is evidence that the role of cyclin D is not restricted to the pRB phosphorylation. Data have been reported showing that some cyclin D1 activities are unrelated to its function both as a cdk regulatory subunit and as a regulator of pRB phosphorylation. In fact, cyclin D has been shown to interact with several transcription factors as well as regulating their activity, independently of the kinase function of cdk4 (Moroy and Geisen, 2005). Moreover, cyclin D1 over-expression is associated with a slow-growing, more differentiated phenotype of breast cancer (Sutherland and Musgrove, 2004), and, accordingly, in the present study we found that cyclin D1 expression levels were inversely related to both cell proliferation rate and ER expression level. Therefore, in human breast cancer the level of cyclin D expression may be related to other biological functions independently of its effect on pRB phosphorylation. In any case, in light of the available evidence, we are unable to explain the present finding of an inverse relationship between cyclin D expression and ppRB-LI.

Cyclin E, the regulatory component of the cyclin/cyclin-dependent kinase 2 complex, is overexpressed in different tumor types and is often associated with a poor clinical outcome (Han et al., 2003; Hunt and Keyomarsi, 2005). The importance of cyclin E in cell cycle progression is thought to be due to the fact that the cyclin E/Cdk2 complexes are responsible for the complete phosphorylation of the RB protein. The contribution of cyclin E to tumorigenesis has been studied most in breast cancers where over-expression is: i) apparent in 10% to 25% of tumors; ii) a strong predictor of endocrine therapy failure; iii) associated with high cell proliferation rate; and iv) an accurate predictor of a poor clinical outcome (Jung et al., 1995; Alkarain and Slingerland, 2004). In our series, we found that the expression of cyclin E was positively related to the Ki67 LI. However, a lack of relationship was noticed between cyclin E expression level and ppRB-LI. This discrepancy may be explained considering that in addition to the specific regulatory function of S-phase-entry by RB protein phosphorylation, cyclin E plays a direct role in triggering DNA replication, as well as in the control of both the genomic stability, and the centrosome cycle (Moroy and Geisen, 2004).

On the other hand, as expected from the available evidence regarding the well-established function of p16 INK4a and p27 in the control of cell cycle progression, in the present study we found that the ppRB-LI was inversely related to both p16INK4a and p27 expression. Indeed, there is evidence that p16 INK4a, the product of the INK4a gene, controls RB phosphorylation by inhibiting the binding of CDK-4 and CDK-6 to cyclin D1 (Sherr and Roberts, 1999) and, consequently, the formation of cyclin/kinase active complexes. Mutation, deletion, and epigenetic silencing of INK4a are responsible for the functional inactivation of p16INK4a in about 30% of breast cancers (Sherr and McCormick, 2002). These changes may be responsible for the highly variable p16 INK4a levels observed in the present study. Since we excluded from the present study all the tumors that did not express RB protein, our findings demonstrate that an inverse relationship actually exists in vivo between p16INK4a expression and RB protein phosphorylation level.

RB protein phosphorylation is also negatively controlled by p27Kip1, an inhibitor of cyclin E-dependent CDK2. Accordingly, in our series, the p27-LI appeared to be negatively related to RB protein phosphorylation level. Our results might therefore explain why primary breast cancers with a high level of p27 have shown a better prognostic outcome than those with low p27 expression (Alkarain and Slingerland, 2004). However, it is worth remembering that no agreement has been reached on the usefulness of p27 as a prognostic marker in breast cancer (Alkarain and Slingerland, 2004).

In conclusion, our results show that, in human primary breast cancers, the expression levels of those factors which are known to facilitate the cell cycle progression by RB protein phosphorylation were not positively related to ppRB-LI. This does not mean that cyclin D, cdk4, and cyclin E do not play a key role in the phosphorylation of RB protein in breast cancers. As it has been recently suggested for cyclin D (Ewen and Lamb, 2004), cdk4, and cyclin E also, when over-expressed, may have only a permissive role, indistinguishable from that played by the physiological levels of these factors. Pathological increases of cyclin D, cdk4, and cyclin E are very likely associated with other biological functions other than their well-established action on cell cycle progression.

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References


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