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The Retinoblastoma Gene in Breast Cancer: Allele Loss Is Not Correlated with Loss of Gene Protein Expression

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Abstract

The significance of the retinoblastoma gene (RB) in the development of human breast cancer remains unclear. In the present study, loss of heterozygosity (LOH) in RB was found in 26% of 90 informative primary breast tumors and was correlated to DNA nondiploidy, a high S-phase fraction, and LOH at chromosome 17p13.3. However, allele loss was not associated with loss of RB protein (pRB) expression. Low to absent levels of pRB were found in 15% of 73 immunoblot analyzed tumors, most of which manifested retained heterozygosity in RB. Conversely, tumors exhibiting LOH showed high pRB expression. Our data suggest that RB may be involved in the pathogenesis of some breast tumors, as evidenced by the absence of pRB, but that this alteration is acquired by mechanisms other than the unmasking of a recessive mutation by allele loss. LOH in RB may be merely a stochastic event in the unstable genome of aneuploid, rapidly proliferating cells or, alternatively, reflect the presence of an adjacent tumor suppressor gene.

Introduction

The RB gene has become a paradigm for the class of recessively acting cancer genes termed "tumor suppressor genes" (1). The early hypothesis that both sporadic and familial forms of retinoblastoma arise due to lesions of a common gene, altered by a "two-hit" mechanism (2), was eventually confirmed by cloning of the gene at chromosome 13q14 (3). It was found to encompass more than 200 kilobases of genomic DNA, including 27 exons which are fused in a 4.7-kilobase transcript encoding a M, 105,000 protein (pRB) with nuclear localization (4, 5). It is presumed to be crucially involved in normal cell growth regulation, its phosphorylation status being highly cell cycle phase dependent (6). The unphosphorylated form of pRB is found in quiescent and G1-phase cells, restricting cell cycle progression in G1 by an interaction with the E2F transcription factor (7). It is a target of complex formation with several viral oncoproteins known to have an immortalizing effect on infected cells (8), an inactivation mechanism functionally similar to pRB phosphorylation or to its loss by gene mutation or deletion, resulting in unregulated cell proliferation. Introduction of a RB gene into cancer cells lacking a functional endogenous RB gene was found to revert their transformed phenotype and tumorigenicity, a finding constituting conclusive evidence of its tumor-suppressing capability (9).

Patients with hereditary retinoblastoma are at greater risk of developing second primary tumors later in life, most notably osteosarcoma and soft tissue sarcoma (10), and indeed the presence of RB gene abnormalities has been demonstrated in such tumors (4). RB is also frequently altered in small cell lung carcinoma and bladder carcinoma, although these tumor types have not been associated with a predisposition to retinoblastoma (11). Nor has any relationship been observed between the occurrence of retinoblastoma and breast cancer, although mothers of children with osteosarcoma or soft tissue sarcoma are at high risk of developing breast cancer (12). Several groups have reported the presence of chromosome 13 and RB gene alterations in breast cancer, both in cell lines and primary tumors (13-18), findings confirmed in the present study of primary breast cancer. However, although a reduced pRB expression was observed in several tumors, there was no correlation to LOH, suggesting that RB inactivation in breast cancer is acquired by means other than allele loss.

Materials and Methods

Patients and Sample Handling. This study comprised 150 female and 5 male patients from the Southern Swedish Health Care Region, who were surgically treated for primary breast cancer between 1987 and 1989. Patient age ranged from 26 to 90 years (median, 60 years). Data on tumor size, lymph node status, and histology type were collected from pathology examination records. Sixty-two percent of the patients were lymph node positive; and of a total of 155 tumors, 115 were infiltrating ductal, 4 colloid, 2 medullary, 15 infiltrating lobular, and 19 unclassified invasive carcinoma. Tumor tissue specimens were analyzed for estrogen receptor and progesterone receptor content within 2 weeks of surgery using enzyme immunoassays (Abbott Laboratories) (19). Flow cytometry analysis of DNA ploidy and S-phase fraction was performed (20) on residual tumor tissue that was stored frozen at −70°C and used for DNA or protein extraction and cryostat cutting (21, 22). Peripheral blood was collected within a month after operation, blood cells being separated from plasma and kept frozen until used for DNA extraction (21). Data on patient follow-up were obtained from the Regional Tumor Registry in Lund. Survival was expressed as the number of months from the date of primary surgery to the occurrence of an event (distant, local, or regional recurrence) or to the date of the most recent follow-up and analyzed in terms of disease-free survival (the median duration of follow-up was 28 months).

DNA Probes and Southern Hybridization. The clone p68RS2.0 of genomic DNA, detecting a Rsal polymorphism (variable number of tandem repeats of a 50-53-base pair segment) within the large intron between exons 17 and 18 of the RB gene (5), was used in the pairwise screening of Rsal-digested tumor and leukocyte DNA in Southern hybridization, as described earlier (21). The same panel of samples was also screened for variable number of tandem repeat markers located on other chromosomes [e.g., the pYNS22.1, p144-D6, pMUC10, pYNS2, and p1-79 probes for the chromosomal loci D17S5, D17S34 (chromosome 17p13.3), MUC1 (1q21), D1S57 (1p33-p35), and D1Z2 (1p36), respectively], providing a reference for the equal loading of DNA, for normal cell interference, and for the interpretation of allele imbalance. Tumor DNA was also analyzed with slot blot hybridization and probes for the ERBB2, INTR2, and MYC protooncogenes, as described earlier (22).

Immunoblotting. Tumor protein (200 μg of hot sodium dodecyl sulfate extracts) was electrophoresed on a 7.5% polyacrylamide gel

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To whom requests for reprints should be addressed.

The abbreviations used are: RB, retinoblastoma; pRB, RB protein; LOH, loss of heterozygosity.
under denaturing and reducing conditions, and electroblotted onto nitrocellulose membrane, as described previously (22). Membranes were blocked and incubated overnight at 4°C under constant shaking with primary antibody (5 μg/ml rabbit polyclonal IgG, raised against a COOH-terminal epitope (Ab-2) or residues 248–262 (Ab-3) of the pRB; Oncogene Science) in block buffer. Membranes were washed, incubated with 100,000 cpm/ml 125I-labeled donkey anti-rabbit antibody (Amer-sham, Inc.) for 2 hours at room temperature, washed, and subjected to autoradiography. Band specificity, as recognized by the antibodies, was determined by a peptide neutralization procedure. Primary antibody was incubated for 2 hours at room temperature with a 10-fold (by weight) excess of peptide antigen (Oncogene Science) in block buffer, before being used according to the protocol outlined above.

Immunohistochemistry. Cryostat sections (thickness 7 μm) were air-dried, fixed in Zamboni's mixture for 10 min at room temperature, rinsed in 0.1 M phosphate-buffered saline (pH 7.4), and incubated in the presence of a mouse monoclonal antibody to pRB (NCL-RB; Novocastra, Ltd; diluted 1:80 in phosphate-buffered saline) overnight and at room temperature. The antigen-antibody complex was detected by use of the avidin-biotin technique and diaminobenzidine staining as described previously (23). The specificity of the immunostaining reaction was confirmed by substituting the primary antibody with phosphate-buffered saline.

Results

Allele Imbalance at the RB Gene. Southern hybridization was used to screen 155 pairs of RsaI-digested tumor and leukocyte DNA with the p68RS2.0 probe, 90 (58%) of which were constitutionally heterozygous and thus informative. LOH was found in 23 (26%) of these 90 tumors, as evidenced by the manifestly reduced intensity of one allele (Fig. 1). Four of the 23 tumors with LOH had a duplication of the remaining allele, whereas 14 tumors without LOH manifested allele duplication, resulting in a total of 18 (20%) tumors with allele duplication and 37 (41%) with allele imbalance (loss and/or gain in intensity of alleles) in RB.

LOH in the RB Gene Correlates with DNA Nondiploidy and High S-Phase Fraction. To elucidate the significance of LOH as a step in RB gene inactivation and breast cancer development, comparisons were made with other factors reflecting the process of tumor progression. LOH was found to be unrelated to tumor size, lymph node status, the presence of estrogen receptor or progesterone receptor, or patient age. Although all but two of the tumors with manifest LOH were of the histologically ductal type (the remaining two being lobular carcinomas), this relationship was not statistically significant. LOH was unrelated to amplification of the ERBB2 protooncogene, the 11q13 region (INT2), and the MYC protooncogene, but was significantly more prevalent in tumors with a nondiploid DNA pattern (i.e., more than one stem cell population) than in diploid tumors (P = 0.0099, χ² analysis). LOH was seen in all ploidy subtypes, although most frequently in tumors with a hypertriploid/hypertetraploid or hypotetraploid pattern (the single hypodiploid tumor in the present series also having lost one allele of the RB gene).

A significant relationship was also found between LOH in RB and a high S-phase fraction (≥12%). Nine (53%) of 17 cases with LOH were found among the most rapidly proliferating tumors (S-phase fraction ≥12%), as compared to 9 (18%) of 50 cases without LOH (P = 0.0050). Moreover, LOH was associated with early disease recurrence, although this relationship was not statistically significant (P = 0.17, log-rank test). Finally, one of three informative cases of male breast cancer had lost one allele of the RB gene.

Coexistence of LOH at 13q14 and 17p13.3. Twenty tumors with LOH in RB were informative (constitutionally heterozygous) at either of two loci at 17p13.3 (D17S5 and D17S34; Table 1). In 15 of these 20 cases, LOH was also manifest at either or both loci on chromosome 17. The three remaining tumors with LOH in RB were homozgyous and uninformative at both 17p13.3 loci, although two of these tumors manifested

<table>
<thead>
<tr>
<th>13q14 RB</th>
<th>1q33-p36 (D15S7, D17Z2)</th>
<th>1q21 (MUC1)</th>
<th>17p13.3 (D17S55, D17S34)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No LOH</td>
<td>34</td>
<td>13</td>
<td>30</td>
</tr>
<tr>
<td>LOH</td>
<td>10</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>P</td>
<td>0.20</td>
<td>0.16</td>
<td>0.012</td>
</tr>
</tbody>
</table>

Fig. 1. Southern hybridization of paired RsaI-digested tumor (T) and leukocyte (N) DNA with a probe for the retinoblastoma gene (RB, clone p68RS2.0) detecting a variable number of tandem repeats with bands 1.5–2.0 kilobases in size (top), and rehybridization with a probe for the D17S34 locus (clone p144D6) detecting a variable number of tandem repeats of 2.0–5.0 kilobases in size (bottom). LOH, of approximately equal degree for both loci, is seen in samples 6942, 7035, 6728, 7293, and 6537, whereas sample 6307 manifests LOH in RB but heterozygosity for D17S34. Samples 6366 and 6721 also manifest LOH in RB but are homozgyous for the D17S34 locus; the latter, however, show a clearly reduced signal in tumor DNA.
a clear reduction in intensity of the single band (representing the D17S34 locus) in tumor DNA, as compared to leukocyte DNA, reflecting that allele loss had indeed occurred. Thus, coexisting allele loss at 17p13.3 was seen in 17 (74%) of 23 tumors with LOH in RB. By comparing the degree of reduction in allele intensity, attempts were made to elucidate whether LOH at either of the two chromosomes is acquired in a specific order in the succession of genetic alterations during tumor development. However, as exemplified in Fig. 1, most tumors manifested approximately the same degree of allele loss in RB as at 17p13.3. By contrast, LOH in RB was not statistically related to LOH at the short or long arm of chromosome 1 (Table 1).

Loss of RB Protein Expression Is Not Associated with LOH. A polyclonal antibody raised against a COOH-terminal epitope of pRB (Ab-2) was used in Western blot analysis of 73 tumors and recognized a single M., 105,000–110,000 band (Fig. 2), which was readily outcompeted in peptide neutralization experiments. A polyclonal antibody against an internal epitope of pRB (Ab-3), used in the control analysis of 16 tumors, bound to a protein band of a size and intensity similar to that of the band bound to by Ab-2. Moreover, immunohistochemical analysis of five tumors, of varying pRB status according to immunoblot analysis, verified the nuclear localization and concentration of pRB. One tumor, scored as pRB-negative by immunoblotting, showed no or only a diffuse immunoreactivity in the majority of cancer cells, with the exception of a small cluster of cancer cells being clearly pRB-positive.

The content of pRB, assessed by immunoblot analysis, was semiquantified (see Table 2), 11 (15%) of the 73 tumors being scored as pRB-negative owing to an absence or only a faint band on the blots (Fig. 2). Loss of pRB expression was not correlated to LOH; in fact, only one of nine nonexpressing informative tumors manifested LOH (Table 2). Nor was loss of pRB expression associated with other prognostic factors (with the exception of ERBB2 amplification), with none of the pRB-negative tumors manifesting gene amplification, as compared to 15 (29%) of 52 tumors scored as pRB-positive (P = 0.060). Finally, two of three cases of male breast cancer were scored as pRB-negative, and in one of these one allele of the gene had also been lost.

![Image](6616 6628 6674 6677 6697 6710 6629 6720 6721)

**Fig. 2.** Western blot analysis of pRB, a M., 105,000–110,000 phosphoprotein, using a polyclonal antibody raised against a carboxyterminal epitope (Ab-2). An absence or low level of pRB is seen in samples 6697 and 6629, which were scored as pRB-negative.

### Table 2 Relationship between allele status and protein expression of the RB gene

<table>
<thead>
<tr>
<th>RB allele status</th>
<th>pRB expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absent-low</td>
</tr>
<tr>
<td>Not informative</td>
<td>2</td>
</tr>
<tr>
<td>No LOH</td>
<td>8</td>
</tr>
<tr>
<td>LOH</td>
<td>1</td>
</tr>
<tr>
<td>Total (%)</td>
<td>11 (15)</td>
</tr>
</tbody>
</table>

### Discussion

The two-step mechanism of RB gene inactivation in retinoblastoma, proposed by Knudsen (2), has been accorded almost universal acceptance as the general process by which tumor suppressor genes are affected in tumorigenesis (24). The first event may include rearrangements or translocations, detectable in Southern blot analysis using probes from the cloned gene, but more often takes the form of such subtle alterations as minor deletions or point mutations in the coding or splice junction regions (25). The second event is usually a larger deletion, as a result of mitotic recombination or nondisjunction, leading to LOH in tumors from individuals who are constitutionally heterozygous for the locus (26).

Accordingly, the frequent deletion of a chromosomal locus is generally suggestive of a gene whose loss of function is important in tumor development, a concept also interpreted as suggesting the involvement of the RB gene in breast cancer. In the study by Lundberg et al. (13) LOH was demonstrated at several loci on 13q in 4 of 10 ductal tumors, chosen as being representative of familial forms of the disease, although Friend et al. (4), analyzing the same panel of tumors, were unable to detect any structural changes in the remaining allele. The RB gene was also excluded as a primary lesion in familial breast cancer (27), but findings in several subsequent studies, both on cell lines and primary tumors, support its involvement in a subset of breast cancer (14–16). Rearrangement in RB and loss of pRB expression were reported to have occurred in 2 of 9 breast cancer cell lines (14), whereas in another study 4 of 16 breast cancer cell lines and 3 of 41 primary breast tumors were reported to manifest homozygous structural aberrations, including internal or total deletions and duplication of a portion of the RB gene (15). Moreover, Varley et al. (16) found gene abnormalities or loss in 19% of 77 primary breast cancers, while their immunohistochemical analysis of pRB showed reduced expression in all but one of the tumors with verified DNA alterations and in five additional tumors without detectable DNA alterations; in all, 29% of the tumors analyzed manifested loss of pRB expression. Some of these tumors showed unequivocal structural gene changes, although they contained a mixture of RB-expressing and -nonexpressing cancer cells (16).

This reported lack of complete correlation between RB gene alterations and loss of protein expression derives support from the findings in the present study. We found LOH in 26% of 90 informative cases and reduced or absent pRB expression in 15% of 73 immunoblot-analyzed tumors; nonetheless, most of the nonexpressing informative tumors showed a retained allele heterozygosity. Similar findings were recently reported from a study of primary bladder carcinomas where loss of pRB expression was found irrespective of allele status (28), thus raising some doubt as to the significance of allele loss in such tumors. It might be argued that using a probe for sequences in the huge intron downstream of exon 17 would be inappropriate, since a minor deletion within this intron would result in LOH but leave the coding regions intact. In breast cancer, however, LOH in RB is usually accomplished by a more or less complete deletion.
of the gene (29) or loss of a whole chromosome, support for the latter mechanism deriving from the frequent cytogenetic findings of chromosome 13 monosomy both in near-diploid and tri-tetraploid tumor cells (30) and by findings in molecular studies using probes for several loci along 13q (13, 17). Thus, considering the likelihood of chromosome loss, LOH in the RB gene might be merely incidental and reflect the genomic instability of the aneuploid, rapidly proliferating tumor cells, in which it is most frequently manifest, and raises the question of cause or effect (31). Alternatively, LOH at 13q14 may be suggestive of an adjacent tumor suppressor gene of importance in breast cancer development. We found a significant correlation between LOH in RB (13q14) and LOH at 17p13, a chromosomal region harboring the P53 tumor suppressor gene. Coincident instances of LOH have earlier been described in breast cancer (18), as well as in lung, gastric, adenocortical, and hepatocellular carcinoma; soft tissue sarcoma; and osteosarcoma (32), implying a concerted effect of alterations in the two chromosomal loci during tumorigenesis.

Clearly, alterations other than the unmasking of a recessive mutation by allele loss must be implicated to explain the mechanisms behind RB inactivation in breast cancer. Loss of pRB expression is a universal finding in retinoblastoma cell lines and a frequent finding in small cell lung carcinomas (11), both being of neuroendocrine cell origin. Our data support the possible significance of the RB gene in a subset of breast cancer, as evidenced by its loss of expression in 10–20% of the tumors. We found no correlation between loss of pRB expression and clinical features of aggressive breast cancer, but in agreement with Varley et al. (16) we found the loss of pRB expression to be inversely related to ERBB2 amplification, which is found predominantly in advanced and autonomously growing invasive tumors (22). Thus, RB gene inactivation may be restricted to a certain subtype of breast cancer and may provide a clue in the mechanisms behind RB inactivation in breast cancer.

The RB DNA probe was kindly provided by Thaddeus P. Dryja (Boston, MA). We also thank Torgil Möller, of the Regional Tumor Registry in Lund, for providing the clinical follow-up data, and Bo Baldetorp and Mårten Fernö, of the Department of Oncology, Lund University, for providing the flow cytometry data.

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