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Short Communication

High Frequency of Aberrant p16^{INK4A} Expression in Human Breast Cancer

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The product of the CDKN2/MTS1 gene, p16^{INK4A} (p16), inhibits phosphorylation of the retinoblastoma protein, pRB, and thus acts as a negative cell cycle regulator. It is inactivated in a wide range of human malignancies, including breast cancer. Using an immunohistochemical approach, we studied the expression of both p16 and pRB in 104 archival breast tumors, including 63 ductal, 33 lobular, and 8 mixed carcinomas. All specimens except one were evaluable for pRB expression, but only 87 were interpretable for p16 expression, reflecting the lower abundance and greater lability of this protein. Only six tumors showed abnormal RB expression. However, 43 carcinomas (49%) were completely (35) or focally (8) negative for p16. Abnormal p16 expression did not significantly correlate with several histopathological parameters. These findings provide evidence that aberrant p16^{INK4A} expression is one of the most common abnormalities in human breast cancer. (Am J Pathol 1996, 149:15–20)

There is mounting evidence that disruption of cell cycle regulatory pathways is a common phenomenon in human neoplasia. One such pathway comprising p16^{INK4A} (p16), the product of the CDKN2/MTS1 gene, CDK4, cyclin D1, and pRB, the product of the retinoblastoma (RB) gene, is disrupted in the majority of lung,[1] mesothelial,[2] pancreatic,[3] and certain brain tumors,[4] among others. Similar alterations have been described in human breast neoplasia. The RB gene was found to be nonfunctional in up to 25% of human breast cancer cell lines and primary tumors.[5–8] Abnormalities involving the CDKN2/MTS1 gene have been described in 30–60% of breast cancer cell lines,[9–12] but at significantly lower frequency in primary tumors,[9–11,13,14] and the importance of this gene in human mammary tumorigenesis has been questioned. We have established immunohistochemical assays that allow us to study expression of pRB and p16 specifically in neoplastic cells in archival tissues. We were able to demonstrate that practically all mesotheliomas studied are devoid of p16.[2] Moreover, in a preliminary study showing the applicability of our method to archival tumors in general, we provided evidence that aberrant expression of p16 is frequent in several common human malignancies including breast cancer.[15] We have now extended this series to a larger number of archival breast cancer specimens to arrive at a better estimate of the frequency of p16 and pRB alterations and to examine the correlation with histopathological parameters.

Materials and Methods

Tissues

All specimens were primary breast carcinomas that had been fixed in 10% buffered formalin and embedded in paraffin. Twenty cases were randomly selected from the tissue files of the UNC Carolina Breast Cancer Study. These cases were included in our previous pilot study on common human malignancies.[15] Eighty-four additional samples were re-

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Table 1. *Immunohistochemical Staining Pattern for p16 and pRB in Infiltrating Ductal, Lobular, and Mixed Carcinomas of the Breast*

Type	N	p16			pRB		
		Normal	Abnormal	Inconclusive	Normal	Abnormal	Inconclusive
Ductal	63	28	10	6	57	5*	1
Lobular	33	10	12	11	32	1†	0
Mixed	8	6	2	0	8	0	0
Total	104	44	43	17	97	6	1

*All nuclear grade 3.
†Pleomorphic lobular carcinoma.

trieved from the files of the UNC Division of Surgical Pathology. Both excisional biopsies and mastectomies were included. Selection was based on the original pathology reports and was not random: a disproportionately large number of lobular carcinomas were selected because of our interest in identifying molecular genetic and immunophenotypic differences between ductal and lobular carcinomas. Among the ductal carcinomas, roughly equal numbers were retrieved for the three grades. After one paraffin block for each of the 84 cases was selected, they were independently categorized and graded by the two study pathologists, as were the 20 UNC Carolina Breast Cancer Study cases. In the grading of the ductal carcinomas, the scoring system proposed by Elston and Ellis[16] was strictly followed. Lobular and mixed carcinomas were not subclassified. Differences in classification were resolved in conference.

Materials

Mouse monoclonal anti-RB antibody 3C8 was purchased from QED (San Diego, CA).[17] Mouse monoclonal anti-RB antibody PMG 3–245 and rabbit polyclonal anti-p16 antiserum were obtained from PharMingen (San Diego, CA).[2,18] Nonspecific mouse IgG1 and rabbit serum, respectively, were used as negative control reagents. The detection reaction utilized the Vector (Burlingame, CA) Elite ABC kit.

Immunohistochemistry

The immunohistochemical assays for detecting pRB and p16 in fixed and paraffin embedded tissues have been described in detail elsewhere.[15,18,19] We used five micrometer sections that had been freshly cut or stored at 4°C. Briefly, for detecting pRB, sections from all 104 cases were reacted with antibody 3C8 at 1 μg/ml for 1 hour. A subset of cases, including all RB negative tumors, were also reacted with PMG 3–245 at 2 μg/ml for 2 hours. In both instances, the primary incubation followed a 20 minute antigen retrieval step in hot citrate buffer. Negative control slides were treated with nonspecific mouse IgG1 at equivalent conditions. The staining protocol for p16 did not include an antigen retrieval step. Sections were reacted with p16 antiserum (negative controls: preimmune rabbit serum) at a 1:400 dilution at 4°C overnight. The Vector Elite ABC kit provided the secondary reagents. For color development of both pRB and p16 stains, we used diaminobenzidine with hematoxylin as counterstain.

Evaluation of Immunostains

For scoring the pRB and p16 staining patterns as normal or abnormal, we used previously published criteria.[15,18,19] Cytoplasmic reactivity was disregarded, and only nuclear staining above any cytoplastic background was considered evidence of expression of either tumor suppressor gene. If stained nuclei were present in all areas of the tumor, it was considered normal. If there was absence of nuclear staining in a portion of or in an entire tumor section while admixed non-neoplastic cells did show nuclear reactivity, the lesion was considered abnormal. If there was no discernible nuclear reactivity in either tumor or stroma, the stain was deemed inconclusive.

Results

After histopathological review of the 104 archival breast cancer specimens, 63 carcinomas showed ductal, 33 showed lobular, and 8 showed mixed ductal and lobular differentiation. The former included a number of special subtypes such as mucinous and tubular carcinomas, which can be graded analogously to ordinary ductal carcinomas and usually fall in the low grade category. The three grades of ductal carcinoma were represented in approximately equal numbers (Table 1). The mixed carcinomas were either clearly biphasic with well developed

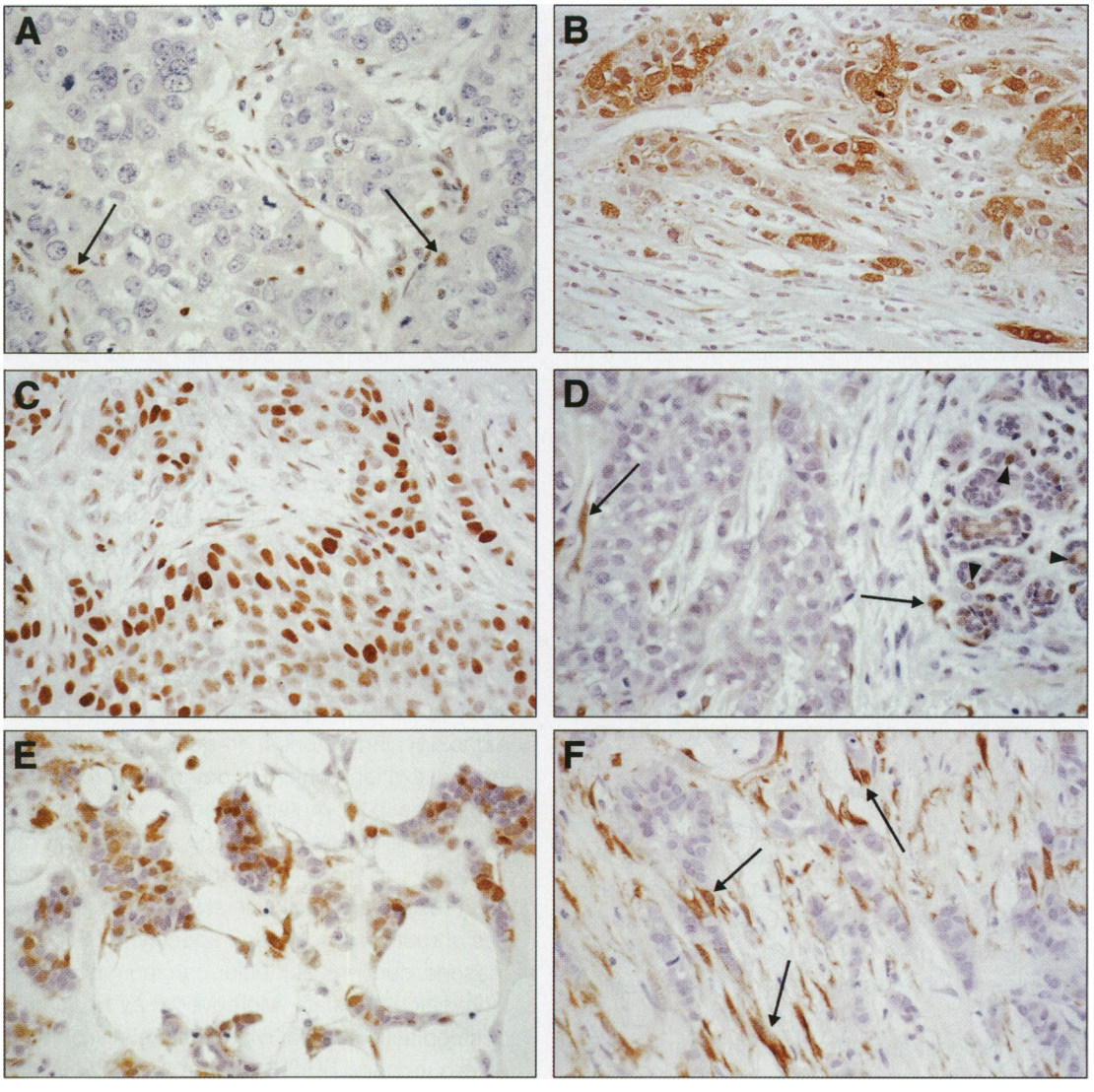

Figure 1. *Immunohistochemical staining patterns for pRB and p16.* A, C: *pRB stains.* B, D–F: *p16 stains.* A, B: *RB-/p16+ tumor. The tumor nests displays strong nuclear and weaker cytoplasmic reactivity for p16; they are completely negative for pRB, whereas admixed stromal cells are clearly reactive* (arrows). C, D: *RB+/p16− tumor. There is moderate to strong nuclear staining for pRB in most of the tumor cells; on p16 stain, the nonreactivity of the tumor is in contrast to distinct staining in stromal cells* (arrows) *and in the normal lobular epithelium* (arrowheads). E, F: *Focally p16 negative tumor. In some areas, the tumor displays distinct nuclear staining for p16* (E)*; in other areas the neoplastic cells are entirely nonreactive, while admixed stromal cells* (arrows) *stain strongly* (F). *Original magnification: 400X* (A–F).

tubule forming and lobular components, or had a phenotype intermediate between typical ductal and lobular carcinomas.

As previously noted,[15,18] the immunohistochemical RB and p16 assays have certain technical limitations, some of which are exacerbated by the fatty nature of many breast tumor specimens. Somewhat surprisingly, we were able to determine the RB status of all but one of the cases. The one failure was due to detachment of very fatty tissue from the slide during the antigen retrieval step on several attempts. p16 stains had variable intensity but generally displayed weak nuclear reactivity in normal cells and in positive tumors. A number of sections showed rather strong staining (Figure 1). In 17 cases, the p16 status could not be determined, although the stains were performed at least three times. In the great majority of these, there was complete absence of nuclear reactivity in both tumor and non-neoplastic tissue. Occasionally, there was strong background staining, which rendered evaluation of the nuclei impossible. Inadequate staining was a particular problem in lobular carcinomas, one-third of which were not evaluable for p16 (Table 1). Sections reacted with nonspecific antibodies were completely unstained.

Only 6 of 103 evaluable cases displayed aberrant RB expression (Figure 1A). Antibodies 3C8 and PMG 3–245 yielded identical staining results. All tumors

Table 2. *p16 Status in Ductal Carcinomas: Correlation with Mitotic, Nuclear, and Overall Grade*

	Mitotic grade			Nuclear grade			Overall grade		
	1	2	3	1	2	3	1	2	3
p16 normal	15	3	10	5	13	10	9	9	10
p16 abnormal	9	5	15	4	16	9	8	11	10
Total	24	8	25	9	29	19	17	20	20

Evaluable cases, n = 57.

with abnormal RB expression had a high nuclear grade: 1 pleomorphic lobular carcinoma and 5 out of 20 ductal carcinomas with a nuclear grade of 3. In contrast, all evaluable ductal carcinomas with nuclear grades of 1 (n = 11) or 2 (n = 31) showed a normal pattern of RB expression.

Forty-three of 87 interpretable cases (49%) abnormally expressed p16 (Figure 1, D and F; Table 1). Ductal and lobular carcinomas showed aberrant p16 expression in equal proportions (Table 1). Two patterns of abnormal gene expression were observed. Four tumors were completely negative for pRB and 35 neoplasms were diffusely negative for p16; admixed normal cells had distinct nuclear staining (Figure 1, A and D). Two carcinomas had both RB positive and RB negative areas and eight tumors showed a similar pattern on p16 stains (Figure 1, E and F). No tumor displayed aberrant staining for both tumor suppressor genes. In the two mixed carcinomas with aberrant p16 expression, the ductal and lobular areas reacted similarly. Several cases with abnormal p16 or pRB status had evaluable *in situ* components, which reacted similarly to the invasive tumor.

Table 2 demonstrates that p16 status in invasive ductal carcinomas was not significantly correlated with mitotic activity or with nuclear or combined grade. Among the different nuclear and overall grades, there were similar numbers of p16 positive and abnormal tumors.

Discussion

Immunohistochemistry is an attractive method to study the expression of oncogenes and tumor suppressor genes in human neoplasia because it allows one to differentially assess the level of the corresponding proteins in the various cell types. Mutations in the RB gene usually cause complete absence of immunoreactive pRB.[18,19] The two most common mechanisms of p16 inactivation are homozygous deletion or hypermethylation of the gene.[12,20,21] Immunohistochemically, these changes are manifest as absence of nuclear staining in the neoplastic cells (Figure 1, A and D). Admixed non-neoplastic cells serve as an internal positive control. If these do not show nuclear reactivity, absence of staining in the tumor cells cannot be taken as evidence of aberrant gene expression. There is marked variation in the level of pRB and p16 in normal tissues,[15,22,23] and generally only a minority of cells will stain. p16 seems to be expressed at markedly lower levels than pRB in all tissues we have examined so far. The human breast belongs to those organs with the lowest levels of both pRB and p16, and thus sensitive methods are required for their detection. Assessment of these two tumor suppressor genes in breast tissue is additionally challenging because the latter is notoriously difficult to cut and process in pathology laboratories, frequently leading to prolonged fixation, which is detrimental to many antigens. The p16 protein appears to be particularly susceptible to fixation damage and other processing artifacts. This may explain the relatively high proportion (16%) of inconclusive staining results in our series of routinely processed specimens. For unknown reasons, sections of lobular carcinomas are particularly difficult to stain. Moreover, they frequently lack desmoplastic stroma, which has relatively the greatest abundance of p16 (Figure 1, B, D and F), and hence a good positive internal control. Because lobular carcinomas were over-represented in our series, the inconclusive rate for random breast cancers may actually be lower.

Immunohistochemical staining protocols, such as the one for pRB, that require an antigen retrieval step in near-boiling citrate buffer are also difficult to perform on breast tissue, because the latter frequently detaches from the slide because of its often fatty nature. Other organs such as lung and bladder are significantly easier to analyze (J. Geradts, unpublished). These problems may partially explain why no other immunohistochemical data on p16 expression in breast neoplasms have been published, and very few such data exist on other types of tumors.[2,15,24] In addition, there are methodological problems associated specifically with the immunohistochemical p16 and RB assays, which are discussed in detail elsewhere.[15,18]

Despite these limitations, our data shed light on the importance of alterations in the RB and CDKN2/MTS1 genes in human breast neoplasia. Only 6% of our tumors were clearly abnormal for pRB. This number is in line with the rate of structural RB alterations found by T'Ang et al,[5] but is lower than that reported in three other studies of primary breast tumors (15 to 24%)[6–8] and that in breast cancer cell lines.[5] It is conceivable that some of our RB positive breast cancers harbor missense mutations, or small insertions or deletions, which may not be distinguishable from normal pRB by immunohistochemistry.[18] Interestingly, all RB negative tumors in our series had grade 3 nuclei (Figure 1A). By extrapolation, it is possible that some 25% of breast cancers with high nuclear grade contain an RB mutation, whereas such an event may be distinctly uncommon in low grade tumors.

The most significant finding in our study is that half of the evaluable breast cancers fail to express p16, either completely (n = 35) or focally (n = 8). This is in stark contrast to conclusions from five previous studies which detected very few deletions or mutations in primary breast cancers.[9–11,13,14] However, these studies employed PCR-SSCP on homogenates of fresh or frozen breast tissue. This technique is unsuitable to reliably detect homozygous deletions in tumors contaminated by non-neoplastic cells and may also fail to identify gene silencing by DNA hypermethylation. Our data indicate that the frequency of p16 deletions in human breast cancer cell lines[9–12] and the incidence of loss of heterozygosity at 9p in breast tumors[20] may in fact mirror the *in vivo* rate of p16 inactivation. Based on a much smaller series of primary breast cancers, Herman et al[21] also recently suggested that the CDKN2/MTS1 gene may be targeted in breast tumors at a higher than presumed rate. In our study, p16 abnormalities were not correlated with important histological parameters such as histological type or, for ductal carcinomas, nuclear or combined grade, or mitotic activity. Whether p16 status predicts for patient survival or response to therapy remains to be determined. Those tumors that fail to express p16 as a consequence of homozygous deletion of the gene may be appropriate targets for gene replacement therapy.

A novel observation is the presence of p16 negative areas within a p16 positive tumor (Figure 1, E and F). The underlying mechanism is unclear, but the findings suggest the emergence of a population of tumor cells that have lost p16 secondary to a genetic event (eg, homozygous deletion) or because of an epigenetic phenomenon (eg, hypermethylation). Similar observations apply to pRB in two out of six aberrant breast cancers, as well as in other types of tumors (J. Geradts, unpublished). This phenomenon may be similar to the presence of estrogen or progesterone receptor negative cell populations in receptor positive tumors. Lastly, our data show that loss of both p16 and pRB seems to be a distinctly uncommon event in human mammary tumorigenesis.

In conclusion, we have shown that immunohistochemistry is a suitable modality to screen for abnormal RB and p16 expression in formalin fixed, paraffin embedded breast tumors; that such tumors may be completely or, less commonly, focally negative for either pRB or p16; that abnormal expression of RB is relatively uncommon; that aberrant p16 expression is much more frequent that previously thought and may in fact be one of the most common abnormalities in human breast cancer; and that p16 status is not significantly correlated with several histopathological parameters.

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