

HLA Class I Antigen and Transporter Associated with Antigen Processing (TAP1 and TAP2) Down-Regulation in High-Grade Primary Breast Carcinoma Lesions¹

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ABSTRACT

Five specimens of normal mammary tissue and 53 primary breast carcinoma lesions were tested for expression of HLA antigens and components of the antigen-processing machinery by immunohistochemical staining. The expression of transporter associated with antigen processing (TAP) 1, TAP2, and HLA class I antigens in breast carcinoma lesions was significantly associated with tumor grading. Like normal mammary tissue, the 16 low-grade (G1) breast carcinoma lesions showed strong staining for TAP1, TAP2, and HLA class I antigens. In contrast, only 12 (32%) of 37 high-grade (G2 and G3) breast carcinoma lesions displayed the normal staining pattern. In 14 (38%) of 37 high-grade lesions, HLA class I antigen down-regulation was observed without loss of low molecular mass polypeptide and/or TAP staining. Congruent down-regulation of HLA class I antigen and TAP1 or TAP2 was found in 8 (22%) of 37 high-grade lesions. Complete loss of HLA class I antigens, TAP1, and TAP2 was observed in 3 (8%) of 37 high-grade lesions. No lesion was negative for TAP1 and/or TAP2 staining while positive for HLA class I antigen staining. These data demonstrate an association of HLA class I antigen and TAP down-regulation with tumor progression in breast carcinoma. This association suggests that loss of HLA and/or TAP may represent an escape from the host's immune pressure or may reflect the accumulation of abnormalities associated with neoplastic progression. This accumulation of defects in antigen processing and presentation may in turn be responsible for reduced recognition of malignant cells by putative clinically relevant tumor-specific T cells.

INTRODUCTION

In recent years, the molecular steps which lead to the generation and presentation of peptides to CTLs have been defined (reviewed in Ref. 1). An important role in this series of events is played by the multisubunit 20S proteasome complex, which generates peptides from endogenous proteins, and by the TAP,³ which transports peptides to the endoplasmic reticulum where they are loaded on HLA class I heavy chains in association with β_2m . Two subunits of the 20S proteasome, the LMPs LMP2 and LMP7, are encoded by genes closely linked to those that encode the TAP1 and TAP2 subunits of the TAP heterodimer. Once assembled, the HLA class I peptide complex is transported to the cell surface for presentation to CTL.

The role played by CTL in immunosurveillance and the interest in the application of T cell-based immunotherapy for the treatment of malignant diseases (2) have stimulated interest in the analysis of the

antigen-processing machinery in malignant cells. Structural and functional abnormalities in HLA class I-dependent antigen-processing may provide malignant cells with a mechanism to escape from immune surveillance and may have a negative impact on the outcome of T cell-based immunotherapy.

A number of studies have described HLA class I antigen down-regulation in breast carcinoma lesions (3-8). To the best of our knowledge, only Kaklamani *et al.* (9) have investigated TAP1 expression in breast carcinoma lesions. They have found loss of this protein in about 30 and 40% of primary and metastatic lesions, respectively. TAP1 loss was associated with HLA class I antigen loss but was not related to tumor stage, grade, or histology. No information is available about the expression of other components of the antigen-processing machinery in breast carcinoma lesions. Therefore, in the present study we have analyzed the expression of LMP2, LMP7, TAP1, and TAP2 in surgically removed breast carcinoma lesions. Furthermore, we have correlated these results with HLA class I antigen expression, with the histopathological characteristics of the lesions, and with clinical parameters.

MATERIALS AND METHODS

Tissue. Tissues were obtained from 53 patients with diagnosed breast carcinoma. Representative paraffin tissue blocks were selected from all tumor specimens and serially cut into 5- μ m sections. Serial sections from each specimen were routinely stained with H&E for histological examination and tumor grading. The lesions were graded following the modified Bloom-Richardson criteria (10).

Antibodies. Affinity-purified rabbit anti-LMP2 and anti-LMP7 (11), anti-TAP1 and anti-TAP2 (12) antibodies, mAb HC-10 to a determinant expressed on β_2m -free HLA class I heavy chains (13) and anti-HLA-DR, -DQ, -DP mAb H2-27 (14) were developed and characterized as described. Biotinylated rabbit anti-mouse immunoglobulin and goat anti-rabbit immunoglobulin xenoantibodies were purchased from Dako A/S (Gostrup, Denmark).

Immunohistochemical Staining. Paraffin sections were deparaffinized with xylene and rehydrated by passage through decreasing concentrations of ethanol (from 100 to 80%); endogenous peroxidase activity was blocked by a 30-min incubation at room temperature with methanol containing 3% H₂O₂. Sections used for staining with polyclonal antibodies were then microwaved in citrate solution at 750 W for 10 min. After rinsing in Tris-buffered saline (TBS; pH 7.4), sections were preincubated for 30 min at room temperature with normal rabbit serum or normal goat serum diluted 1:5, prior to incubation in a humidified chamber for 90 min at room temperature with anti-HLA mAb or overnight at 4°C with polyclonal anti-TAP or anti-LMP antibodies, respectively. Sections were washed twice in TBS and then incubated for 30 min with either biotinylated rabbit anti-mouse IgG or biotinylated goat anti-rabbit IgG antibodies. Sections were washed again in TBS and incubated for 60 min at room temperature with avidin-biotin peroxidase complex. Peroxidase activity was detected by incubating sections for 10-15 min with a stock solution of 1 mg/ml 3,3'-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) 3% H₂O₂. Sections were counterstained with Mayer's hemalum (Sigma). Staining intensity was graded as - when no staining was detected, +/- when staining was faint or barely detectable, + when staining was weakly positive, ++ when staining was moderately positive, and +++ when staining was strong. Ex-

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³ The abbreviations used are: TAP, transporter associated with antigen processing; β_2m , β_2 -microglobulin; mAb, monoclonal antibody; LMP, low molecular mass polypeptide.

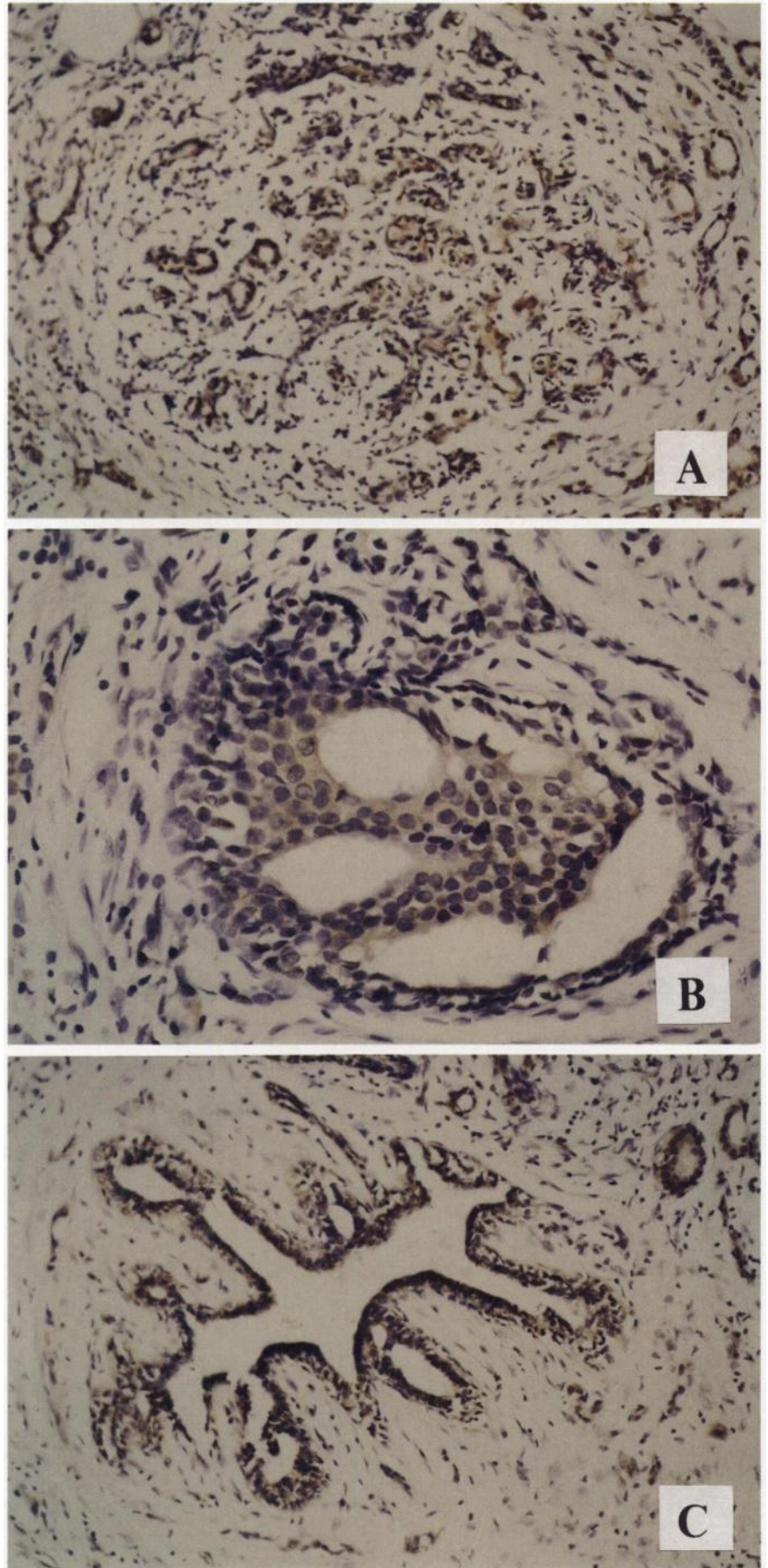


Fig. 1. HLA class I antigen, TAP1, and TAP2 expression in normal mammary tissue. Sections of a formalin-fixed, paraffin-embedded normal mammary tissue were stained in the immunoperoxidase reaction with mouse anti-HLA Class I mAb HC-10 (A; $\times 120$), with affinity-purified anti-TAP1 antibodies (B; $\times 300$) and with affinity-purified anti-TAP2 antibodies (C; $\times 120$). Anti-HLA class I antigen, anti-TAP1, and anti-TAP2 immunoreactivity is located in the cytoplasm, and staining varies from moderate to strong intensity.

pression of a marker was scored as down-regulated when staining was +/- or -. Normal stroma and infiltrating lymphocytes were examined in each specimen as internal controls. Negative controls were performed by omitting primary antibodies. Samples were analyzed and scored blindly.

Flow Cytometry. DNA content was measured by flow cytometry in 50- μ m-thick formalin-fixed, paraffin-embedded tissue sections, using the methodology described by Hedley *et al.* (15). Briefly, tissue sections were sequentially deparaffinized with xylene, rehydrated by passage through decreasing ethanol concentrations, and washed twice in distilled water. Tissue was then enzymatically disrupted by resuspending in 1 ml of 0.5% pepsin, 0.9% NaCl, adjusted to pH 1.5 with 2 N HCl, and incubated at room temperature for 90 min with intermittent mixing. Enzymatic digestion was stopped by adding an excess of Tris buffer (pH 8.6). Disaggregated samples were washed in PBS, filtered to remove aggregates, and stained with 0.6 μ g/ml 4',6-diamidino-2-phenylindole (Calbiochem). DNA ploidy was measured by a Partec Cull flow cytometer (Partec GMBH, Munster, Germany). Human lymphocytes were used as an internal control.

RESULTS

Preliminary assays tested the staining of formalin-fixed, paraffin-embedded normal mammary tissues with the panel of antibodies to be used in the present investigation. Anti-HLA class I mAb HC-10, as well as affinity-purified anti-LMP2, anti-LMP7, anti-TAP1, and anti-TAP2 antibodies, stained alveolar cells and epithelial cells of ducts in the five normal mammary tissue samples tested (Fig. 1). The staining intensity with anti-HLA class I mAb and with anti-LMP subunit antibodies was strong, whereas that with anti-TAP subunit antibodies was moderate to strong. The five antibodies stained also the interlobular tissue but with weak intensity. Anti-HLA class II mAb H2-27 stained alveoli and ductal tissues with a focal pattern and strong intensity.

Fifty-three primary breast carcinoma lesions were tested for HLA class I antigen, HLA class II antigen, and TAP1 and TAP2 expression. Twenty-two lesions were also tested for LMP2 and LMP7 subunit expression. Anti-HLA class I mAb and anti-TAP1 and anti-TAP2 antibodies stained 28 (53%), 46 (87%), and 46 (87%) of the 53 lesions, respectively. Expression of HLA class I antigens, TAP1, and TAP2 was coordinate in 28 lesions and was not detectable in 3 others. Among the 22 remaining lesions with HLA class I antigen down-regulation, 14 expressed both TAP1 and TAP2, 4 only TAP1, and 4 only TAP2 (Table 1).

HLA class I antigen and TAP subunit down-regulation in breast carcinoma lesions were significantly ($P < 0.001$ and $P < 0.02$, respectively) correlated with tumor grading. Down-regulation of HLA class I antigen, TAP1, or TAP2 was not found in any of the 16 low-grade (G1) lesions tested (Fig. 2). In contrast, TAP1 and/or TAP2 down-regulation and HLA class I antigen down-regulation occurred in 29 and 68% of high-grade (G2 and G3) lesions, respectively (Fig. 3). The intensity of staining for HLA class I antigens and TAP subunits did not differ significantly between low- and high-grade lesions, because the intensity was moderate to strong in greater than 50% of low- and high-grade lesions. Lesions negative for HLA class I antigens and either one of the TAP subunits stained only with weak

intensity for the other TAP subunit. Neither HLA class I antigen nor TAP down-regulation are significantly associated with histopathological characteristics of the lesions, *i.e.*, lesion dimensions, tumor ploidy, cellular proliferation, and lymph node metastases. Data on survival were available for only 13 patients in this study. In this small number of patients, no relationship was found between HLA class I antigen and TAP subunit down-regulation and patients' survival.

LMP2 and LMP7 were expressed in the 9 low-grade and 13 high-grade lesions tested. Intensity of staining for LMP2 and LMP7 ranged from weak to strongly positive with no relationship to tumor grading. HLA class II antigen expression was detected in 37 (70%) of the lesions. Positive lesions were stained strongly for HLA class II antigen expression with a focal pattern of staining similar to that found in normal alveoli and ductal breast tissue. HLA class II antigen expression and staining intensity did not correlate with the degree of differentiation of the lesions (data not shown), because HLA class II antigens are expressed in 11 (69%) of 16 low-grade lesions and in 26 (70%) of 37 high-grade lesions.

DISCUSSION

The present study has shown down-regulation of HLA class I antigens in 47% and down-regulation of TAP1 and/or TAP2 in 21% of primary breast carcinoma lesions. HLA class I antigen and TAP down-regulation are both associated with tumor grading. The results of our study differ in several respects from those published by Kaklamani *et al.* (9). The frequency of TAP1 down-regulation found in the present study is lower than that described by Kaklamani *et al.* (9), who had not detected TAP1 in 33% of primary breast carcinoma lesions. Furthermore, Kaklamani *et al.* (9) found coordinate expression of TAP1 and HLA class I antigens, whereas we have found a significantly more frequent loss of HLA class I antigens than of TAP1 or TAP2. Lastly, Kaklamani *et al.* (9) found no significant association between HLA class I antigen or TAP1 down-regulation in primary breast carcinoma lesions and tumor grading, whereas we did. Whether these differences reflect the characteristics of the anti-TAP1 xenoantibodies used in the two studies cannot be assessed, because no information is available about the anti-TAP1 xenoantibodies used by Kaklamani *et al.* (9). Furthermore, we cannot exclude a role of the criteria to classify the results and/or of the characteristics of the lesions used in the two investigations, because the earlier study (9) provides only limited information about these parameters. On the other hand, two lines of evidence argue against the use of different substrates in the immunohistochemical reactions, *i.e.*, frozen tissue sections by Kaklamani *et al.* (9) and formalin-fixed tissue sections by ourselves, as a major reason for the conflicting results. In the earlier analysis of a large number of breast carcinoma lesions (9), identical results were obtained when the staining of sections from frozen and formalin-fixed portions of each lesion were compared with anti-HLA class I mAb HCA2 and mAb W6/32, respectively. mAb HCA2 (13) and mAb W6/32 (16) recognize a linear and a conformational antigenic determinant of HLA class I antigens, respectively. Furthermore, we have not found significant differences in the immunohistochemical staining of frozen and autologous formalin-fixed sections of a number of melanoma lesions (17 and unpublished results) with the mAb HC-10, which recognizes a linear determinant of HLA class I heavy chains (13), and with anti-TAP1 and anti-TAP2 xenoantibodies. The discrepancy between the earlier results (9) and our own is also not likely to be caused by the different specificity and characteristics of the anti-HLA class I mAb used in the two studies, because Kaklamani *et al.* (9) reported that the results of immunostaining of breast carcinoma lesions with mAb W6/32 were identical to those obtained

Table 1 Phenotype of HLA class I, TAP1, and TAP2 in primary breast carcinoma lesions

TAP1	TAP2	HLA I	Grade ^a	No. of lesions	%
+	+	+	Low	16/16	100
+	+	+	High	12/37	32
+	+	-	High	14/37	38
-	+	-	High	4/37	11
+	-	-	High	4/37	11
-	-	-	High	3/37	8

^a Low-grade lesion, G1; high-grade lesion, G2 and G3.

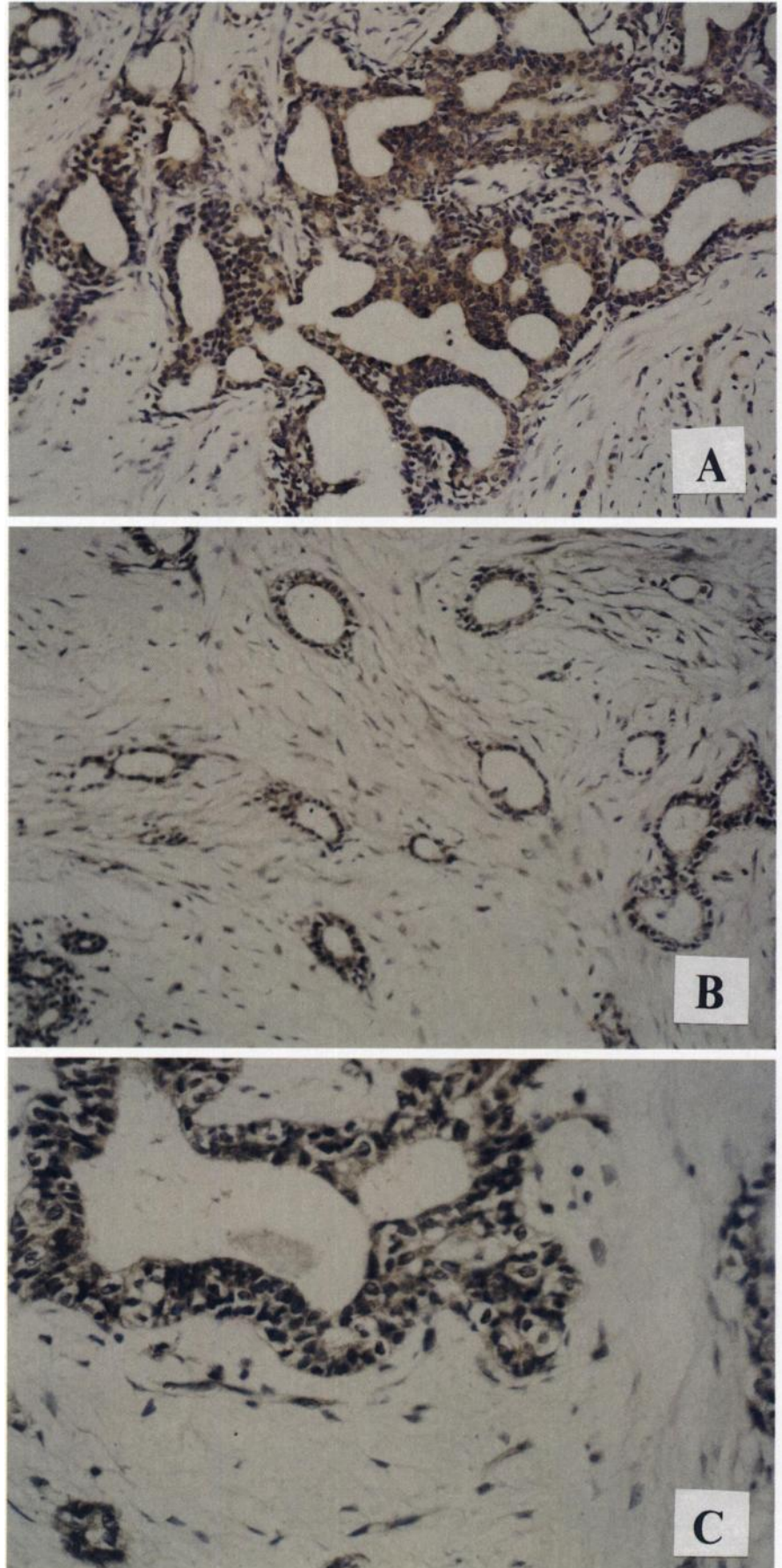


Fig. 2. HLA class I antigen, TAP1, and TAP2 expression in a low-grade (G1) breast carcinoma lesion. Sections of a formalin-fixed, paraffin-embedded low-grade (G1) breast carcinoma lesion were stained in the immunoperoxidase reaction with mouse anti-HLA class I mAb HC-10 (A; $\times 120$), with affinity-purified anti-TAP1 antibodies (B; $\times 120$), and with affinity-purified anti-TAP2 antibodies (C; $\times 300$). Staining intensity with anti-HLA class I mAb is moderate to strong, whereas that with anti-TAP1 and anti-TAP2 antibodies is weak to moderate.

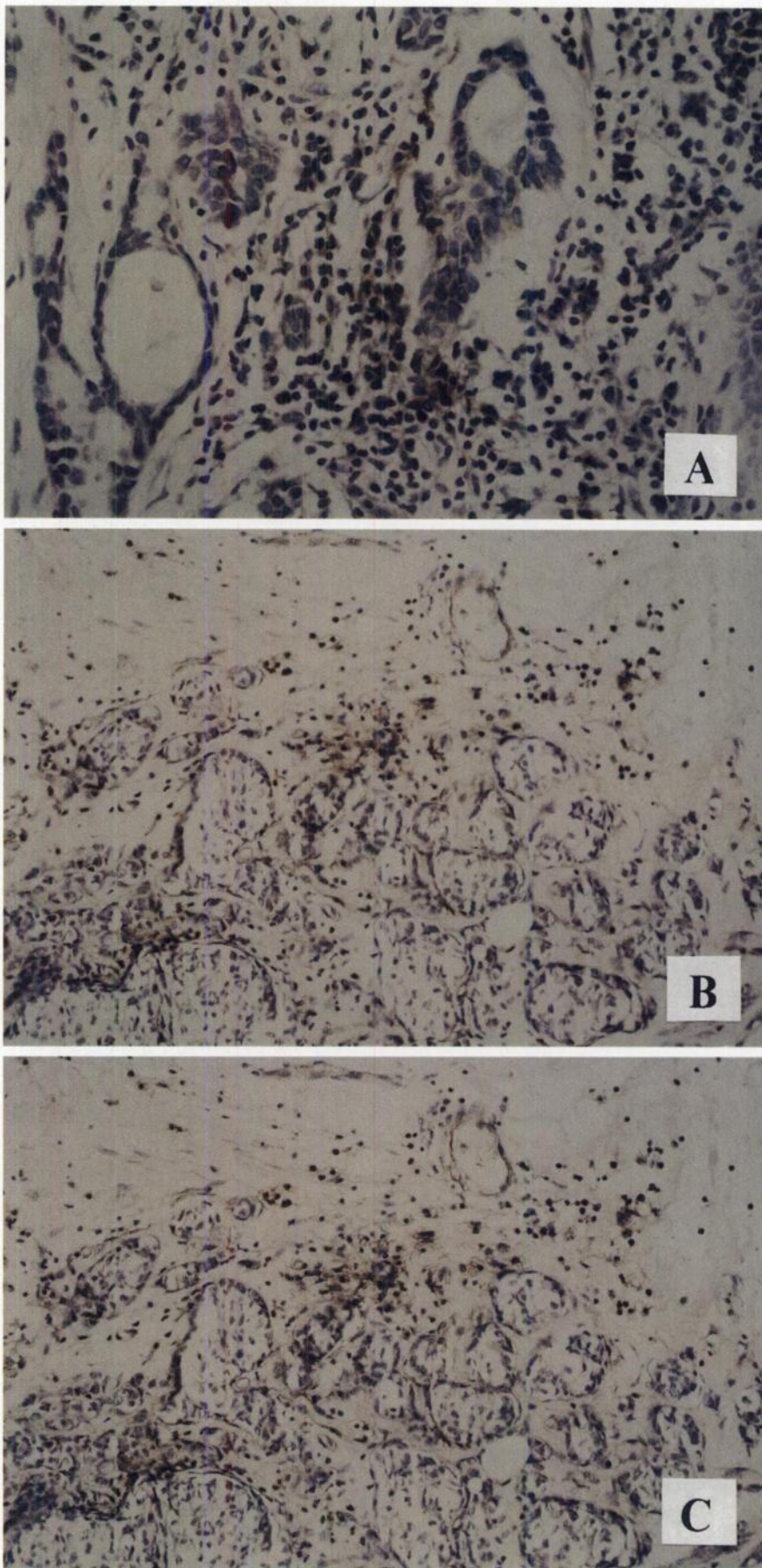


Fig. 3. Reduced HLA class I antigen, TAP1, and TAP2 expression in a high-grade (G2) breast carcinoma lesion. Sections of a formalin-fixed, paraffin-embedded high-grade (G2) breast carcinoma lesion were stained in the immunoperoxidase reaction with mouse anti-HLA class I mAb HC-10 (A; $\times 300$), affinity-purified anti-TAP1 antibodies (B; $\times 120$), and affinity-purified anti-TAP2 antibodies (C; $\times 120$). Note weak, heterogeneous staining for HLA class I antigens, TAP1, and TAP2.

with mAb HCA2. The latter mAb has characteristics similar to those of mAb HC-10, which we have used in our studies.

HLA class I antigen down-regulation, which in this study as in previous ones has been found in a high percentage of breast carcinoma lesions, was associated with TAP down-regulation. This association parallels similar findings in cervical carcinoma lesions (18, 19). In both types of carcinoma, the molecular mechanisms underlying this defect have not been identified. The association between HLA class I antigen and TAP down-regulation is not a cause-effect relationship, because we have measured the expression of β_2m -free HLA class I heavy chains. Their level, in contrast to that of the HLA class I heavy chain: β_2m :peptide complex, is not affected by TAP defects. In addition, the association between HLA class I antigen and TAP down-regulation does not appear to be a fortuitous event, because it appears to be selective both in breast and cervical carcinomas. It is our working hypothesis that the association of HLA class I antigen and TAP down-regulation reflects the accumulation of distinct defects in malignant cells because of genetic instability. These defects may include genomic loss of one or both haplotypes, mutations in HLA class I heavy chain and TAP subunit genes, and/or abnormalities in the mechanism controlling their transcription.

TAP1 expression has been investigated in various types of solid tumors (18–22). The frequency of TAP1 down-regulation in breast carcinoma lesions is similar to that found in colorectal carcinoma and lower than that found in non-small cell lung carcinoma, cervical carcinoma, and melanoma. Prior to the present study, TAP2 expression has been investigated only in melanoma lesions (22). The frequency of TAP2 down-regulation in primary melanoma lesions is higher than that we have found in breast carcinoma lesions.

The increasing body of evidence in the literature demonstrating loss of TAP expression in human cancers strongly suggests that malignant transformation of cells is associated with defects in HLA class I-dependent antigen processing. The down-regulation of HLA class I antigen and TAP expression in high-grade, primary breast carcinoma raises the question of whether this abnormality plays a role in disease progression. TAP1 and/or TAP2 down-regulation in breast carcinoma lesions were not associated with patients' survival. This finding parallels similar results in colon carcinoma (18) but is at variance with those in malignant melanoma.⁴ In melanoma, TAP1 and/or TAP2 down-regulation was associated with a statistically significant decrease in survival. It is tempting to speculate that the different results obtained in the three types of malignancies may reflect the different role that immunological events play in the pathogenesis and in the clinical course of the disease.

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