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Cyclins as markers of tumor proliferation: Immunocytochemical studies in breast cancer

(proliferation index/angiogenesis/cell cycle/immunohistochemistry/pathology)

ANINDYA DUTTA*, REENA CHANDRA, LORENE M. LEITER, AND SUSAN LESTER

Division of Molecular Oncology, Department of Pathology, Brigham & Women's Hospital, Harvard Medical School, 75 Francis Street, Boston, MA 02115

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ABSTRACT **We have developed methods to use anti-cyclin A, B, and E antibodies as reagents to specifically detect proliferating cells in specific phases of the cell cycle in formalin-fixed, paraffin-embedded sections of tissues and cells. Staining of 48 archival cases of breast cancer showed that these antibodies estimate the tumor proliferation fraction and therefore are potentially useful for the prediction of prognosis. A subset of cancers had a high frequency of tumor cells expressing cyclins A and E, out of proportion to other proliferation markers, suggesting that these tumors may have deregulated cyclin expression. In addition to recognizing authentic cyclin E in the nucleus of proliferating cells, anti-cyclin E antibody cross-reacted with a cytoplasmic protein in nonproliferating endothelial cells. This cross-reaction allows the simultaneous visualization and quantitation of microvessels in the tumors, measuring a second potential predictor of breast cancer prognosis, tumor angiogenesis.**

Cyclins are proteins that vary in abundance and associate with and activate different cyclin-dependent kinases (cdk) at different stages of the cell cycle, a given cyclin-cdk complex being essential for passage through a specific stage in the cycle. The periodic appearance of the cyclins in distinct phases of the cell cycle suggests that they can be used as markers for proliferation of tissues. Because the three different cyclins mark different phases of the cell cycle—E for G_1 and early S, A for S and G_2, and B for late G_2 (1–3)—the fraction of cells positive for a given cyclin should predict the fraction in a given phase of the cell cycle. Further, increased expression of various cyclins has been noted in extracts of human cancers, cyclin E in particular being markedly elevated in multiple tumors of different origins (4, 5), and an easy method for detecting such tumors will allow their biological behavior to be separately followed in clinical studies.

Estimation of the proliferative behavior of a tumor is important for the management and prognosis of breast cancers. After local excision of node-negative tumors, two-thirds of the patients are expected to do well without further adjuvant therapy, and one-third are expected to relapse. Since chemotherapy is most effective before widespread metastases, it is useful to be able to predict which cancers are likely to recur in order to avoid undertreating these patients or overtreating patients whose tumors are not expected to recur (6, 7). One such prognostic factor is the S phase fraction (SPF) of the tumor estimated by fluorescence-activated cell sorting (FACS) analysis for DNA content of singly suspended tumor cells (8–10). Patients with a large fraction of tumor cells in S and G_2 have a poorer prognosis and SPF has been used to guide the selection of patients for adjuvant therapy.

Despite its value, flow cytometry of breast cancers is not performed widely, mostly because such analyses require fresh, relatively large tumors with enough tumor left over after tissue is taken for histopathology and because of the frequent intermixing of tumor with normal stromal tissue. Newer markers for the easy estimation of growth fraction of tumors on histopathology sections will be of great value, particularly if multiple markers give internally consistent results and increase the reliability of the measured proliferation index or if they provide new information on cell-cycle kinetics. Here we report that staining for cyclin expression detects (*i*) tumor proliferation, (*ii*) deregulated cyclin expression, and (*iii*) microvessel density (tumor angiogenesis), all of which could be important for predicting clinical outcome.

METHODS

Immunohistochemistry. Monoclonal antibodies for Ki-67 (MIB 1) (11) were purchased from AMAC (Westbrook, ME). The other antibodies used were polyclonal rabbit antisera against bacterially expressed recombinant cyclin A (from Jonathon Pines, Wellcome CRC Institute), mouse monoclonal antibodies to cyclin B and cyclin E (from Ed Harlow, Massachusetts General Hospital Cancer Center), and monoclonal antibody to RPA p70 (12), p70-9 (from Bruce Stillman, Cold Spring Harbor Laboratory).

Paraffin sections were prepared from tissue fixed in formalin for 5–24 hr. Sections were cut at 3–5 μm, and the slides were dried at room temperature for 2 days, baked at 60°C for 20 min, deparaffinized in xylene, and then rehydrated in alcohol and water. They were then heated in 10 mM sodium citrate in a microwave oven (pH 6.0) at 93°C for 30 min. After blocking by incubation with either 2% horse serum in phosphate-buffered saline (PBS) (p70-9, MIB 1, cyclin B, and cyclin E) or 2% goat serum in PBS (cyclin A) for 15 min, the slides were incubated for 1 hr with the following antibodies: p70-9 ascites (1:500 dilution), MIB 1 (1:100), anti-cyclin A serum (1:1000), anti-cyclin B culture supernatant (1:100), or anti-cyclin E culture supernatant (1:100). The ABC Elite kit (avidin-biotin complex; Vector Laboratories) was used with the chromagen 3′,3′-diaminobenzidine to develop brown color (1–3 min). The sections were counterstained with 2% methyl green nuclear stain (Cell Analysis Systems) for 25 min before dehydration in acetone and xylene.

Staining Indices. All stained sections were surveyed microscopically at 100× for areas with highest density of positive tumor cells. At least two or three fields (usually adjacent) in these areas were examined at 400× and a total of 500–1000 tumor cells was counted to estimate the percent of cells positive for a given antigen. A staining index is the percentage of tumor cells stained with the antibody.

Cell-Cycle Synchronization. CV1 cells (monkey kidney epithelial cell line) were blocked in mitosis with nocodazole (40 ng/ml) for 22 hr and then released from the block. As the cells progressed synchronously through the subsequent stages in the

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Abbreviations: FACS, fluorescence-activated cell sorting; SPF, S phase fraction.

*To whom reprint requests should be addressed.

cell cycle (G_1, S, G_2), aliquots were incubated with bromodeoxyuridine (BrdUrd) for 30 min and harvested at the indicated time points. Cell pellets from each point were fixed in formalin and embedded in wax, and sections were stained with various antibodies.

Flow Cytometry and Immunoblotting. All samples were processed by the flow cytometry laboratory of our department as part of the routine evaluation of breast cancer biopsies (13, 14). Protein extracts from fresh frozen tissues were prepared according to published protocols and 200–300 μg of protein was immunoblotted using standard protocols (5, 15).

RESULTS

Anti-Cyclin Antibodies Detect Proliferating Cells of Normal Tissues. Normal colon mucosa and tonsils were stained with various anti-cyclin antibodies to determine if proliferating cells were specifically detected using the MIB-1 antibody as a positive control. As expected, MIB-1 detected proliferating cells located in the bases of colonic mucosal crypts, while nonproliferating surface mucosal cells were not detected (Fig. 1*b*). The anti-cyclin A antibody stained the nuclei of a subset of cells that were positive for MIB-1 (Fig. 1*c*). This is expected because the Ki-67 antigen is present in cells in G_1, S, G_2, and M (11, 16), while the cyclin A protein is present only in the nuclei of cells in S and G_2. Anti-cyclin B also predictably stained a subset of Ki-67-positive cells in the cytoplasm (Fig. 1*d*). p70-9 antibody, in contrast, stained postmitotic cells in the surface of the colon mucosa and serves as a negative control (Fig. 1*a*). Cyclin E exhibited an unexpected pattern (Fig. 1 *e–h*). The nuclei in the epithelium were negative but the cytoplasm of vascular endothelial cells in the lamina propria and submucosa were stained strongly. This result is even more unusual because of the negative staining of vascular endothelium for the other three proliferation markers (cyclins A and B and Ki-67). Staining of tonsils showed the presence of nuclear cyclin A and cytoplasmic cyclin B in the germinal centers (staining a subset of Ki-67-positive cells, data not shown) and anti-cyclin E-reactive protein in the nuclei of few cells in the germinal center and in the cytoplasm of endothelial cells (data not shown and Fig. 1*g*).

The cytoplasmic staining of nonproliferating endothelial cells with anti-cyclin E antibody was observed in kidneys, prostate, and placenta, though not in the lungs (Fig. 1*f* and data not shown). The endothelial staining was also seen with a polyclonal antibody to bacterially expressed GST-cyclin E. None of the three other monoclonal antibodies to cyclin E, HE67, HE111, and HE172, gave specific immunohistochemical staining in paraffin sections. The endothelial staining with monoclonal antibody HE12 was blocked by preincubation of antibody with bacterially expressed GST-cyclin E but not with GST (Fig. 1 *h* and *g*). Nonproliferating decidual cells associated with a term placenta were also strongly stained in the cytoplasm with anti-cyclin E antibody (see Fig. 4*f*).

In summary, antibodies to cyclins A and B specifically stained a subset of proliferating cells, while anti-cyclin E antibody stained the nuclei of a subset of proliferating cells in the tonsils but also strongly stained the cytoplasm of nonproliferating endothelial cells and uterine decidual cells. As we shall show, only nuclear staining with anti-cyclin E antibody corresponded to authentic 55-kDa cyclin E protein (see Fig. 3).

Cell-Cycle Stage-Specific Staining. CV1 cells released from a nocodazole-induced mitotic block were followed at various time points for staining with the anti-cyclin antibodies in comparison to their passage through S phase (Fig. 2). BrdUrd uptake into nuclei indicated the synchronous passage of the cells through S phase. Nuclear staining with anti-cyclin A and anti-cyclin E antibodies and cytoplasmic staining with anti-cyclin B antibody peaked at approximately the time points where the corresponding cyclins are expressed. These results confirm the utility of the anti-cyclin antibodies (particularly the nuclear staining with anti-cyclin E) as cell-cycle stage-specific proliferation markers. The differences in the peak percentage of cells staining with each antibody are probably due to different titers of the antibodies and due to different sensitivities of the antigens to processing. This should not lessen the utility of the antibodies for comparisons between tumors.

Anti-Cyclin-Reactive Antigens in the Cells. Immunoblots of cell extracts with anti-cyclin A and anti-cyclin B antibodies

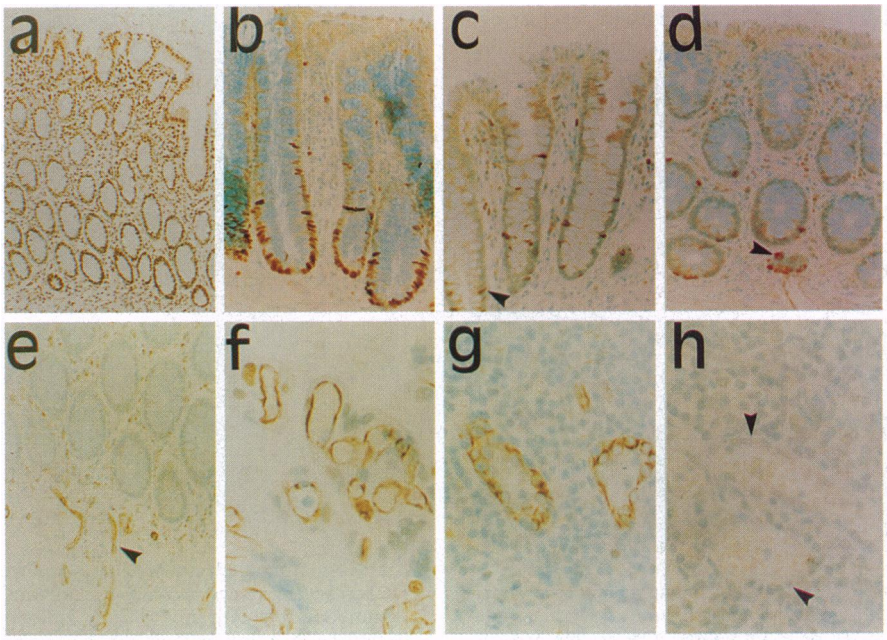

FIG. 1. Immunohistochemical staining of normal tissues with anti-cyclin antibodies. (*a–e*) Colon mucosa oriented with the luminal surface toward the top. (*f*) Placental villi. (*g* and *h*) Tonsils. The antibodies used are anti-RPA 70, p70-9 (*a*), anti-Ki-67, MIB1 (*b*), anti-cyclin A (*c*), anti-cyclin B, GNS (*d*), and anti-cyclin E, HE12 (*e–h*). The antibody was blocked by preincubation with bacterially expressed glutathione *S*-transferase (GST) (*g*) or GST-cyclin E (*h*). Arrowheads in *d* and *e* indicate positive cells and in *h* indicate unstained endothelium. (*a*, ×65; *b–e*, ×130; *f*, ×50; *g* and *h*, ×200.)

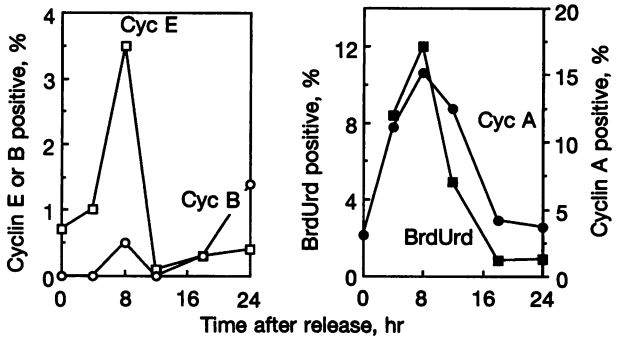

FIG. 2. Quantitation of immunohistochemistry on cell-cycle-synchronized CV1 cells. The *x* axes show the time after release from a mitotic block with nocodazole. The *y* axes show the percentage of nuclei labeled with anti-BrdUrd, anti-cyclin A, and anti-cyclin E and percentage of cytoplasts labeled with anti-cyclin B antibodies. Cells were exposed to BrdUrd for 30 min before harvesting at each time point.

showed that they specifically recognized the expected 60- and 62-kDa proteins (Fig. 3). In contrast, anti-cyclin E antibody recognized two polypeptides of 75 and 85 kDa in addition to the expected 55-kDa cyclin E protein.

We examined different tissues and cells by immunostaining and immunoblotting with anti-cyclin E antibody HE12 to prove that the presence of authentic 55-kDa cyclin E protein produced nuclear staining. HE12-reactive antigen was exclusively present in the cytoplasm of endothelium and absent from all nuclei in normal colon mucosa and submucosa, kidneys, and prostate (Fig. 1 and data not shown), present in the cytoplasm of vascular cells and in nuclei of trophoblasts in placenta (Fig. 1), and present mostly in the nuclei, but occasionally also in the cytoplasm of HeLa and 293 cells (data not shown). Protein extracts from these tissues and cells were immunoblotted with HE12 antibody (Fig. 3). A polypeptide of 55 kDa corresponding in size to authentic cyclin E (2, 3) was present in extracts from placenta, HeLa, and 293 cells, the only tissue/cells with nuclear staining. In contrast, only polypeptides of 75 and/or 85 kDa were recognized in extracts of tissues with exclusively vascular cytoplasmic staining. Therefore, the nuclear staining with anti-cyclin E antibody is due to the authentic 55-kDa cyclin E protein, while the cytoplasmic staining of the vascular endothelium correlates with the presence of the cross-reacting 75-kDa polypeptide.

Thus, three different lines of investigation validated that anti-cyclin A (nuclear), anti-cyclin E (nuclear), and anti-cyclin B (cytoplasmic) antibodies recognize the "correct" cell-cycle stage-specific proliferation markers.

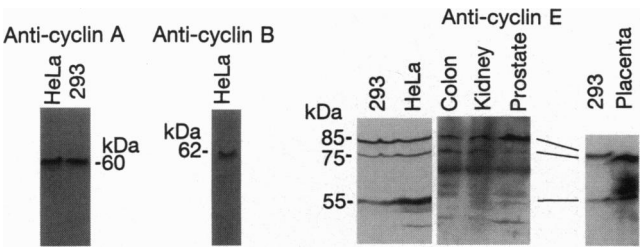

FIG. 3. Immunoblot of protein extracts from human HeLa and 293 cells, human placenta, colon mucosa and submucosa, kidney, and prostate with anti-cyclin A, B, or E antibodies. The protein extracts were electrophoresed on a 15% SDS/polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with the relevant antibodies. Horseradish peroxidase-conjugated secondary antibody was used and positive bands were visualized by the enhanced chemiluminescence reaction (Amersham).

Table 1. Mean cyclin and Ki-67 staining indices for each of the prognostically different S phase grades in 48 invasive breast cancers

S phase grade	Cyclin A	Cyclin B	Cyclin E	Ki-67
1	4.9 ± 4.6	0.9 ± 1.0	7.0 ± 11.6	19.2 ± 20.2
2	8.9 ± 9.1	1.7 ± 1.2	11.5 ± 19.1	23.5 ± 15.0
3	14.1 ± 12.3	2.8 ± 2.7	18.1 ± 20.5	37.0 ± 30.9
P	0.026	0.022	0.21	0.084

Forty-eight breast cancers were each stained with antibodies for the indicated antigens and the staining indices were calculated. SPF% was determined for each cancer by FACS for DNA content. Tumors were assigned to S phase grades: grade 1 (<7.0 for aneuploid and <3.5 for diploid tumors), grade 2 (7.0–16.0 for aneuploid and 3.5–7.0 for diploid), grade 3 (>16.0 for aneuploid and >7.0 for diploid). Each grade contains 16 tumors, half aneuploid and half diploid. The mean ± SD of each grade is shown. An analysis of variance was done to test if the indices for a given antigen were significantly different between the three grades, and the *P* value of this comparison is also shown. Significant difference between grades: $P < 0.05$.

Staining of Breast Cancers. Forty-eight cases of human breast cancer were selected to obtain a representation of aneuploid and diploid tumors from all three S phase grades (described in Table 1) and representative results of staining are presented in Fig. 4. Anti-cyclin A showed strong nuclear staining (Fig. 4 *a* and *b*) and anti-cyclin B showed cytoplasmic staining (Fig. 4*c*). Unlike normal tissues, nuclear staining was easily detected in tumor cells with anti-cyclin E antibody (Fig. 4 *d* and *e*). The cyclin and Ki-67 staining indices and the flow cytometrically determined SPF were determined for each of the tumors (data not shown but available from the authors). The tumors were divided into three prognostic groups depending on their SPF (9, 14), and the mean ± SD of these indices in each of the groups is shown in Table 1. The mean staining indices increased progressively as the tumors fell into progres-

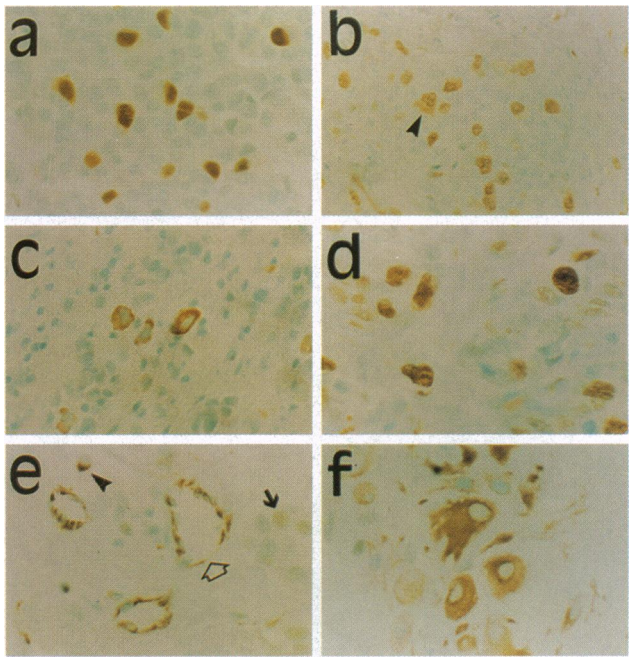

FIG. 4. Immunohistochemical stain with anti-cyclin antibodies. Breast cancers stained with anti-cyclin A antibody (*a* and *b*), anti-cyclin B antibody (*c*), and anti-cyclin E antibody (*d* and *e*). (*f*) Uterine decidual cells stained with anti-cyclin E antibody. Arrowheads in *b* and *e* indicate aberrant cytoplasmic staining of tumor cells for cyclin A and cyclin E, respectively. (*e*) The solid arrow indicates weak nuclear staining for cyclin E in tumor cells; the open arrow indicates cytoplasmic endothelial staining in tumor-associated blood vessels. (×200.)

sively worse prognostic groups (increasing SPF), and for cyclin A and B the means were significantly different between the three groups.

If the cyclin staining is correlated with cell proliferation, we should expect to see a positive correlation between these indices and with the staining index for another proliferation marker, Ki-67. The best correlation was between cyclin A and Ki-67 staining (Fig. 5 and Table 2), although the cyclins E vs. A and E vs. Ki-67 indices in a given tumor were also well correlated with each other. We also compared the cyclin staining indices with a different measure of the growth fraction of tumors: FACS-determined SPF. Here too, cyclin A staining was best correlated to the FACS-determined SPF. Therefore the staining of tumor sections with anti-cyclin antibodies correlated with two different criteria of proliferation.

The cyclin staining indices were better correlated to each other and to the Ki-67 staining index than to the FACS-determined SPF. This could be due to the smaller number of cells assayed by immunohistochemistry, due to contaminating stromal cells confounding SPF measurements, or due to the greater technical difficulty of FACS for the measurement of SPF. In addition, staining for the cyclins would be affected by differences in the cell-cycle kinetics between tumors, so that it is not surprising that, although the cyclin staining indices are positively correlated with the Ki-67 staining index and the SPF, they are not perfectly correlated (correlation coefficient of 1). The results, however, suggest that immunostaining with the anti-cyclin antibodies complements FACS analysis for the determination of proliferation and cell-cycle kinetics in tumors.

Deregulation of Cyclin Expression in Breast Cancer. Since deregulation of cyclin physiology could be directly involved in the oncogenic process, we specifically looked for cases that either had a much higher fraction of cells staining for cyclin E and/or cyclin A or did not follow the normal strong correlation between the cyclin staining indices and the Ki-67 staining index. For cyclins A and E, we could detect tumors with staining indices that were higher than expected from the Ki-67 staining (tumors in group c, Fig. 5). We also calculated an arbitrary index by multiplying the percentage of cyclin E-positive cells and the percentage of cyclin A-positive cells (the EA index) to produce a composite representation of cyclins E and A expression in a tumor (Fig. 5). We could detect tumors that had staining indices significantly greater than the general population—e.g., there were eight tumors with an EA index above a threshold of 400. As with A or E alone, there were at least four tumors with EA indices that were greater than expected from their Ki-67 staining (group c, Fig. 5). We hypothesize that tumors with a staining index either (*i*) much higher than the general population (above a threshold) or (*ii*) greater than expected from the Ki-67 index (group c, Fig. 5) may have deregulated cyclin overexpression.

Table 2. Correlation of staining indices with each other and with FACS-determined SPF in breast cancers

	Ki-67	Cyclin A	Cyclin B	Cyclin E
Cyclin A	0.76 (<0.001)			
Cyclin B	0.57 (<0.001)	0.51 (<0.001)		
Cyclin E	0.67 (<0.001)	0.73 (<0.001)	0.36 (0.013)	
SPF	0.44 (0.002)	0.53 (<0.001)	0.43 (0.003)	0.35 (0.014)

The correlation coefficient for each comparison is shown for the entire set of breast cancers. For example, the correlation of the cyclin A staining indices with the Ki-67 staining indices for each of the 48 breast cancers had a correlation coefficient of 0.76. For each of the comparisons the correlation coefficient is accompanied (in parentheses) with the *P* value for the two-tailed significance. Statistically significant correlation: $P < 0.05$.

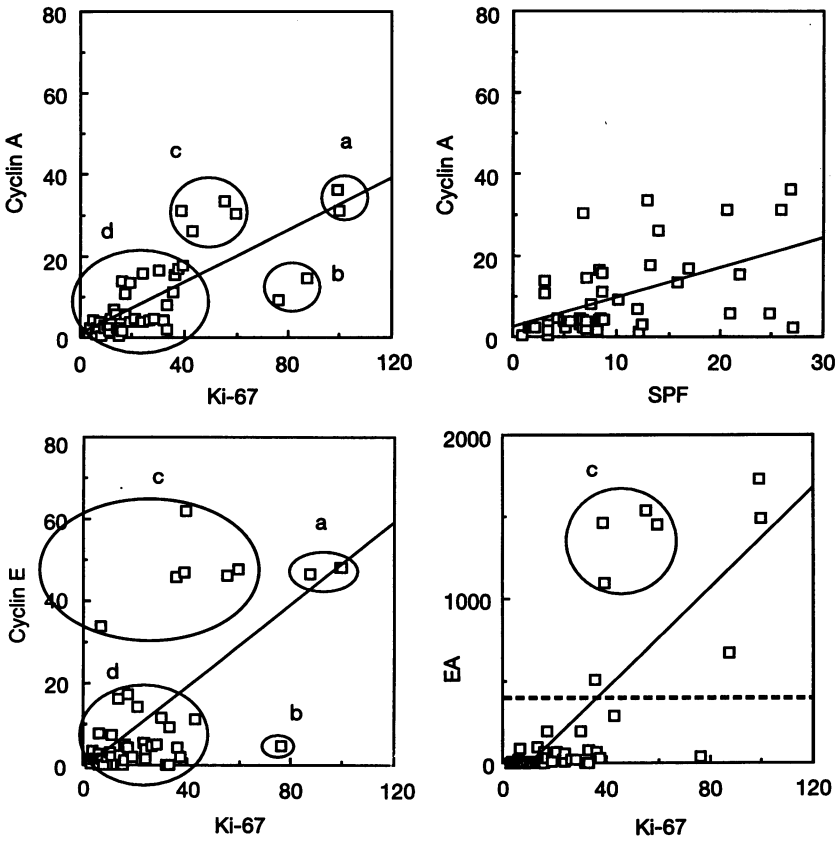

FIG. 5. Selected correlations of staining indices with each other and with SPF. The EA index is described in the text. The tumors can be divided into four groups, a–d, as shown for the cyclin A index vs. Ki-67 index plot. For the EA index vs. Ki-67 plot, the dashed line indicates an arbitrary threshold of 400 and shows that eight cancers had EA indices above that threshold.

DISCUSSION

We have demonstrated that immunocytochemical detection of cyclins assesses the proliferation rates of breast cancers and therefore has the potential to be used as a prognostic indicator. The majority of the tumors with elevated fraction of cells expressing cyclins A, E, and B also showed a corresponding increase in the proliferative status of tumor cells, reflected in the high correlations between the various staining indices (group a, Fig. 5). Such internal consistency in the staining indices is an added virtue of this method of proliferation measurement, because it rules out an increase in the level of a particular cyclin due to gene amplification or some other mode of deregulation. This immunocytochemical approach is applicable to small tumor specimens and to archival specimens, allowing retrospective studies relating to tumor growth and prognosis. The method has the added advantage of not being influenced by the proliferative status of stromal cells and infiltrating lymphocytes.

The advantages outlined above are, of course, equally applicable to assessment of proliferation by staining for Ki-67, which has been used successfully as a prognostic marker in breast cancers (17, 18). However, besides enhancing the reliability of the proliferation index, staining for cyclins provides further information about cell-cycle kinetics. Unlike Ki-67, staining for cyclins A and B (and possibly E) selectively detects tumor cells committed to cell division (late G_1 and beyond) (19). Such improvement in tumor phenotyping may be a special advantage of the cyclins over Ki-67, because it could potentially predict responsiveness to chemotherapy targeted at cells in the S and M phases of the cell cycle.

We also detect breast cancers with elevated fractions of tumor cells expressing cyclins E, A, or both, a small subset of which is also inconsistent with the Ki-67 staining index. The elevated staining indices could be due to overexpression of the cyclins, due to amplification of the cyclin genes, or due to the failure to appropriately degrade the cyclin messages in the cell cycle (4). Immunoblotting of tumor extracts suggested that breast cancers with a high level of cyclin E are likely to have a poorer prognosis (5). Perhaps these are the tumors being identified as "group c" in the plots in Fig. 5. If this proves to be true, we can now identify such tumors in retrospective studies and determine their biological behavior. Immunoblotting experiments also suggested that expression of the 55-kDa cyclin E protein was specific to tumors and not seen in adjacent normal tissue, suggesting that elevation of cyclin E was a marker for oncogenesis and not just proliferation (5). This would explain our observation that there was scant nuclear staining with anti-cyclin E antibody in normal tissues (which corresponds to the 55-kDa cyclin E), while such staining was easily detected in tumor tissue.

The strong staining of the cytoplasm of normal endothelial cells with anti-cyclin E antibodies (HE12) is probably due to a cross-reacting 75-kDa polypeptide, distinct from the authentic 55-kDa cyclin E polypeptide present in the nucleus of proliferating cells. This unexpected cross-reaction potentially expands the utility of the HE12 antibody because it can be used to quantitate the density of small vessels in breast cancers. Microvessel density has been used to measure tumor angiogenesis and shown to be a predictor of tumor metastasis, independent of the proliferative status of tumor cells (20–22).

Despite rapid improvements in our knowledge of the regulation of the eukaryotic cell cycle, few of these discoveries have yet been applied clinically. This study underlines the considerable heterogeneity in the cell-cycle parameters of individual breast cancers, a heterogeneity likely to be reflected in the biological behavior of the tumors and responsiveness to chemotherapy. An immediate question that will be addressed is whether staining for cyclins will reliably predict the prognosis of breast cancer patients and thereby help select patients who will benefit from adjuvant therapy.

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