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Short Communication

Expression of the G2-M Checkpoint Regulators Cyclin B1 and cdc2 in Nonmalignant and Malignant Human Breast Lesions

Immunocytochemical and Quantitative Image Analyses

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We investigated the* in vivo *expression of cyclin B1 and Cdc2 (key molecules for G2-M transition during the cell cycle) in nonmalignant and cancerous human breast lesions using immunohistochemistry and quantitative proliferative index (PI) analysis. Breast epithelial cells co-expressed cyclin B1 and Cdc2 in their cytoplasm in the G2 phase and in their nuclei in the M phase. Cyclin B1, but not Cdc2, immunostaining rapidly disappeared from the nuclei during the mitotic metaphase to anaphase transition. Static image analysis revealed the mean proliferative index for cyclin B1/cdc2 for each type of lesion to be as follows: normal glands (n = 20), 2.0/2.5%; benign lesions, including typical ductal hyperplasia (n = 76), 2.5/5.8%; atypical ductal hyperplasia (n = 21), 3.0/6.6%; carcinomas* in situ *(n = 70), 7.4/14.0%; and invasive carcinomas (n = 58), 10.0/22.9%. Proliferative index data for atypical hyperplasia were virtually identical to those for benign lesions and were significantly lower than those for breast cancer, suggesting that expression levels of cyclin B1 and Cdc2 may be used to distinguish premalignant human breast lesions from advanced disease. Furthermore, the proliferative index for cyclin B1 for comedo-type ductal carcinomas* in situ *agreed with that for invasive ductal carcinomas (mean, 10.1%* versus *9.5%), apparently explaining the clinicopathological aggressiveness of this tumor at the molecular level. (Am J Pathol 1997, 150:15–23)

Cells in tissues have only three options: they can grow and divide, remain dormant, or die by apoptosis. Tumors arise, therefore, either by inappropriate growth and division or by cells failing to die when they should.[1] Cell division results from DNA replication and mitosis, the two key cell-cycle events, the onsets of which are triggered by successive waves of cyclin-dependent protein kinases (CDKs), comprising a cyclin regulatory subunit and a Cdc2-family kinase subunit (reviewed in Refs. 2–4). Of the various cyclin/cdk complexes involved in cell-cycle regulation, cyclin D1/CDK4 and -6 and cyclin B1/Cdc2 are of particular interest because the former directs G1-S and the latter the G2-M checkpoint surveillance mechanisms, which are in turn essential for DNA synthesis and mitosis, respectively.[1–4] It is therefore reasonable to speculate that dysregulated expression of these cyclins and/or cdks may lead to disrupted cell growth control and tumor development.[4–6] Indeed, a number of breast cancer studies have reported overexpression of cyclin D1 mRNA or its protein product in tumor cell lines and pathological tissue specimens.[7–11] Moreover, a recent study using *in situ* hybridization[12] has shown that premalignant atypical ductal hyperplasia (ADH), a lesion

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with cytological and morphological features of early noninvasive carcinoma, but limited in extent,[13–16] expresses cyclin D1 mRNA at a level similar to benign breast lesions, such as typical ductal hyperplasia. This study indicated that ADH and breast cancer may thus be distinguishable on the basis of molecular assay.[12] In contrast to this accumulating knowledge regarding altered cyclin D1 expression in various breast lesions, only a few studies have reported cyclin B1 overexpression in pathological breast cancer specimens,[10,17] and any quantitative changes in its expression in a range of early breast diseases including carcinoma *in situ* remain unclear. Moreover, to our knowledge, there is no information concerning Cdc2 expression in the human breast and simultaneous monitoring of the expression of the two proteins in any human tissues. In this communication we show the immunohistochemical detection and quantitative analysis of cyclin B1 and Cdc2 expression in a wide spectrum of human breast diseases.

Materials and Methods

Formalin-fixed, paraffin-embedded surgical specimens, including portions of normal ductal and lobular units (n = 20); benign proliferative lesions (sclerosing adenosis, n = 50; typical ductal hyperplasia, n = 26); ADH lesions (n = 21); ductal carcinomas *in situ* (DCIS; n = 61; 37 noncomedo and 24 comedo types); lobular carcinomas *in situ* (LCIS; n = 9); and invasive carcinomas (n = 58; 40 ductal and 18 lobular types) were examined. The pathological diagnosis of each case was evaluated independently by two expert pathologists, and only those with an agreed diagnosis were enrolled in the study. The diagnosis of DCIS, noncomedo type, and ADH was based on the criteria of Page et al,[13,18,19] ie, noncomedo DCIS has a population of evenly spaced, uniform cells with uniformly oval to rounded nuclear features, comprising without doubt the entire population of cells throughout two basement membrane-bound spaces. ADH has histological and cytological features predominantly of the noncomedo DCIS but also some features of proliferative disease consisting of nonatypical cell populations. Three nonmalignant and two malignant frozen breast tissue samples were also surveyed for comparison of the immunostaining results. Three-micron sections or one-micron serial sections were placed onto saline-coated slides, deparaffinized, immersed in phosphate-buffered saline (PBS) with 0.3% (v/v) hydrogen peroxide, and subjected to microwave oven treatment (10 mmol/L sodium citrate buffer, pH 6.5, for 15 minutes at 700 W).[20] After blocking with 1% (w/v) bovine serum albumin in PBS containing 0.05% (v/v) Tween 20 for 30 minutes, the slides were incubated at 4°C overnight with mouse anti-human recombinant cyclin B1 monoclonal antibody (GNS1, IgG_1, 0.4 μg/ml) or mouse anti-*Xenopus* Cdc2 p34 monoclonal antibody known to react with the human homologue (17, IgG_{2a}, 0.2 μg/ml), both of which were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Immunostaining was performed using the streptavidin-biotin peroxidase complex method (LSAB universal kit, Dako, Carpinteria, CA). In negative controls, the antibodies were replaced by equivalent amounts of the subtype-matched normal mouse IgG. The final reaction product was visualized with either a 3-amino-9-ethylcarbozole solution (Dako) for immunolocalization analysis or 0.03% (w/v) 3,3′-diaminobenzidine tetrahydrochloride for digital image analysis. Frozen sections were fixed with 4% (w/v) paraformaldehyde in PBS for 15 minutes and processed exactly as described for the paraffin sections, except that microwaving was omitted. This resulted in comparable cyclin B1 and Cdc2 immunostaining to that in the paraffin sections, although their morphology was less well preserved.

Computerized static image analysis was carried out using a CAS 200 Image Analysis System in combination with the Quantitative Proliferation Index CAS Software Program (Becton Dickinson, Cell Analysis Systems, Elmhurst, IL), as described previously for tumor tissue sections.[21,22] Data obtained from this analysis were expressed as the proliferative index (PI), or the percentage of cells positively stained with the cyclin B1 or Cdc2 antibody relative to the total cell number. Ten random fields or more than 500 mammary cells were analyzed at a magnification of ×400. Care was taken not to include stromal cells in the cell counts. The automated mean cyclin B1 or Cdc2 level was scored as the representative $PI_{cyclin\ B1}$ or PI_{Cdc2}, respectively, for each specimen. Negative control sections were analyzed in each case to establish background immunostaining thresholds. The $PI_{cyclin\ B1}$ and PI_{Cdc2} values measured in each group of lesions were expressed as the mean ± SD, and the difference between these means was analyzed using the Mann-Whitney unpaired *U* test. The level of significance was set at $P < 0.05$.

Results

In all types of breast lesions, i.e., normal glands, benign changes (adenosis and typical ductal hyperplasia), ADH and *in situ* and invasive carcinomas,

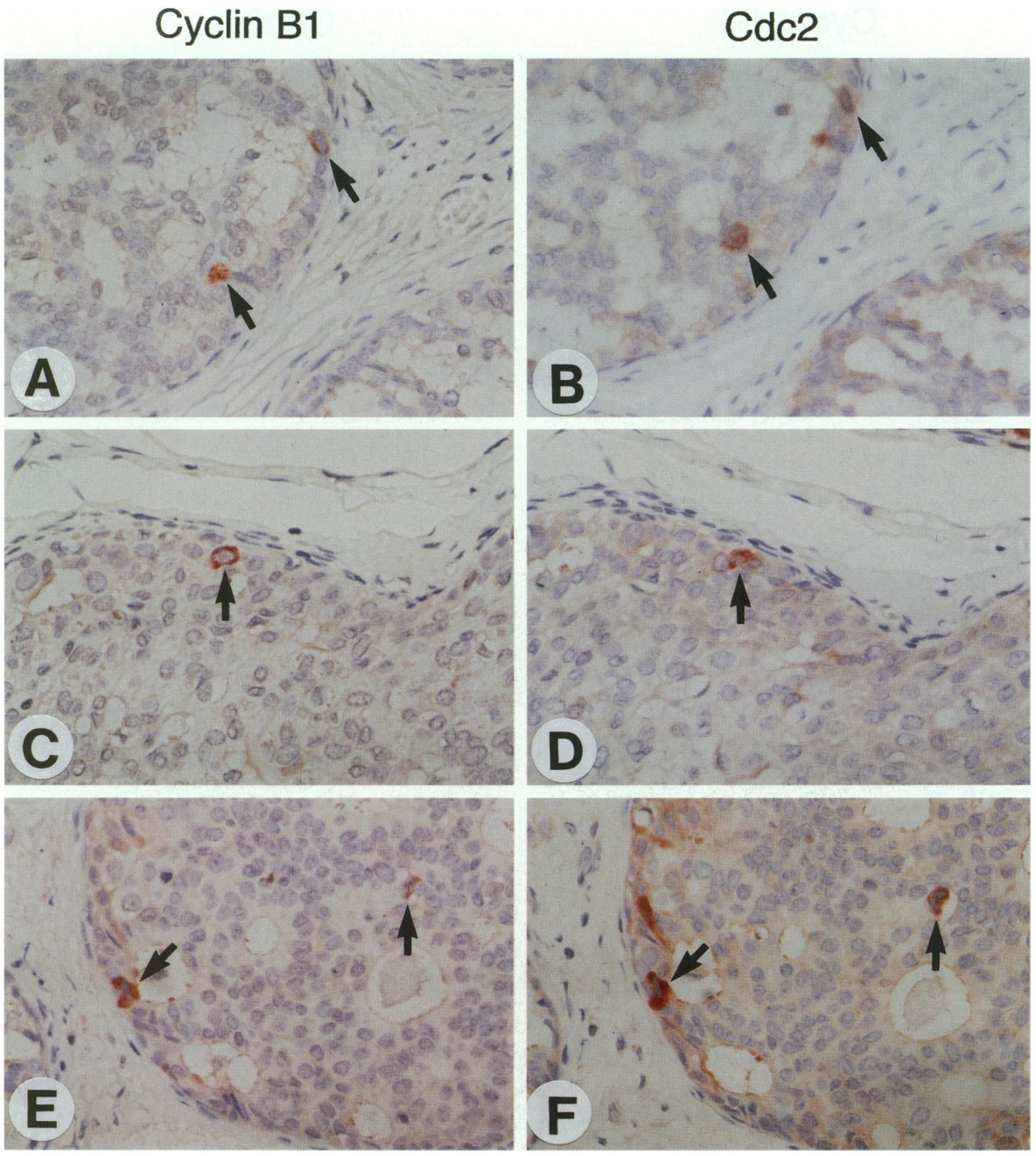

Figure 1. *Expression of cyclin B1 and Cdc2 in nonmalignant lesions and noninvasive carcinoma.* **A, C,** *and* **E:** *Cyclin B1.* **B, D,** *and* **F:** *Cdc2.* **A** *and* **B:** *Typical ductal hyperplasia.* **C** *and* **D:** *ADH. Cyclin B1 and Cdc2 are expressed by few breast epithelial cells in these lesions.* **Arrows** *indicate the mammary cells co-expressing the proteins. No cyclin B1 and Cdc2 immunostaining is present in the myoepithelium.* **E** *and* **F:** *Ductal carcinoma* in situ, *noncomedo type. The protein co-expressing cells are indicated by* **arrows.** *Serial sections; original magnification,* ×*400.*

cyclin B1 and Cdc2 immunostaining was detected in the cytoplasm and some nuclei of various numbers of luminal or proliferative epithelial cells. In every lesion, cyclin-B1-immunoreactive cells were less numerous than Cdc2-positive cells. The myoepithelium hardly showed immunoreactivity for the proteins (Figure 1). The most abundant immunostaining for both proteins was seen in invasive breast carcinomas (Figure 2; see PI data below). Cyclin-B1-positive cells co-expressed Cdc2, but not *vice versa*, in all breast lesions, as revealed by immunostaining in serial sections (Figures 1 and 2). As it was known that cells *in vitro* expressed Cdc2 in all but the G0 phase and cyclin B1 after the G2 phase,[2,3,23,24] this

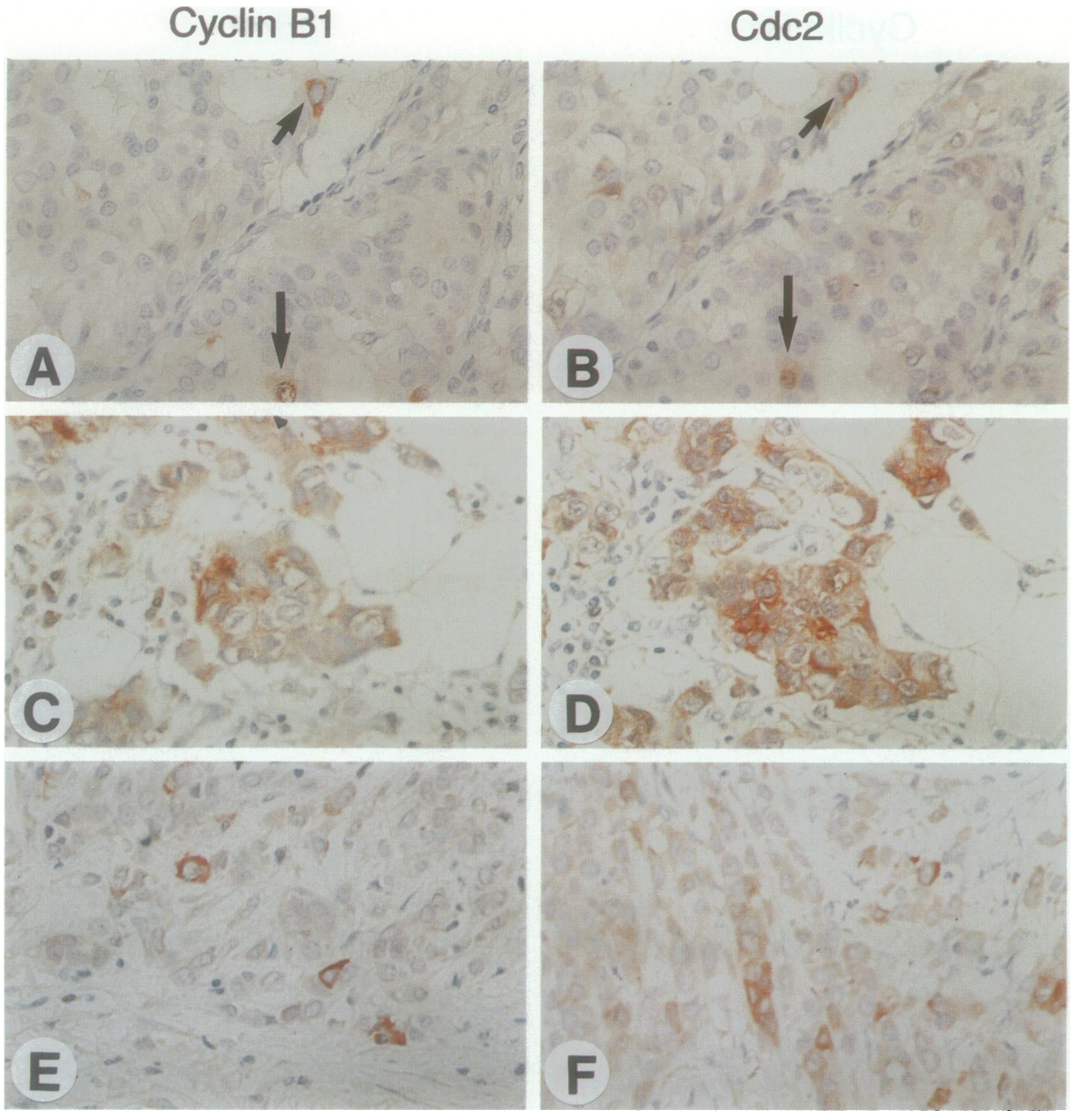

Figure 2. *Expression of cyclin B1 and Cdc2 in invasive breast carcinomas.* **A, C,** *and* **E:** *Cyclin B1.* **B, D,** *and* **F:** *Cdc2.* **A** *and* **B:** *Invasive ductal carcinoma. Some cancer cells co-express Cdc2 B1 and Cdc2 in the cytoplasm* (**short arrow**) *and in both the cytoplasm and nuclei* (**long arrow**). *Serial sections.* **C** *to* **F:** *Scattered portions of invasive ductal* (**C** *and* **D**) *and lobular* (**E** *and* **F**) *carcinomas. Intense immunostaining for cyclin B1 and Cdc2 is seen in cancer cell clusters diffusely infiltrating the adipose tissue or stroma. Magnification,* ×*400.*

suggested that the mammary cells co-expressing the proteins were at G2 or, if mitotic, M phase, whereas those solely expressing Cdc2 were at the pre-G2 (G1 or S) phase. To analyze precisely the intercellular localization of cyclin B1 and Cdc2 at various cell-cycle phases, immunohistochemistry with and without hematoxylin counterstaining was then performed on five specimens of each type of breast lesion, including normal glands. This analysis clearly demonstrated the occurrence of cell-cycle-dependent translocation and disappearance of the protein(s) in every tested tissue with the frequency depending on the proliferative activity of each lesion. Figure 3 shows the representative results obtained for an invasive ductal carcinoma. Whereas nonmitotic cells thought to be in the G2 phase co-expressed cyclin B1 and Cdc2 in the cytoplasm, those at mitotic prophase additionally exhibited intense nuclear staining for both proteins. The same immunolocalization of cyclin B1 and Cdc2 was observed in cells at prometaphase and metaphase, except that their cytoplasmic staining was weaker. In anaphase

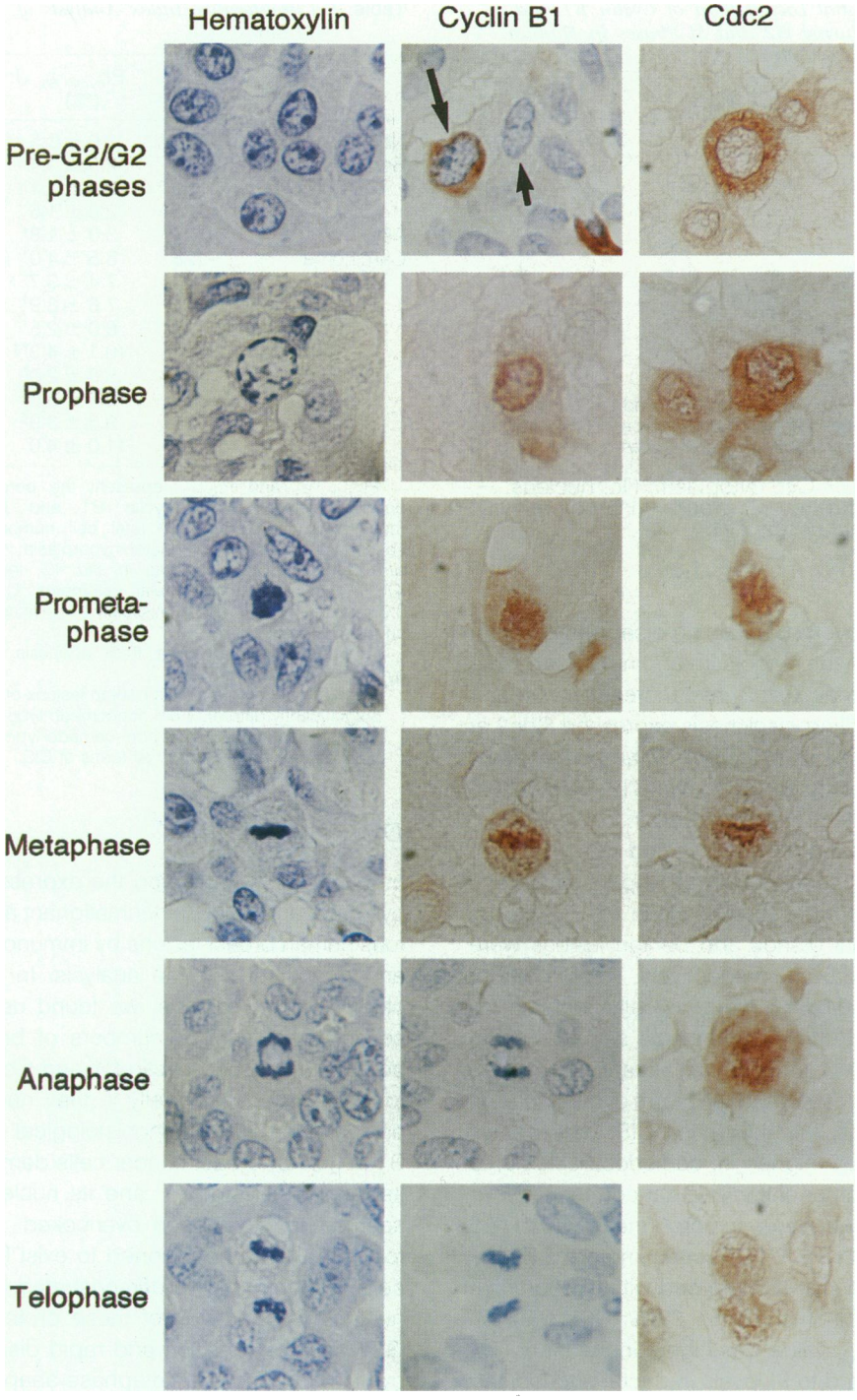

Figure 3. *Differential localization of cyclin B1 and Cdc2 during the cell cycle.* **Left:** *Hematoxylin staining.* **Middle:** *Cyclin B1.* **Right:** *Cdc2. Cyclin B1 immunoreactivity is detected strongly in the cytoplasm of G2-phase mammary cell* (**long arrow**) *but not in pre-G2-phase one* (**short arrow**). *Cdc2 constantly exists during these phases in the cytoplasm and nucleus. Both proteins are additionally detected in the nucleus during mitotic prophase, prometaphase, and metaphase, although their cytoplasmic staining is weaker in these periods. Cyclin B1 immunostaining has completely disappeared from mammary cells in anaphase and telophase, whereas Cdc2 staining is still detectable in the nucleus and cytoplasm. There was no counterstaining except for cyclin B1 immunostaining during the protein-undetectable phases. Invasive ductal carcinoma; magnification, ×1000.*

Table 1. *Differential Localization of Cyclin B1 and Cdc2 during G2 and M Phases in Human Mammary Cells*

Phases	Cyclin B1		Cdc2	
	Cyt	Nc	Cyt	Nc
Pre-G2	−	−	++	+*
G2	++	−	++	+*
M				
Prophase	+	++	+	++
Prometaphase	+	++	+	++
Metaphase	+	++	+	++
Anaphase	−	−	+	+
Telophase	−	−	+	+
Cytokinesis	−	−	++	+*

Mammary cells co-expressing cyclin B1 and cdc2 and lacking mitotic nuclear changes were considered to be in G2 phase and those expressing only cdc2 in pre-G2 phases (see text). M subphases were determined on the basis of standard morphological criteria.[29] Cyt, cytoplasm; Nc, nucleus. −, no immunoreactivity; +, weak immunoreactivity; ++, strong immuno- reactivity.
*Speckled staining.

and telophase, cyclin B1 staining disappeared completely from both the cytoplasm and nuclei, whereas both showed weak Cdc2 immunoreactivity. Mammary cells exhibiting cytokinesis expressed Cdc2 at a level similar to those in the pre-G2 and G2 phases, although they totally lacked cyclin B1 immunostaining. These results are summarized in Table 1.

Next, we measured $PI_{cyclin\ B1}$ and PI_{Cdc2} in relation to the type of lesion using computerized static image analysis (Table 2). The mean $PI_{cyclin\ B1/Cdc2}$ values for normal glands and benign lesions were 2.0/2.5% and 2.5/5.8%, respectively. The PI data for ADH (mean, 3.0/6.6%) were virtually identical to those for typical ductal hyperplasia and sclerosing adenosis ($P > 0.1$) and were significantly lower than the $PI_{cyclin\ B1/Cdc2}$ values for any form of carcinoma *in situ* (mean overall, 7.4/14.0%). In DCIS, the PI values were significantly higher in comedocarcinoma, a presumed precursor of invasive cancer, than in low-grade, noncomedo-type tumors (mean, 10.1/17.8 *versus* 6.0/12.7%; $P < 0.001$). Intriguingly, the $PI_{cyclin\ B1}$ value for comedo DCIS agreed with that for invasive ductal carcinoma (mean, 9.5%; $P = 0.9$), although the PI_{Cdc2} value was higher in the latter (Table 2). With regard to PI levels in ductal and lobular-type tumors, comedo and both noncomedo and comedo-type DCIS showed significantly higher values for $PI_{cyclin\ B1}$ and PI_{Cdc2}, respectively, than LCIS, although their invasive forms did not show such a difference in PI data. There was no significant relationship between $PI_{cyclin\ B1/Cdc2}$ values and other pathological features of breast cancer, ie, tumor differentiation grade, tumor size, and nodal involvement (data not shown).

Table 2. *Proliferative Index Analysis of Human Breast Lesions*

	n	$PI_{cyclin\ B1}$ (%)	PI_{Cdc2} (%)
Normal	20	2.0 ± 1.5	2.5 ± 1.4
Benign	76	2.5 ± 1.4	5.8 ± 3.2
Adenosis	50	2.4 ± 1.4	5.6 ± 3.3
TDH	26	2.8 ± 1.6	6.1 ± 2.9
ADH	21	3.0 ± 1.8*	6.6 ± 2.9*
Carcinoma	128	8.5 ± 4.0†	18.0 ± 8.6†
CIS	70	7.4 ± 3.7†	14.0 ± 5.6†
DCIS	61	7.6 ± 3.9†	14.7 ± 5.5†
Noncomedo	37	6.0 ± 2.5†	12.7 ± 4.2†
Comedo	24	10.1 ± 4.3†‡	17.8 ± 5.9†‡
LCIS	9	5.8 ± 2.0†	9.3 ± 3.5†
IC	58	10.0 ± 3.9§	22.9 ± 9.2‖
IDC	40	9.5 ± 3.9§	23.5 ± 9.6‖
ILC	18	11.0 ± 4.0	21.4 ± 8.4

$PI_{cyclin\ B1}$ and PI_{Cdc2} represent the percentages of cells positively stained with cyclin B1 and Cdc2 antibodies, respectively, relative to the total cell number. n, number of specimens; TDH: typical ductal hyperplasia; CIS, carcinoma *in situ*; LCIS, lobular carcinoma *in situ*; IC, invasive carcinoma; IDC/ILC, invasive ductal/lobular carcinoma. Differences at $P < 0.05$ were considered statistically significant (Mann-Whitney unpaired *U* test).
*Not statistically different from adenosis or typical ductal hyperplasia ($P > 0.1$).
†Significantly different from benign lesions or ADH.
‡Significantly different from noncomedo type.
§Not significantly different from comedo-type DCIS ($P > 0.5$).
‖Significantly different from all forms of CIS.

Discussion

In this study, we clarified the expression profiles of cyclin B1 and Cdc2 in nonmalignant and carcinomatous human breast lesions by immunohistochemistry and quantitative image analysis. In all lesions, including normal glands, we found using thin serial sections that various numbers of breast epithelial cells co-expressed cyclin B1 and Cdc2 in their cytoplasm and occasionally in their nuclei (Figures 1 and 2). A recent immunohistological study of cyclin B1 in human breast cancer cells demonstrated only its cytoplasmic form,[10] and its nuclear localization seemed to have been overlooked, although both forms of Cdc2 are reported to exist in oral mucosa cells.[25] Moreover, we demonstrated a cytoplasm to nucleus translocation of these proteins during the G2-M phase transition and rapid disappearance of cyclin B1 during the metaphase-anaphase transition in mammary cells of all types of lesions examined (Figure 3). Similar relocation of cyclin B1,[26] Cdc2,[27] and the cyclin B1/Cdc2 complex[28] has been shown *in vitro*, and our data provide the first evidence of its *in vivo* occurrence in human cells. Our results appear to agree with the postulated kinetics of the cyclin B1/Cdc2 complex in proliferative cells,[28] that is, an inactive complex is present exclusively in the cytoplasm, in association with the cytoskeleton, in the G2

phase. Upon activation during the G2-M phase transition, part of the complex moves to the nucleus to condense the chromosomes and cause mitosis. Therefore, it is conceivable that our defined prophase mammary cells exhibiting nuclear translocation of the proteins included those in the G2-M transition phase, which is not a sharply defined stage by light microscopic observation.[29] The disappearance of cyclin B1 immunostaining during the metaphase-anaphase transition obviously represents the proteolytic breakdown of this protein, which involves Cdc2 dephosphorylation and is the mechanism leading to M phase termination.[30,31] The consistent presence of Cdc2 in the cytoplasm and nuclei of post-metaphase and pre-G2-phase mammary cells is in good agreement with its expression pattern in HeLa cells.[27]

Several findings were obtained by the PI analysis (Table 2). First, the mean $PI_{cyclin\ B1}$:PI_{Cdc2} ratio was approximately 1:2 in most of the lesions. Clearly, this reflects restriction of cyclin B1 expression in the G2 and M phases and of Cdc2 in all but the quiescent phases of the cell cycle.[23,24] Alternatively, the results may indicate that the number of cells in the G1 to S and G2 to M phases is maintained in an almost equal balance, and therefore cells undergoing G1 or G2 arrest[32,33] are rare in human breast tissue regardless of the type of lesion. Second, ADH exhibited similar $PI_{cyclin\ B1/Cdc2}$ values to those observed in benign proliferative lesions, and these PI data were significantly lower than those for any form of breast carcinoma. This implies that quantitative expression analysis of cyclin B1 and/or Cdc2 may allow the distinction of premalignant human breast lesion from breast carcinoma, although their histological characterization is sometimes quite difficult.[15,16] Weinstat-Saslow et al[12] proposed a similar possibility in a recent study of cyclin D1. Using *in situ* hybridization, they found that only 18% of patients with benign and ADH lesions overexpressed cyclin D1 mRNA, whereas ~80% of those with breast carcinomas did so. Intriguingly, they showed that cyclin A, another Cdc2 partner active at the G2-M checkpoint, did not show such clear differences in its expression pattern. Taken together, the fact that the benign and premalignant hyperplastic lesions exhibited almost identical PI values and the incidence of cyclin D1 overexpression[12] suggests that phenotypic differences between these types of lesion[34] may be attributable to nonproliferative effects (eg, cell dedifferentiation) caused by certain genetic abnormalities, such as oncogene activation.[35,36] If so, the postulated genetic disorder and subsequently occurring overexpression of the G1-S and G2-M checkpoint regulators may provide multistep hits in the process of breast ductal carcinogenesis. Third, there was a significant difference in $PI_{cyclin\ B1/Cdc2}$ values between well differentiated/noncomedo DCIS and high-grade/comedo DCIS, and furthermore, the latter showed a $PI_{cyclin\ B1}$ value almost identical to that for invasive ductal carcinoma (mean, 10.1% *versus* 9.5%). Again, the results are in accord with the reported cyclin D1 expression patterns in these cancerous lesions.[12] The fact that comedo-type DCIS strikingly overexpresses the central cyclins indicates that this tumor is already more advanced in terms of cell division activity. It is therefore reasonable to speculate that some minimal additional alteration, eg, protease production, occurring in comedo DCIS may easily result in progression to invasive cancer, which may explain the clinicopathological aggressiveness of this tumor. Finally, the mean $PI_{cyclin\ B1}$ for breast carcinomas overall showed a 4.3-fold increase over that for normal glands, which closely agrees with the relative increase in the ratio of cyclin D1 mRNA between these tissues revealed by Northern blot analysis.[7] Taking into consideration the very similar expression profiles of these cyclins in various breast lesions, we propose that the expression of cyclins B1 and D1 may be up-regulated by a common mechanism, such as transcriptional regulation, in human mammary cells.

In conclusion, the results of this study clarify the subcellular localization of cyclin B1 and Cdc2 in human mammary cells and their degrees of expression in a wide spectrum of breast diseases. The significant difference in $PI_{cyclin\ B1}$ and PI_{Cdc2} values between benign/premalignant lesions and breast carcinomas indicates a consequential role of cyclin B1 and Cdc2 overexpression in the malignant transformation of breast epithelial cells. We suggest that PI analysis, given the relative simplicity and reproducibility of immunohistochemical examination, might provide a practical approach for solving the diagnostic problems frequently encountered in surgical pathology of the breast.

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