Cyclin A and E2F1 Overexpression Correlate with Reduced Disease-free Survival in Node-negative Breast Cancer Patients

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Abstract. *Background: Available prognostic factors do not accurately identify node-negative breast cancer patients at high risk of disease recurrence and progression. Patients and Methods: Cyclin A and E2F1 expression levels were evaluated in 75 consecutive node-negative breast cancer patients with a median follow-up of 10 years. Both parameters were tested for correlation with all the available clinicopathological parameters and with the clinical evolution of the disease. Results: Cyclin A was overexpressed in 45.3% of patients and significantly related to large tumor size, high Ki67 and high E2F1 expression levels. No relationship was observed between cyclin A and tumor estrogen receptor (ER) status, grading or patient age. Seventeen patients relapsed within 5 years from diagnosis. Twelve (71%) of them showed cyclin A overexpression in comparison with 22 (38%) out of the 58 who did not relapse (p=0.02). Disease-free survival (DFS) was significantly shorter in patients with cyclin A-overexpressing tumors compared to non-overexpressing ones (p=0.01). DFS was also significantly longer in low vs. high Ki67 expression (p=0.003) and in low vs. high E2F1 expression (p=0.02). On multivariate analysis, the simultaneous high expression of all three parameters (cyclin A, Ki67 and E2F1) was a strong independent prognostic factor for shorter DFS (HR 13.4). Conclusion: These findings suggest that assessment of cyclin A and/or E2F1 expression levels, associated with Ki67, might be useful for a better prognostic evaluation of node-negative breast cancer patients and support the need for further studies to evaluate their suitability for use in the routine clinical management of these patients.*

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Key Words: Cyclin A, E2F1, Ki67, breast cancer, disease free survival.

Breast cancer is the leading type of cancer and the second leading cause of cancer death in women in the United States. Adjuvant treatments produce significant benefits in operable breast cancer (1). However, despite surgical resection and subsequent adjuvant therapy, about 20-25% of node-negative breast cancer patients still develop recurrence and/or distant metastases within ten years from diagnosis (1-3). Available risk factors, such as tumor size, estrogen receptor (ER) status, grading and age, often fail to clearly identify patients at high risk of disease recurrence and progression. Thus, the search for new molecular markers continues to be a hot topic in breast cancer research.

Perturbations in cell cycle control mechanisms are frequently reported in human neoplasms and the investigation of their prognostic significance is an interesting field of research. On these grounds, cyclin A seems to be a promising new molecular marker related to the human multistep tumorigenesis process. In mammalian cells, the cell cycle is tightly regulated by a family of kinases (cyclin-dependent kinases, Cdks) which are activated by different cyclins and in turn regulate cell cycle progression. Cyclin A first appears in late G1-phase, is expressed during S- and G2-phases, peaks in late G2 and is degraded when cells enter pro-metaphase. Cyclin A binds to Cdk2 in the G1 to S transition, in the S-phase as well, and with Cdc2 (Cdk1) in the G2/M-phase (4). The specific expression of cyclin A is essential to control the correct sequence of events that brings the cell through S-phase to the G2-M checkpoint (5). Given its role in controlling the DNA replication machinery and the G2-M checkpoint, cyclin A plays an important role in cell proliferation control and, probably, in cancer growth (6).

Overexpression of cyclin A accelerates pRb phosphorylation (7), promotes entry into the S-phase (8) and is associated with the ability of cells to grow in suspension (a classic feature of cancer cells) (9); in addition, the cyclin A/Cdk2 complex is a target of the adenovirus E1A oncoprotein (10). The overexpression of cyclin A has been found in various human tumors and cancer cell lines (7, 11). High levels of cyclin A have been associated with tumor size in colorectal and pancreatic cancer (12, 13) and with poor differentiation in thyroid carcinomas and pancreatic adenocarcinomas (13). Recently, an increased expression of cyclin A has been reported to correlate with poor prognosis in breast, colorectal and laryngeal squamous cell carcinomas and childhood acute lymphoblastic leukemia (12, 14-17).

E2F1 transcription factor plays a dual role in cell cycle control acting both as a pro-apoptotic agent (18) and enhancing the cell proliferation rate (19, 20). E2F1 positively regulates cyclin D1, E and A expression levels through a direct interaction with their promoters (21, 22) and plays a role in tumor development (23, 24).

In this study, the expression of cyclin A and E2F1 was evaluated in a group of operable node-negative breast cancer patients and their relationship with main biological and clinical parameters was investigated.

Patients and Methods

Patient characteristics and tissue samples. Tumor samples were obtained from 75 consecutive node-negative breast cancer patients who had undergone surgery at the Santa Chiara University-Hospital in Pisa (Italy) from 1989 to 1995. Patients receiving pre-operative chemotherapy or having a family history of breast cancer were excluded. Patients who had undergone quadrantectomy received complementary radiotherapy. Chemotherapy and/or hormonal therapy were administered according to individual risk factors. The percentage of patients who received chemotherapy, hormonal therapy or no therapy were similar in the relapsed and non-relapsed group. In particular, patients receiving chemotherapy were treated either with a combination of methotrexate, cyclophosphamide and 5-fluorouracil (CMF) or with an anthracycline-containing regimen at a similar percentage for both relapsed and non-relapsed patient groups. Hormonal treatment consisted mainly of tamoxifen with a small number of patients also being treated with progestins. Median age at diagnosis was 50 years (range 36 to 81 years) and, at the time of the present report, median follow-up was 10.6 years (range 9-15 years). Seventeen out of 75 patients experienced a disease relapse within 5 years from diagnosis. The selection of patients did not require approval by the Institutional Ethics Committee because the samples were coded and the names of the patients were not revealed. All available clinicopathological data were collected and stored in a database: age, tumor grade and stage (25), size, histotype and estrogen receptor (ER) status were considered.

Immunohistochemistry. Cyclin A, Ki67, ER and E2F1 expression was evaluated using immunohistochemistry (IHC). All analyses were performed on routinely processed, formalin-fixed and paraffin-embedded tissue samples, as previously described (26, 27). Briefly, representative tumor sections (3 μm) were deparaffinized, rehydrated and immunostained using antigen retrieval with a microwave technique at 750W for 15 min in 10 mM citrate buffer (pH 6). After cooling at room temperature, the endogenous peroxidase was blocked with 1% H_2O_2 in methanol for 5 min. Sections were incubated for 1 hour at room temperature with anti-human cyclin A (clone BF683, Pharmingen, USA), anti-human E2F1 (clone KH95/E2F, Pharmingen) and anti-estrogen receptor (ER ID5, DAKO, Glostrup, Denmark) monoclonal antibodies diluted 1:100 and anti-Ki67 (MIB-1, Immunotech, Marseille, France) monoclonal antibody diluted 1:200. After washing, sections were immunostained using the avidin-biotin-peroxidase complex method (Vectastain Elite ABC kit; Vector, Burlingame, CA, USA). The peroxidase activity was visualized using 0.05% 3,3-diaminobenzidine (Sigma Chemical Co., St Louis, MO, USA) and 0.2% H_2O_2. The slides were rinsed with PBS and counterstained with hematoxylin. Sections of positive mammary carcinoma were used as positive controls. Negative controls were obtained by omitting the primary antibodies from the procedure. Nuclear staining in the absence of cytoplasmic background coloration was considered positive. The immunostained nuclei were evaluated by two observers independently and the discrepant cases were jointly re-evaluated. A minimum of 1,000 cells were counted for each tumor and immunoreactivity was expressed as a percentage of stained cells on the total number of tumor cells. A value of 3% of positive cells, corresponding to the median values of cyclin A and E2F1, was used as cut-off point to distinguish high and low expressing tumors. A cut-off of 10% positive cells was used to identify ER-negative (ER–) and ER-positive (ER+), or high and low Ki67 expressing tumors. No suitable paraffin material was available for immunohistochemical analysis of Ki67 and E2F1 of two tumors, thus, 73 out of the 75 cases were stained for both parameters.

Statistical analysis. The relationship between cyclin A and other molecular and clinical parameters were assessed using contingency table methods and tested for significance using the Pearson's chi-square test. Mean values were compared using the Student's *t*-test. Disease-free survival (DFS) was defined as the interval between surgery and the first documented evidence of disease in the local-regional area, distant sites, contralateral breast or a combination of the above. Relapses were accurately assessed by clinical, radiological and, whenever feasible, histopathological examination. DFS curves were calculated using the Kaplan-Meier method and the log-rank test was used to compare survival curves. Statistical analyses were performed using the Statistical Package for Social Science rel. 8.00 (SPSS, Chicago, IL, USA). All tests were two-tailed and $p<0.05$ was considered to be significant.

Results

Expression of cyclin A, Ki67 and E2F1 were evaluated using immunohistochemistry in a series of 75 node-negative breast cancers. Median values of the percentage of positive cells were used as cut-off points to distinguish high and low expressing tumors.

Cyclin A expression ranged from 0% to 80% of immunostained nuclei with a median expression value of 3%. Using this value as cut-off, high expression of cyclin A

Table I. *Cyclin A expression in relation to clinical and molecular parameters in a series of node-negative breast cancer patients.*

	Total	Cyclin A expression		*p*-value
		Low (%)	High (%)	
Age (yr)				
≤50	29	16 (55.2)	13 (44.8)	n.s.
>50	46	25 (54.3)	21 (45.7)	
Tumor size*#				
pT1	48	39 (81.3)	9 (18.7)	0.0009
pT2	17	2 (11.8)	15 (88.2)	
Ki67 expression*				
High	35	4 (11.4)	31 (88.6)	0.0001
Low	39	36 (92.3)	3 (7.7)	
E2F1 expression*				
High	40	17 (42.5)	23 (57.5)	0.04
Low	33	23 (69.7)	10 (30.3)	
Grading*				
I	16	9 (56.2)	7 (43.8)	n.s.
II	32	17 (55.0)	15 (45.0)	
III	23	11 (45.5)	12 (54.5)	
ER expression				
Positive	55	33 (60.0)	20 (40.0)	n.s.
Negative	20	8 (40.0)	12 (60.0)	

n.s. = not significant.
*not all parameters were available for all cases.
#pT1 ≤2 cm, pT2 >2 cm.

Table II. *Relationship between disease-free survival (DFS) and various clinical and molecular parameters in a series of node negative breast cancer patients.*

	DFS (months) mean (95% CI)	*p*-value
Age (yr)		
≤50	144 (118-169)	n.s.
>50	117 (102-131)	
Tumor size#		
pT1	149 (132-165)	n.s.
pT2	106 (82-131)	
Cyclin A expression		
High	115 (95-136)	0.02
Low	161 (146-177)	
Ki67 expression		
High	110 (89-132)	0.004
Low	166 (152-179)	
E2F1 expression		
High	115 (95-135)	0.02
Low	165 (150-179)	
ER expression		
Positive	145 (129-162)	n.s.
Negative	132 (107-157)	

n.s. = not significant.
#pT1 ≤2 cm, pT2 >2 cm.

was observed in 34 out of 75 (45.3%) tumors in our series. No relationship was observed between cyclin A expression levels and age, ER status or tumor grading (Table I). Conversely, cyclin A expression was strongly related to tumor size: the mean value of the percentage of positive cells for cyclin A staining was 4.26±0.74 (mean±SE) and 16.0±5.03 in tumor ≤2 cm and in those larger than 2 cm, respectively; high levels of cyclin A were significantly more frequent in pT2 than in pT1 tumors ($p=0.0009$). Likewise, a similar relationship was found between Ki67 and tumor size: the mean value of the percentage of positive cells for Ki67 staining was 9.93±1.4 in tumor ≤2 cm and 28.9±6.9 in tumor larger than >2 cm ($p=0.0002$).

High levels of cyclin A were strongly related to high Ki67 expression. Thirty one (91.2%) out of the 34 tumors that overexpressed cyclin A also showed high Ki67 levels, and 36 (90%) out of 40 specimens that did not overexpress cyclin A showed Ki67 levels that were under the cut-off value ($p=0.0001$; $\chi^2=45.38$). The correlation between cyclin A and Ki67 was independent from tumor size and was observed also when tumors ≤ 2 cm ($p=0.0001$; $\chi^2=30.73$) or larger than 2 cm ($p=0.0098$; $\chi^2=6.67$) were considered separately. On the other hand, no relationship was observed between cyclin A and Ki67 when ER-positive or ER-negative tumors were analyzed separately (data not shown).

Cyclin A was also strongly related to E2F1 levels: twenty-three (69.7%) out of 33 cyclin A overexpressing tumors showed high levels of E2F1, and 23 (57.5%) out of 40 tumors displaying low levels of cyclin A also showed low levels of E2F1 ($p=0.04$). A significant correlation was also observed between Ki67 and E2F1: 73.53% of tumors (25 out of 40) expressed high levels of both E2F1 and Ki67 while 61.54% of tumors (24 out of 39) expressed low levels of both molecules ($p=0.0057$; $\chi^2=7.65$).

Seventeen (22.7%) out of the 75 patients relapsed within 5 years from initial diagnosis while 58 (77.3%) did not. Cyclin A was overexpressed in 12 out of 17 (71%) cases that recurred during the period of follow-up and only in 22 (38%) out of the 58 cases did not. Thus, recurrence was more frequent in high cyclin A expressing tumors and this difference was significant ($p=0.02$).

Moreover, a shorter mean length of DFS was significantly related to high cyclin A ($p=0.02$), high Ki67 ($p=0.004$) and high E2F1 ($p=0.02$) expression levels (Table II). As expected, DFS plots were significantly different in patients with high levels of cyclin A *versus* low expressing tumors ($p=0.012$ using the log-rank test) (Figure 1A). DFS analysis using the Kaplan-Meier method also showed a significant separation between tumors with high *vs.* low Ki67 expression ($p=0.003$ using

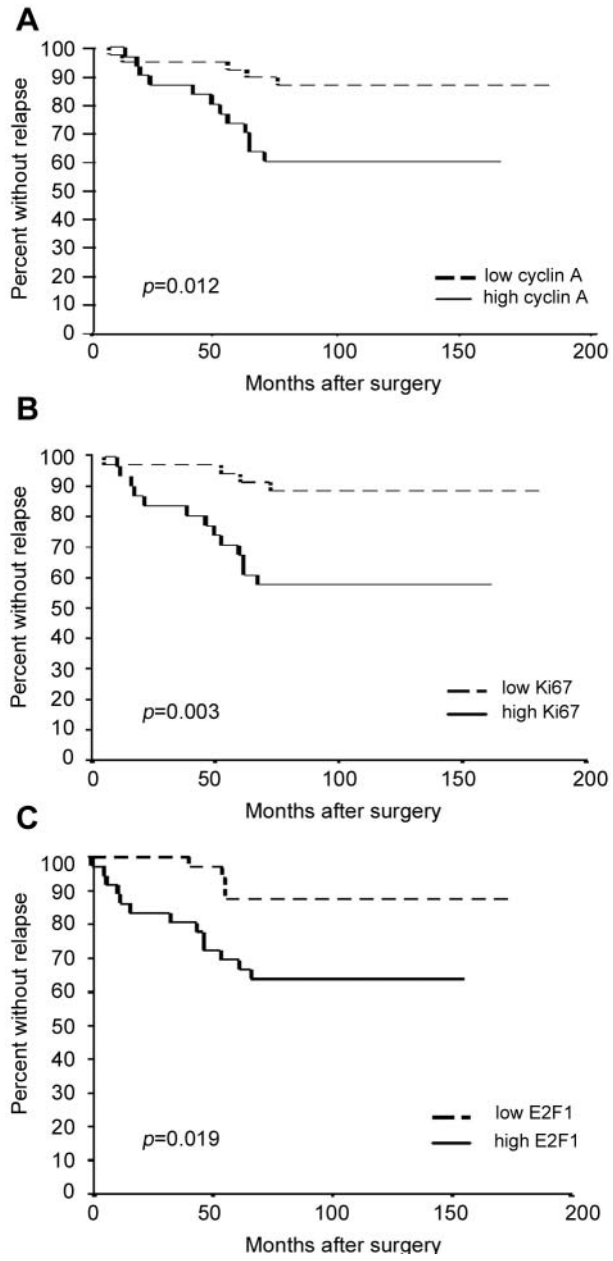

Figure 1. *Kaplan-Meier curves for disease-free survival in 73 node-negative breast cancer patients stratified according to cyclin A (A), Ki67 (B) and E2F1 (C) expression levels.*

the log-rank test) (Figure 1B) and between tumors with high *vs.* low expression of E2F1 ($p=0.019$ using the log-rank test) (Figure 1C).

The potential cooperative significance of these parameters was then evaluated and patients were stratified into four groups: high expressor tumors for all three parameters (cyclin A, E2F1 and Ki67), only two (Ki67 and E2F1), only one (Ki67) or none. Interestingly, 9 (52.9%), 4 (23.5%), 3 (17.6%) and only 1 (5.9%) out of 17 relapsing cancers fell in each of the groups, respectively. When all four groups were included in the analysis, the Kaplan-Meier curves of DFS also showed a significant separation ($p=0.029$ using the log-rank test) (Figure 2).

On multivariate analysis, high expression of cyclin A, Ki67 and E2F1 were not independent predictors of DFS when a Cox proportional hazard model was constructed including all available variables (data not shown). However, when a second model was built taking into account the simultaneous presence of two of the three markers (high cyclin A plus high Ki67, high cyclin A plus high E2F1 and high E2F1 plus Ki67) or all three (high levels of cyclin A, Ki67 and E2F1), a progressive rise in the hazard ratio (HR) for disease recurrence was observed in relation to the high expression from one to three of the analyzed parameters. In fact, the high expression level of only Ki67, which gave the best results compared to the other two parameters when considered alone, was associated with an HR of 4.86 ($p=0.17$, not significant); the simultaneous presence of high levels of cyclin A and Ki67 was associated with an HR of 4.19 ($p=0.038$); the contemporary overexpression of E2F1 and Ki67 was associated with an HR of 12.45 ($p=0.016$); and, finally, the simultaneous overexpression of all three parameters was associated with an HR of 13.42 ($p=0.01$). The simultaneous overexpression of E2F1 and cyclin A was not an independent predictor of shorter DFS (Table III).

Discussion

In this study, the expression levels of cyclin A, Ki67 and E2F1 were evaluated using immunohistochemistry in a series of node-negative breast cancers, and their relationship and potential prognostic significance, alone or in combination, were analyzed.

Cell proliferation markers are helpful in identifying tumors with aggressive biological behavior and could be useful prognostic markers of clinical outcome. Ki67 protein is a nuclear protein, of approximately 395 kDa, which is essential for cell proliferation (28). The functions and molecular targets of this protein are still unknown even if a possible role in DNA/RNA binding and ribosomal RNA processing (29, 30) or in centromeric and satellite DNA organization (31) has been suggested. Ki67 expression, detected with the widely used monoclonal antibody MIB-1, showed prognostic significance in various human neoplasms, including breast cancer; in the latter case, several studies have clearly demonstrated that high Ki67 expression levels are associated with reduced disease-free and overall survival (32). The relationship between high Ki67 expression and reduced DFS observed in our series of breast cancer patients is consistent with the data reported in the literature (Figure 1B).

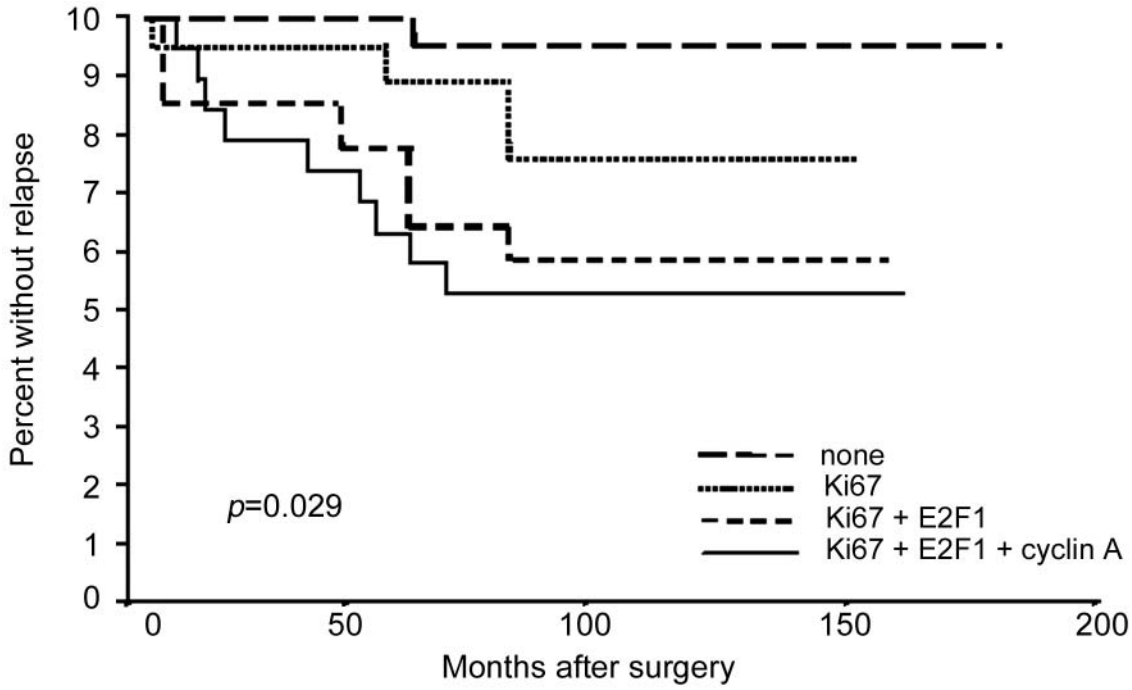

Figure 2. *Kaplan-Meier curves for disease-free survival in 73 node-negative breast cancer patients stratified according to the contemporary overexpression of three (Ki67, E2F1 and cyclin A), two (Ki67 and E2F1), one (Ki67), and none of the parameters analyzed.*

Cyclin A, synthesized during DNA replication and G2/M transition, promotes cell replication and its expression level may reflect the proliferative activity of tumor cells. Thus, the significant correlation observed in our series of node-negative breast cancer patients between cyclin A expression and Ki67 could be related to the role of cyclin A in controlling cell cycle progression. The cyclin A/Cdk2 active complex directly phosphorylates, among others, Rb family proteins (5, 6), thus, permitting the progression through the S-phase and the G2 to M transition. Although our results cannot confirm a causal link, they suggest that expression of Ki67 relates to the expression of cyclin A in breast cancer cells. They also suggest that increased cyclin A expression might be, in part, responsible for an increased proliferation rate of breast cancer although deregulation in other cell-cycle related molecules (*i.e.*, cyclin D1 and CDK inhibitors) cannot be excluded.

E2F1 transcription factor is an interesting cell-cycle regulator that can exert both a pro-apoptotic function (18) and a role in enhancing the cell proliferation rate by acting as an oncogene (19, 20). A pro-apoptotic role of E2F-1 is exerted by the induction of p53 (21, 33-35), a well-known negative regulator of cell cycle and pro-apoptotic agent. Positive effects on cell growth seem to be due to a positive loop between E2F1, cyclin D1, cyclin A and cyclin E although other mechanisms cannot be excluded (36). E2F binding sites have been found in both cyclin E and cyclin D1 promoters, and both promoters are activated by E2F gene products (21). Knock-out mice lacking E2F-1 displayed either tumor induction and tissue atrophy or defects in T-lymphocyte development and

Table III. *Contribution of various potential prognostic factors to disease-free survival by Cox regression analysis in human primary node-negative breast cancers.*

Variable	Hazard ratio	95% confidence interval	*p*-value
Age*	0.905	0.316 - 2.592	0.85
Tumor size#	1.713	0.580 - 5.059	0.33
ER status^	0.670	0.212 - 2.210	0.49
High levels of 3 parameters°	13.420	1.691 - 36.660	0.01
High levels of 2 parameters°			
Ki67 + E2F1	12.450	1.815 - 40.947	0.016
Ki67 + cyclin A	4.193	1.321 - 13.313	0.038
E2F1 + cyclin A	12.020	1.541 - 30.734	0.051
High levels of 1 parameter†	4.862	0.502 - 7.058	0.170

*the risk ratio is given as >50 yr *vs.* ≤50 yr.
#the risk ratio is given as pT2 *vs.* pT1.
^the risk ratio is given as positive vs. negative tumors.
°the risk ratio is given as overexpression of the indicated pair of parameters or all three together *vs.* all three negative.
†values calculated for tumors expressing high Ki67 expression (providing the best results among the three parameters considered alone) *versus* low expressing ones.

suppression of cell proliferation (23, 24). E2F-1 also positively regulates cyclin A. Dominant negative E2F mutants led to a decrease in either cyclin A expression levels or cyclin A-associated kinase activity (22). Effect on cyclin A expression seems to be due to the direct action of E2F-1 on cyclin A promoter (22). Given the role of E2F-1 in controlling cyclin A expression and activity, it was of interest to evaluate whether a relationship exists between the expression levels of these molecules in breast cancer cells. To our knowledge, this is the first study reporting an association between high expression levels of E2F-1 and cyclin A in human breast cancer. E2F-1, cyclin A and Ki67 can be considered as parts of the same pathway. From a theoretical point of view, in the initial steps of breast cancer development, high levels of E2F-1 may stimulate cell growth by enhancing, among other effects, the expression and activity of cyclin A and, consequently, of Ki67. The statistically significant relationship observed between E2F-1, cyclin A and Ki67 seems to support this hypothesis (Table I). Studies are ongoing to evaluate the expression levels of other G1 cyclins (D1 and E) and their correlation with E2F-1, cyclin A and proliferation index in the same series of node-negative breast cancer patients.

Seventeen out of 75 patients (22.6%) relapsed within 5 years from diagnosis in our series. These represent a very poor prognostic subgroup of node-negative breast cancer patients which is difficult to identify with the available prognostic markers. We observed that 71% of the patients who experienced an early relapse displayed cyclin A overexpressing tumors, while only 38% of those who remained disease-free showed the same pattern of expression (Figure 1A). This finding suggests that evaluation of cyclin A expression levels using immunostaining might help to identify node-negative breast cancer patients at high risk of recurrence and justifies further evaluation in a larger series of patients. Cyclin A overexpression, being strongly related to the proliferation index, could be used as an additional parameter to better identify tumors with aggressive biological behavior and to select node-negative breast cancer patients with very poor prognosis. As a matter of fact, in our series, when a multivariate analysis on DFS was performed, cyclin A, Ki67 and E2F1 did not seem to be independent predictors of outcome, but when two variables (cyclin A and Ki67 or E2F1 and Ki67) or all three variables were considered together, a very strong relationship with clinical behavior emerged (Table III).

In conclusion, although further studies are needed to confirm the link between cyclin A, E2F1, the proliferation index and prognosis in breast cancer patients, the results of the present study suggest that the simultaneous assessment of cell cycle-related markers (such as cyclin A and E2F1) together with the Ki67 proliferation index might help to identify node-negative breast cancer patients who are at high risk of recurrence and who might benefit from a selective aggressive adjuvant treatment.

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Reeceived May 23, 2006
Revised September 5, 2006
Accepted September 13, 2006