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# Immunohistochemical Evaluation of Cyclin D1 in Breast Cancer

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**Aim** To explore the potential prognostic value of cyclin D1 in invasive breast cancer and its correlation with basic histopathological parameters, hormonal status (estrogen [ER] and progesterone receptor [PR]), and bcl-2.

**Methods** Medical records of 48 patients, diagnosed in 1998, from the Central Database of the Institute of Oncology, Clinical Center University of Sarajevo, were analyzed. The mean follow-up was 61 months (range: 4-103 months). Routine histopathological evaluation was performed for 48 formalin-fixed and paraffin-embedded tissue samples. For immunohistochemical staining, we used monoclonal antibodies for ER, PR, bcl-2, and cyclin D1.

**Results** Cyclin D1 expression inversely correlated with tumor grade ($P=0.010$) and tumor size ($P=0.023$), whereas significant positive association was found with ER ($P=0.001$) and bcl-2 ($P=0.001$) expression. Patients with higher cyclin D1 expression had longer both overall survival ($P=0.014$) and relapse-free survival ($P=0.037$). Cox regression analysis for overall survival (OS) showed that lymph node status, ER expression, therapy, and cyclin D1 expression were independent prognostic factors. ($P$ range from 0.003 to 0.04).

**Conclusion** Expression of cyclin D1 is associated with better disease outcome in breast cancer.

The cyclins are a family of key regulatory proteins that govern the progression of human cells through critical transition points in the cell division cycle. Several classes of cyclins have been identified, displaying sequential expression in different phases of the cell cycle. The D-type of cyclins control the passage through the G1 phase enabling the entry into S phase.

Cyclin D1, the member of D-type cyclins, the key cell cycle regulatory protein, is one of the most commonly expressed oncogenes in breast cancer. It is not still used as a routine prognostic tool in breast cancer, although it has shown its prognostic value in several studies (1-3). It has a pivotal role in the regulation of progression from the G1 to S phase of the cell cycle through the formation of active enzyme complexes with cyclin-dependent kinases Cdk4 and Cdk6. These kinases phosphorylate substrates – retinoblastoma gene product, pRb, and related proteins of the family, p107 and p130, resulting in E2F activation which enables G1/S progression. These activities are named Cdk-dependent activities (1). Cyclin D1 can also form potentially functional interactions with many other molecules independently of the association with cdk4 and cdk6, including estrogen receptor (ER), androgen receptor, DMP1, STAT3, BETA2/NeuroD, C/EBPβ, as well as both histone acetylases and deacetylases (4,5).

The protein cyclin D1 is overexpressed in up to 50% of primary breast cancers, whereas the amplification of gene for cyclin D1 (CCND1)

located on 11q13 chromosome was found in approximately 15% of all cases (4).

Up to now, the prognostic value of cyclin D1 protein has been controversial, with studies reporting both a positive and negative role in breast cancer, whereas amplification of the CCND1 gene is predominantly related with worse outcome in estrogen (ER) positive patients (4).

Our study aimed to clarify the putative prognostic value of cyclin D1 in invasive breast cancer by immunohistochemical staining of cyclin D1 expression.

## Material and Methods

### *Patient Selection*

The biopsy specimens from 48 patients with invasive breast cancer diagnosed at the Department of Pathology, University of Sarajevo School of Medicine, Bosnia and Herzegovina, from January to December 1998 were selected for this study. Clinical data were collected from the Institute of Oncology, University Clinical Center, Sarajevo, Bosnia and Herzegovina. The median follow up time was 67 months (range 4-103). The last follow-up data were obtained in November 2004. All clinicopathological data together with clinical outcome are summarized according to the treatment arm in Table 1.

Breast cancer specimens were reviewed using morphologic and immunohistochemical criteria according to the WHO classification of breast cancer (10).

### *Immunohistochemical Staining*

Formalin-fixed, paraffin-embedded tissue samples were cut at 5 μm, dried overnight at 60°C, and deparaffinized in xylene. Subsequently, sections were rehydrated through graded alcohols into water. Heat-induced epitope retrieval was achieved by boiling sections in an EDTA buffer, pH 8.9 in a microwave oven (Electrolux, Stockholm, Sweden) at 1,000 W for 20 minutes (4 times 5 minutes each). After boiling, sections were permitted to cool at room temperature for 20 minutes, rinsed thoroughly with water, and placed in TRIS-buffered saline (TBS) for 5 minutes. Endogenous peroxidase was blocked with Peroxidase Block solution (provided in the EnVision kit, DakoCytomation, Glostrup, Denmark) for 5 minutes, and slides were rinsed or washed with TBS. Sections were incubated for 30 minutes with primary antibodies, including antibodies anti-cyclin D1 (clone P2D11F11, dilution 1:200, Novocastra Laboratories, Newcastle upon Tyne, UK), anti-ER (clone 1D5, dilution 1:20, DAKO), anti-PR (clone PgR 636, dilution 1:20, DAKO), and-bcl-2 (clone 124-BCL-2, dilution 1:20, DAKO).

The cyclin D1 protein is located in the cellular nucleus of the cells and the stainability was semiquantitatively estimated based on the es-

**Table 1.** Clinical, histopathological, and immunohistochemical data of 48 patients with breast cancer

| Characteristic | Number of patients (%) |
|---|---|
| Age (median, range) | 53 (30-80) |
| Premenopausal | 13 (27) |
| Postmenopausal | 35 (73) |
| Tumor type: | |
| ductal | 29 (60) |
| lobular | 9 (19) |
| other | 10 (21) |
| Tumor size: | |
| <2 cm | 14 (29) |
| 2-5 cm | 23 (48) |
| >5 cm | 8 (17) |
| missing data | 3 (6) |
| Grade:* | |
| 1 | 17 (36) |
| 2 | 16 (33) |
| 3 | 15 (31) |
| Lymph nodes: | |
| negative | 20 (41) |
| 1-3 positive | 17 (35) |
| 4-9 positive | 5 (10) |
| >10 positive | 4 (8) |
| missing data | 2 (8) |
| Overall survival: | 4-103 months (61.17)* |
| no evidence of disease | 31 (65) |
| alive with disease | 5 (10) |
| died of disease | 9 (19) |
| died of other disease | 3 (6) |
| Therapy: | |
| Tamoxifen | 20 (42) |
| chemotherapy | 21 (44) |
| radiotherapy | 7 (15) |
| Cyclin D1:† | |
| 1 | 13 (27) |
| 2 | 21 (44) |
| 3 | 14 (29) |
| Estrogen receptor:‡ | |
| 0 | 10 (21) |
| 1 | 6 (13) |
| 2 | 16 (33) |
| 3 | 16 (33) |
| Progesterone receptor:‡ | |
| 0 | 11 (23) |
| 1 | 10 (21) |
| 2 | 3 (6) |
| 3 | 24 (50) |
| Bcl-2:§ | |
| 0 | 13 (27) |
| 1 | 5 (11) |
| 2 | 4 (8) |
| 3 | 26 (54) |

*According to the WHO classification of breast cancer (10).
†Staining intensity: 1 – <10%, 2 – 10-50%, 3 – >50% of positive cells.
‡Staining intensity: 0 – 0-50 cells, 1 – 51-100 cells, 2 – 101-200 cells, 3 – 201-300 cells with positive staining.
§Staining intensity: 0 – <10%, 1 – 10-20%, 2 – 21-50%, 3 – >50% of positive cells.

timation of the percentage of positive tumor cell nuclei. The stainability was scored as 1 – weakly (<10% positive cells), 2 – moderate (10-50%), and 3 – strong (50-100%). Staining for ER and PR was evaluated semiquantitatively using the H score system (0=negative [0-50 cells], 1=mild reactivity [51-100], 2=moderate ([101-200], and 3=strong reactivity [201-300]) according to the method described by McCarty et al (11). Bcl-2 expression was scored semiquantitatively: score 0 (0-10% of positive cells), score 1 (10-20%), score 2 (21-50%), and score 3 (>50%) (Fig. 1).

### *Statistical Analysis*

The association among the intensity of expression with grade, lymph node status, and tumor size was studied with linear-by-linear association test.

Correlation between cyclin D1, ER, PR, and bcl-2 was analyzed by linear-by-linear association test. For overall survival and relapse-free survival, we used Kaplan Meier test. For multivariate analysis, Cox proportional hazard regression model was used to examine all factors found to be prognostic of survival in univariate analysis simultaneously.

Statistical significance was established at the $P<0.05$ level. Analyses were performed with Statistical Package for Social Sciences, Version 11.5 (SPSS, Inc., Chicago, IL, USA).

## Results

Characteristics of 48 patients with breast cancer are shown in Table 1.

Grade 1 correlated with longer both overall survival and relapse-free survival ($P=0.007$ and $P=0.041$, respectively, Kaplan Meier test).

Thirty one patients (65%) were alive without evidence of disease, 5 (10%) were alive with disease, whereas 12 (25%) died of the disease or other causes.

### *Cyclin D1 Expression*

Thirteen out of the 48 samples (27%) of breast cancer showed weak staining (score 1), 21

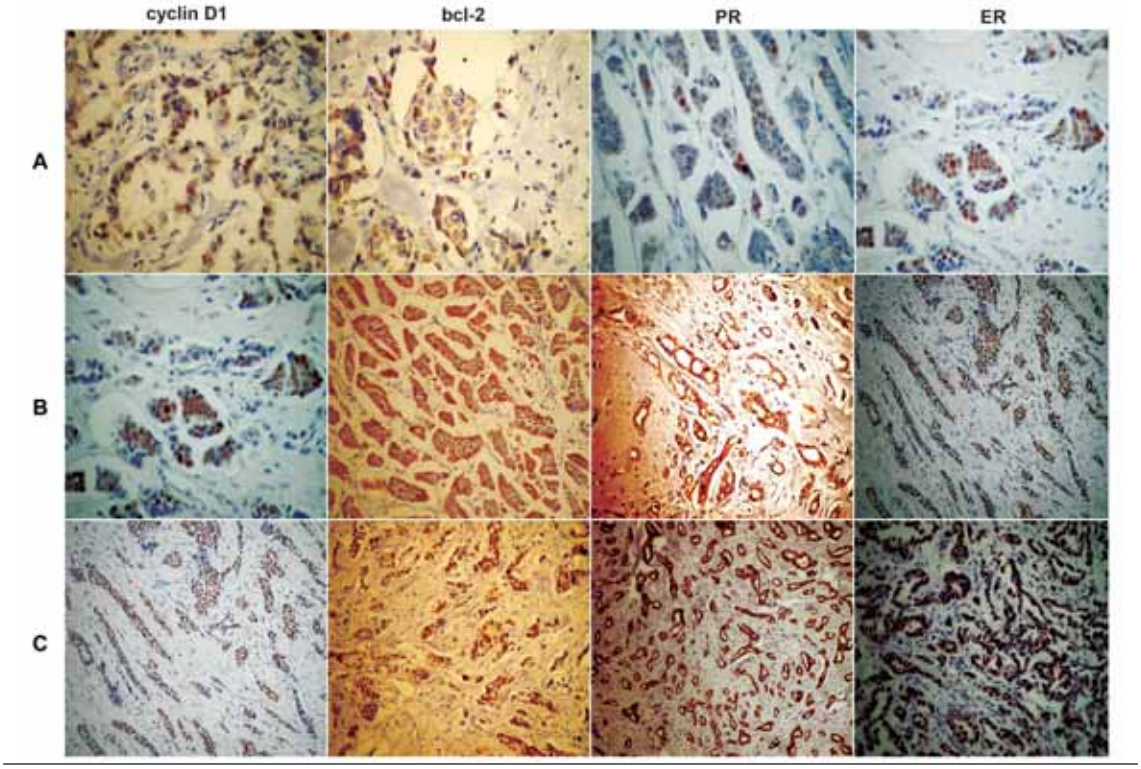


**Figure 1.** Results of immunohistochemical staining for cyclin D1, bcl-2, progesterone receptor (PR), and estrogen receptor (ER) in breast carcinomas with different expression (**A** – weak [1+], **B** – moderate [2+], **C** – strong [3+] expression, hematoxylin and eosin, ×400 magnification; EnVision+method for cyclin D1, ER, and PR ×200 magnification).

(44%) moderate (score 2), and 14 (29%) strong immunohistochemical staining (score 3). Patients with higher cyclin D1 expression had longer both overall survival and relapse-free survival ($P=0.014$ and $P=0.037$, respectively, Kaplan-Meier test) (Fig. 2 and 3). Survival analysis based on a combination of cyclin D1 and estrogen expression revealed that patients who were both ER and cyclin D1 positive had the best prognosis (Fig. 4).

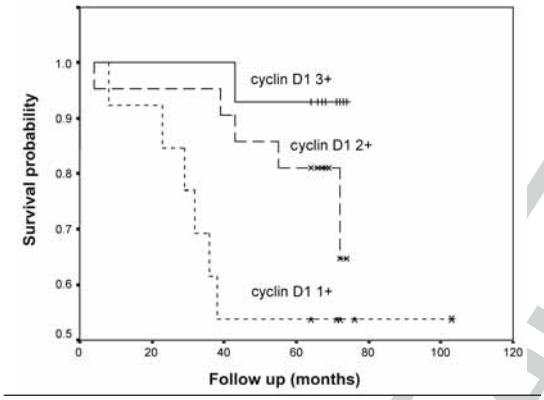


**Figure 2.** Overexpression of cyclin D1 correlates with longer overall survival ($P=0.014$, Kaplan-Meier test).

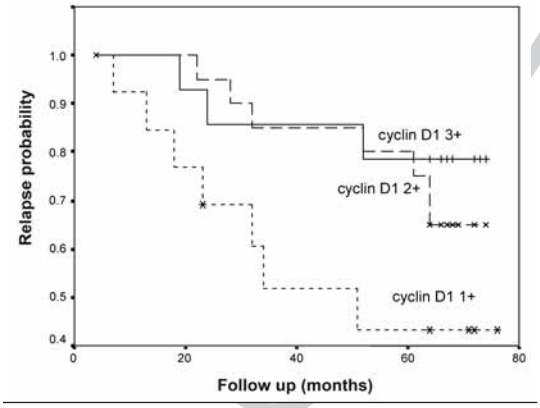


**Figure 3.** Overexpression of cyclin D1 correlates with longer relapse free survival ($P=0.037$, Kaplan-Meier test).

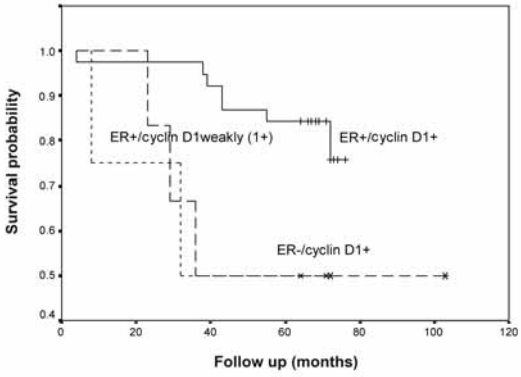


**Figure 4.** Survival analysis performed on combination of cyclin D1 and ER expression showed statistically significant correlation ($P=0.016$, Kaplan-Meier test).

### *Correlation among Expression of Cyclin D1, Estrogen Receptor, and bcl-2*

Cyclin D1 expression was positively associated with ER ($P=0.001$) and bcl-2 ($P=0.001$) expression (Table 2).

### *Correlation with Other Clinical and Pathological Parameters*

Univariate analysis revealed significantly inverse correlation between cyclin D1 and tumor size and grade. ($P=0.023$, 0.010, respectively, linear-by-linear association) (Tables 2 and 3). Other parameters did not show statistically significant correlation with cyclin D1. Additionally, there were negative correlations between ER and PR expressions and tumor grade ($P<0.001$ and $P=0.003$, respectively, linear-by-linear association; Table 3).

We confirmed the prognostic value of tumor size, grade, lymph node status, and cyclin D1 for both overall survival and relapse-free survival whereas ER, PR, and bcl-2 showed prognostic value only for overall survival (Table 4).

Cox multivariate analysis for overall survival showed that lymph node status, ER expres-

**Table 2.** Correlation among cyclin D1, bcl-2, ER expression, and tumor size

| Percent cyclin D1 expression | No. (%) of patients | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Bcl-2 expression† | | | | estrogen receptor expression‡ | | | | tumor size | | |
| | 0 | 1 | 2 | 3 | 0 | 1 | 2 | 3 | <2 cm | 2-5 cm | >5 cm |
| 0-10 | 7 (54) | 2 (40) | 1 (25) | 3 (12) | 8 (80) | 2 (33) | 3 (19) | 0 | 1 (7) | 5 (22) | 5 (63) |
| 11-50 | 6 (46) | 2 (40) | 1 (25) | 12 (46) | 1 (10) | 3 (50) | 6 (38) | 11 (69) | 8 (57) | 11 (48) | 2 (25) |
| 51-100 | 0 | 1 (20) | 2 (50) | 11 (42) | 1 (10) | 1 (17) | 7 (44) | 5 (31) | 5 (36) | 7 (30) | 1 (12) |
| *P** | 0.001 | | | | 0.001 | | | | 0.023 | | |

*Linear-by-linear association test.
†Staining intensity: 1 – <10%, 2 – 10-50%, 3 – >50% of positive cells.
‡Staining intensity: 0 – 0-50 cells, 1 – 51-100 cells, 2 – 101-200 cells, 3 – 201-300 cells with positive staining.

**Table 3.** Correlation among cyclin D1, estrogen and progesterone receptor expression, and tumor grade

| Tumor grade* | No. of patients | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cyclin D1‡ | | | | estrogen receptor expression§ | | | | progesterone receptor expression§ | | | |
| | 0 | 1 | 2 | 3 | 0 | 1 | 2 | 3 | 0 | 1 | 2 | 3 |
| 1 | 0 | 1 (6) | 11 (65) | 5 (29) | 0 | 1 (6) | 7 (41) | 9 (53) | 0 | 4 (23) | 2 (12) | 11 (65) |
| 2 | 0 | 3 (19) | 6 (38) | 7 (43) | 2 (12) | 3 (19) | 8 (50) | 3 (19) | 4 (25) | 2 (13) | 1 (6) | 9 (56) |
| 3 | 0 | 9 (60) | 4 (27) | 2 (13) | 8 (53) | 2 (13) | 1 (7) | 4 (27) | 7 (46) | 0 | 4 (27) | 4 (27) |
| *P*† | 0.010 | | | | <0.001 | | | | 0.003 | | | |

*According to the WHO classification of breast cancer (10).
†Linear-by-linear association test.
‡Staining intensity: 0 – 0-50 cells, 1 – 51-100 cells, 2 – 101-200 cells, 3 – 201-300 cells with positive staining.
§Staining intensity: 0 – <10%, 1 – 10-20%, 2 – 21-50%, 3 – >50% of positive cells.

**Table 4.** Univariate and multivariate analysis of effects of patients and tumor parameters on overall survival and relapse free survival in 48 patients with breast cancer

| Parameter | Log rank (*P*)* | |
|---|---|---|
| | overall survival | relapse free survival |
| Age | 0.21 (0.649) | 2.45 (0.117) |
| Tumor type | 0.04 (0.839) | 3.22 (0.073) |
| Tumor size | 10.01 (0.001) | 6.27 (0.012) |
| Grade | 7.33 (0.007) | 4.20 (0.040) |
| Lymph node | 9.74 (0.002) | 9.23 (0.002) |
| Therapy | 3.36 (0.069) | 1.17 (0.280) |
| ER expression | 4.75 (0.029) | 2.84 (0.092) |
| PR expression | 5.43 (0.019) | 3.24 (0.072) |
| Bcl-2 | 5.12 (0.023) | 2.20 (0.138) |
| Cyclin D1 | 5.99 (0.014) | 5.64 (0.037) |

*Kaplan-Meier test.

**Table 5.** Cox proportional hazard regression analysis of overall survival predictors in patients with breast cancer

| Parameter | Overall survival | | |
|---|---|---|---|
| | hazard ratio | 95 % CI* | *P* |
| Age | 1.100 | 0.982-1.232 | 0.100 |
| Tumor type | 0.463 | 0.119-1.808 | 0.268 |
| Tumor size | 1.020 | 0.975-1.066 | 0.393 |
| Grade | 1.527 | 0.278-8.392 | 0.626 |
| Lymph node | 1.627 | 1.118-2.366 | 0.011 |
| Therapy | 0.770 | 0.598-0.991 | 0.043 |
| ER expression | 1.022 | 1.002-1.044 | 0.033 |
| PR expression | 0.991 | 0.978-1.004 | 0.161 |
| Bcl-2 | 0.790 | 0.373-1.674 | 0.539 |
| Cyclin D1 | 0.018 | 0.001-0.256 | 0.003 |

*CI – confidence interval.

sion, therapy and cyclin D1 expression were independent prognostic factors (Table 5).

## Discussion

Our study demonstrated high expression of cyclin D1 in 73% of the samples with the 2 or 3 expression score. This is in line with some other studies (14). However, there is a wide range of cyclin D1 expression in breast cancer, varying between 35-81% with an average of 50% (4,15).

Up to now, the prognostic value of cyclin D1 expression on disease outcome has been controversial, with studies reporting both positive and negative findings (4).

In our study, cyclin D1 expression was correlated with longer patient survival in general and longer relapse-free survival. This is in concordance with other studies (15-17), although there are studies with opposite results (18). We emphasize that cyclin D1 can have diverse effects, not only those related to the cell cycle machinery and cell progression, depending on its level of expression, specific cell type, and other factors (19,20).

Recently published studies revealed potential functions of cyclin D1 other than those related to cell cycle machinery and proliferation, that is, oncogenic effects. Cyclin D1 may be related to antitumor effects such as the induction of apoptosis, cellular senescence, and cellular growth inhibition (21). In our study, cyclin D1 probably did not show proapoptotic activities because there was a positive correlation between cyclin D1 and bcl-2, a well-defined antiapoptotic regulatory protein. Indeed, under experimental conditions, bcl-2 protein might act as inducer of cyclin D1 activity in human breast epithelial cells, independent of cell anchorage (21). This fact makes cyclin D1 activities more complex since deregulation of apoptosis is a hallmark of cancer (22). A recent study delineated a potential mechanism by which signal pathways that drive cyclin D1 overexpression also influence apoptosis. AKT (protein kinase B) activation leads to cell proliferation by inhibition of glycogen synthase kinase 3 (GSK3) and prevents GSK3-dependent proteolysis of cyclin D1, thereby enabling cell cycle progression via cyclin D1. AKT can induce expression of antiapoptotic protein bcl-2 and inactivate proapoptotic Bad protein (23). This may partially explain positive correlation between cyclin D1 and bcl-2 in our study.

No positive correlation was found in our study between cyclin D1 expression on one side and tumor grade and tumor size on the other. This confirms the results of other studies, which could

not find any positive correlation between cyclin D1 and proliferative markers such as Ki-67 and S-phase fraction (16,24-27). This is also in agreement with studies in which cyclin D1 was predominantly expressed in well-differentiated, low-grade, and slow growing breast cancers (4,15,16,26, 27). This clearly indicates other functions of cyclin D1 which are not related to cell cycle progression and tumor aggressiveness, which may include cell differentiation and growth arrest via p21 induction (26). In some other tumors, like lung cancer and colorectal cancer, expression of cyclin D1 correlated with a worse outcome and a positive correlation with proliferative markers was found. This indicates that cyclin D1 activities might be not only diverse but also tissue specific (28).

We also found a positive correlation between cyclin D1 and ER expression which has already been explained in both experimental and clinical studies, because ER acts as the main mitogen stimulator in breast cancer via cyclin D1 (29).Taken together with correlation of cyclin D1 with tumor size and grade, we assume that ER-cyclin D1 axis might represent a distinct proliferative pathway during breast cancer development comparing with those followed by Ki-67 or other proliferative markers expression. A recently published study explained the mechanism of estrogen interplay with the cyclin D1 gene (CCND1) (30). This partially explains why specific endocrine treatment with tamoxifen has been successful in many breast cancer patients because a high percentage of breast tumors are actually ER positive (4,31). Indeed, the best prognostic group in our study consisted of the patients which were both ER and cyclin D1 positive.

However, recently published studies pointed out cyclin D1 as a putative culprit for acquired resistance to antiestrogen therapy (tamoxifen) in some ER positive breast cancer patients, even though cyclin D1 itself correlated with better outcome in untreated group of postmenopausal female patients (17). These results were confirmed in experimental studies (32).

Finally, we found cyclin D1 to be an independent prognostic factor in Cox multivariate analysis. This fact has already been shown in some other studies (1), thus providing evidence for its use in routine diagnostic evaluation of breast cancer according to proposals suggested by the College of American Pathologists (2).

According to our results, cyclin D1 is a good prognostic factor in invasive breast cancer whose expression is associated with better patient outcome. Further molecular studies are necessary to clarify putative interactions between the regulators of apoptosis and cell cycle regulators like cyclin D1, as well as different signaling pathways involved in mitotic activity and proliferation in tumor cells.

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