Diagnostic role of cyclin D1 as a new marker for early diagnosis of breast cancer

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Breast cancer is the most frequent cancer in women; in Egypt it affects 37.7% of all women and accounts for 29.1% of their cancer-related mortality. Cyclin D1 protein overexpression is found in up to 50% of breast cancers. The aim of this study is to study the correlations between cyclin D1 level and stages of breast cancer (TNM staging) and to study the correlations between cyclin D1 and routine markers used in breast cancer [cancer antigen 15-3 (CA15-3) and carcinoembryonic antigen (CEA)]. This study was performed on 80 female breast cancer patients. Ten healthy women served as controls. The patients were referred from the Assiut University Hospital and South Egypt Cancer Institute. None of the healthy women in the control group had elevated cyclin D1 level above the cutoff value. Elevated levels of cyclin D1 was detected in 80, 90, 95, and 100% of patients in groups II, III, IV, and V, respectively. There was a significant positive correlation between cyclin D1 positivity with CEA and CA15-3 concentrations. The combination of CA15-3, CEA, and cyclin D1 resulted in the highest sensitivity (95.2%), highest specificity (100%), and highest diagnostic accuracy (96%). The cyclin D1 level in samples obtained from Egyptian women with breast cancer is a good marker for the detection of breast cancer, and in the detection of metastasis as it correlates with the clinical staging of the disease. A combination of CA15-3, CEA, and cyclin D1 may be used as a panel for the diagnosis of metastasis among those patients.

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Introduction

Breast cancer is the most frequent cancer in women affecting ~6% of all women. It constitutes almost 20% of all malignancies in women. Despite recent advances in early diagnostic and treatment strategies, breast cancer is still a leading cause of cancer-related deaths among women, with as many as 40% relapsing with metastatic disease [1]. In Egypt, breast cancer affects 37.7% of all women and accounts for 29.1% of their cancer-related mortality with a total of 6546 deaths. Breast cancer may originate either from the ducts, known as ductal carcinomas, or from the lobules, known as lobular carcinomas. There are many different types of breast cancer, with different stages (spread), aggressiveness, and genetic makeup [2].

Carcinoembryonic antigen (CEA) and cancer antigen 15-3 (CA15-3) are the commonly used markers for breast cancer. However, they lack sensitivity and specificity, are rarely elevated prior to gross disease, and are not seen in many patients with metastases [3]. Cancer cells produce CEA in large amounts, but it can also be found in the blood of healthy people. It showed less sensitivity than CA15-3 in both early and advanced breast cancers [4]. CA15-3 lacks sensitivity for early-stage disease combined with a lack of specificity for the early diagnosis of breast cancer. CA15-3 concentrations are increased in 10% of patients with stage I disease, 20% with stage II disease, 40% with stage III disease, and 75% with stage IV disease [5]. The cell cycle is a tightly regulated process, which involves coordinated actions of several proteins, mainly the cyclin-dependent kinases (CDKs) and cyclin proteins [6]. The progression from G1 to S is a critical checkpoint in protecting the cell from abnormal replication, and a key regulator of this process is the cyclin D-CDK 4/6-INK4-Rb pathway [7], upon stimulation of a quiescent cell in G0 by growth factors, the cell enters G1 (gap 1) with the expression of cyclin D1 which promotes the formation of cyclin D1-CDK 4/6 complexes, which in turn phosphorylates its downstream target Rb (which is a canonical tumor suppressor gene in retinoblastoma and in many other cancers as well) [8].

CCND1 is a well-established human oncogene: a recent census concluded that there was substantial evidence for the involvement of *CCND1* amplification and overexpression in breast cancer [9]. The aim of this study was to study the correlations between cyclin D1 level and different stages of breast cancer (TNM staging) and to study the correlations between cyclin D1 as a new marker and routine markers used in breast cancer (CA15-3 and CEA).

Patients and methods

This study included 80 female breast cancer patients aged 25–65 years. Ten age-matched women served as controls. The patients were referred from the General Surgery Department, Assiut University Hospital, South Egypt Cancer Institute over a period of 1 year duration from January 2016 to January 2017. Formal consent was obtained from both patients and controls. The study was approved by the Ethics Committee of Faculty of Medicine, Assiut University. Female patients with any other type of malignant or benign tumors and those with past history of chemotherapy or surgical treatment of cancer were excluded from the study. Staging of breast cancer patients was done according to the American Joint Committee of Cancer staging system and the TNM staging system [10].

Patients and controls were grouped as follows:

Group 1: included 10 controls.
Group 2: included 20 patients with stage I breast cancer.
Group 3: included 20 patients with stage II breast cancer.
Group 4: included 20 patients with stage III breast cancer.
Group 5: included 20 patients with stage IV breast cancer.

Sample collection, storage, and handling

A total of 8 ml of venous blood was collected; 2 ml into an EDTA containing tube for complete blood count, 2 ml into a sodium citrate containing tube for prothrombin time and concentration, and 4 ml into a plain tube without anticoagulants.

The blood was allowed to clot for 15 min at 37°C and serum was separated by centrifugation at 3000 rpm for 10 min. The collected serum was inspected to ensure it was clear and nonhemolyzed or lipemic and then was divided into three aliquots: one for kidney functions, random blood sugar, and liver functions; another for CEA and CA15-3; and the third was stored at –20°C until assay of cyclin D1 level was performed.

Routine investigations

Random blood sugar, serum urea, serum creatinine, and liver functions were done on Dimension RxL Max Integrated Chemistry System (Siemens, Munich Germany). Prothrombin time and concentration were done on Sysmex CA-1500 System (Siemens) [11]. Complete blood count was done on CELL-DYN 3700 (Abbott, New Cairo, Egypt).

Special investigations

Quantitative measurement of CEA and the CA15-3 levels in serum was done using solid-phase, chemiluminescent immunometric assay on Immulite 1000 analyzer (Siemens Medical Solutions Diagnostic Limited, Henkestr, Erlangen, Germany). Normal range for the CEA is 0.11–5.09 ng/ml (cat. no. 363238) and for the CA15-3 is 6.4–58 IU/ml (cat. no. 249755).

Measurement of cyclin D level

Test principle

Purified cyclin D1 is allowed to coat onto a microtiter plate to make solid-phase antibodies. Samples or standards are added to wells with a labeled antibody specific to cyclin D1, then labeled horseradish peroxidase is added to the wells. After washing completely, tetramethylbenzidine substrate solution is added. Tetramethylbenzidine substrate becomes blue color in wells that contain the antibody–antigen–enzyme–antibody complex. Reaction is terminated by the addition of a solution and the color change measured at a wavelength of 450 nm. The concentration of cyclin D1 in the samples is then determined by comparing the optic density of the samples to the standard curve.

Preparation of samples

The samples were diluted 1: 5 in a sample dilution buffer before use (40 μl sample dilution + 10 μl testing sample).

Test procedure

All reagents and samples were stored at room temperature (15–30°C) and mixed well.

(1) The standard solution was diluted to make serial dilutions.
(2) Fifty microliter of the solution was added to wells from both standard and testing samples.
(3) The plate was covered by an adhesive strip and incubated at 37°C for 30 min.
(4) Wash solution was diluted 20-fold with distilled water.
(5) Washing: the adhesive strip was removed, fluid discarded, and the washing buffer was added to each well for 30 s and repeated five times.

(6) Fifty microliter of horseradish peroxidase-conjugate reagent was added to each well except the blank well.
(7) The plate was covered by an adhesive strip and incubated at 37°C for 30 min.
(8) The adhesive strip was removed, fluid discarded, and the washing buffer was added to each well for 30 s and repeated five times.
(9) Fifty microliter of chromogen solution A and B was added to each well for 15 min at 37°C and light was avoided.
(10) Fifty microliter of the stop solution was added to each well to stop the reaction (the blue color was changed to yellow color).

Statistical analysis

The data were tested for normality using the Anderson–Darling test and for homogeneity variances prior to further statistical analysis. Categorical variables were described by number and percent, where continuous variables described by mean and SD. χ^2 and Fisher's exact tests were used to compare between categorical variables where comparison was made between continuous variables by unpaired *t*-test. Pearson's correlation coefficient was used to assess the association between continuous variables. A two-tailed *P* value less than 0.05 was considered statistically significant. All analyses were performed with the IBM SPSS 20.0 software (Armonk, New York, USA).

Results

(1) CEA levels (Table 1).
(2) CA15-3 levels (Table 2).
(3) Cyclin D1 levels (Table 3 and Fig. 1).

Correlation coefficients between CEA and cyclin D1 levels (Table 4).

There is significant positive correlation between CEA and cyclin D1 (r = 0.202 and P = 0.042).

Correlation coefficients between CA15-3 and cyclin D1 levels (Table 5).

There is significant positive correlation between CA15-3 and cyclin D1 (r = 0.339 and P < 0.001) (Table 6).

Table 1 Percentage of cases with normal (≤cutoff) and high (≥cutoff) values of cancer antigen 15-3 in different studied groups

Carcinoembryonic antigen level	Normal [*n* (%)]	High [*n* (%)]
Group 1 (*n*=10)	10 (100)	0
Group 2 (*n*=20)	15 (75.0)	5 (25.0)
Group 3 (*n*=20)	16 (80.0)	4 (20.0)
Group 4 (*n*=20)	5 (25.0)	15 (75.0)
Group 5 (*n*=20)	5 (25.0)	15 (75.0)

Table 2 Percentage of cases with normal (≤cutoff) and high (≥cutoff) values of cancer antigen 15-3 in different studied groups

Cancer antigen 15-3 level	Normal [*n* (%)]	High [*n* (%)]
Group 1 (*n*=10)	10 (100.00)	0
Group 2 (*n*=20)	10 (50.0)	10 (50.0)
Group 3 (*n*=20)	7 (35.0)	13 (65.0)
Group 4 (*n*=20)	3 (15.0)	17 (85.0)
Group 5 (*n*=20)	1 (5.0)	19 (95.0)

Table 3 Number and percentage of cases with normal (≤cutoff) and high (>cutoff) values of cyclin D1 in different studied groups

Cyclin D1 level	Normal [*n* (%)]	High [*n* (%)]
Group 1 (*n*=10)	10 (100.0)	0
Group 2 (*n*=20)	4 (20.0)	16 (80.0)
Group 3 (*n*=20)	2 (10.0)	18 (90.0)
Group 4 (*n*=20)	1 (5.0)	19 (95.0)
Group 5 (*n*=20)	0	20 (100.0)

Table 4 Correlation between carcinoembryonic antigen and cyclin D1

Test	*r*	*P*
Carcinoembryonic antigen and cyclin D1	0.202	0.042*

Pearson's correlation. *Mild statistical significant difference.
**P*<0.05, moderate statistically significant difference .

Table 5 Correlation coefficients between cancer antigen 15-3 and cyclin D1 levels

Test	*r*	*P*
Cancer antigen 15-3 and cyclin D	0.339	0.002**

Pearson's Correlation, **Moderate statistically signficant difference.

Discussion

Breast cancer is the most frequent cancer in women. Its incidence is high and constantly increasing. It constitutes almost 20% of all malignancies in women. Despite recent advances in early diagnostics and treatment strategies, breast cancer is still a leading cause of cancer-related death among women, with as many as 40% relapsing with metastatic disease [1]. Cyclin D1 overexpression has been shown to correlate with early cancer onset and tumor progression, and it can lead to oncogenesis by increasing anchorage-independent growth and angiogenesis via VEGF production [12].

This study was performed on 90 female individuals who were divided into five groups: 10 healthy women as a control group (group 1), 20 patients with stage I (group 2), 20 patients with stage II (group 3), 20 patients with stage III (group 4), and 20 patients with stage IV (group 5).

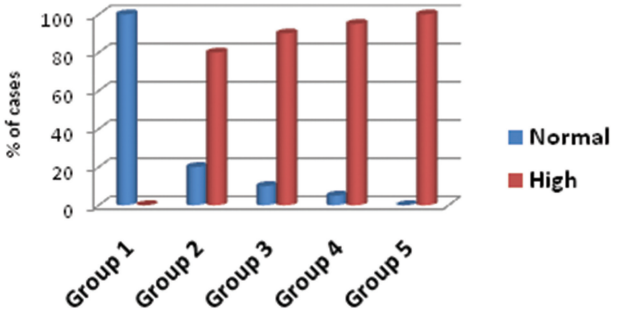
None of the patients gave any history of risk factors (smoking, alcohol consumption, and hormonal replacement therapy). Twenty-three patients have

Table 6 Sensitivity, specificity, and diagnostic accuracy of the markers in all patients' groups

Items	Sensitivity	Specificity	Diagnostic accuracy
CA15-3+CEA	94	95	94
CA15-3+cyclin D1	88.8	100	91
CEA+cyclin D1	63.5	100	71
CA15-3+CEA+cyclin D1	95.2	100	96

CA, cancer antigen; CEA, carcinoembryonic antigen.

Figure 1



The percentage of cases with normal (≤cutoff) and high (>cutoff) values of Cyclin D1 in different studied groups.

positive family history, seven patients in group 2, eight patients in group 3, 10 patients in group 4, and eight patients in group 5.

In this study, we found that there was high statistically significant difference regarding age in a comparison between control group (group 1) and patients' groups (groups 2:5) with a *P* value of 0.000.

Our results are consistent with DeSantis *et al.* [13], who reported increased incidence of breast cancer by increasing age. Also, our results are consistent with Nelson *et al.* [14].

In this study, none of the healthy women in the control group (group 1) (0.0%) had elevated CEA above the cutoff value which was 5.07 ng/ml, but in group 2 (stage I breast cancer) 25% of the patients had elevated levels of CEA, in group 3 (stage II breast cancer) 20% of the patients had elevated levels of CEA, in group 4 (stage III breast cancer) and in group 5 (stage IV breast cancer) 75% of the patients had elevated levels of CEA. Our results are consistent with Guadagni *et al.* [15], who reported that elevated CEA levels were found in 16% of stage I patients, 22% of stage II patients, and 67.5% of stage IV patients. But in stage III, our results are inconsistent with Guadagni *et al.* [15], who reported that elevated CEA levels were found in 37.3% of stage III patients (the cutoff value was 5 ng/ml).

Our results are inconsistent with Gao *et al.* [16], who found that CEA was elevated in 4.7% of stage I female patients with breast cancer, 3.5% of stage II, 0% of stage III, and 38% in stage IV. This could be explained by that CEA can also be elevated in some noncancerous diseases, like cirrhosis, chronic kidney disease, chronic obstructive pulmonary disease, rheumatoid arthritis, and in otherwise healthy smokers [17].

In this study, none of the healthy women in the control group (group 1) (0.0%) had elevated CA15-3 levels above the cutoff value which was 30.09 IU/ml. However, in group 2 (stage I breast cancer) 50% of the patients had elevated levels of CA15-3. In group 3 (stage II breast cancer) 65% of the patients had elevated levels of CA15-3. In group 4 (stage III breast cancer), 85% of the patients had elevated levels of CA15-3. In group 5 (stage IV breast cancer), 95% of the patients had elevated levels of CA15-3. This is consistent with Dai *et al.* [18] who found that CA15-3 levels were elevated in 7.7% of stage I, 20.6% of stage II, 35.7% of stage III, and 63.5% in stage IV. In Dai's study the research was done on a large sample with unequal distribution between the four stages (52 cases in stage I, 131 cases in stage II, 56 cases in stage III, and eight cases in stage IV). This may explain why the percentage of the patients with elevated CA15-3 in his study was less than that in this study.

Higher levels of preoperative CA15-3 represents tumor burden, which is linked to the tumor size and lymph node metastasis and predicts poorer survival in breast cancer. As expected, distinctly higher tumor biomarker levels were noted in the tumor status and TNM staging, suggesting a relationship between high levels of CA15-3 and tumor load [18].

In this study, none of the healthy women in the control group (group 1) (0.0%) had elevated cyclin D1 levels above the cutoff value which was 9.5 ng/ml. However, in group 2 (stage I breast cancer), 80% of the patients had elevated levels of cyclin D1. In group 3 (stage II breast cancer) 90% of the patients had elevated levels of cyclin D1. In group 4 (stage III breast cancer) 95% of the patients had elevated levels of cyclin D1. In group 5 (stage IV breast cancer), 100% of the patients had elevated levels of cyclin D1. This is consistent with Ahlin *et al.* [19] who studied *CCND1* gene amplification in different grades of breast cancer; the gene was amplified in 7% in grade I, 43% in grade II, and 50% in grade III. Our results showed that there was significant positive correlation in a comparison between cyclin D1 positivity with CEA and CA15-3 concentrations.

Our results showed that the combination of CA15-3, CEA, and cyclin D1 resulted in the highest sensitivity (95.2%), the highest specificity (100%), and the highest diagnostic accuracy (96%).

Conclusion

We found that cyclin D1 levels in peripheral blood samples obtained from Egyptian female individuals with breast cancer are good markers for the detection of breast cancer. We also found that there is a positive correlation between the levels of cyclin D1 and the advancement of breast cancer represented by the increase in the clinical staging of the disease, so this marker can be used to aid in the detection of metastasis in patients with breast cancer. A combination of CA15-3, CEA, and cyclin D1 may be used as a panel for the diagnosis of metastasis among breast cancer patients.

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Conflicts of interest

There are no conflicts of interest.

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