Evaluation of Cyclin D1 expression by western blotting methods and immunohistochemistry in breast cancer patients

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Summary

Purpose: Considering that cyclin D1 had a prognostic and clinical value for breast cancer patients, adequate measurement of cyclin D1 is necessary.

Methods: In this investigation, we detect cyclin D1 expression in tumour and peritumoral tissue of breast cancer patients by Western blotting method and by immunohistochemistry.

Results: Cyclin D1 expression decreased significantly with each advanced clinical stage of disease and tumour size. Also, patients without lymph node involvement, with positive hormone receptors and Luminal A type of tumours had significantly increased the expression of cyclin D1. We show that cyclin D1 expression correlates with longer RFS in the entire group of patients, in the group of ER-positive and in the group of HER2-negative patients. Patients who were both ER and cyclin D1 positive had a better prognosis.

Conclusion: Taken together, our results showing correlation of cyclin D1 with clinical stage, tumour size and lymph nodes, suggest that cyclin D1 expression detected by Western blotting could be considered as an additional marker for the staging of breast cancer, as well as a marker for longer RFS and survival in ER-positive breast cancer patients.

Key words: cyclin D1, breast cancer patients, estrogen receptor, progression-free survival, overall survival

Introduction

Novel tumour markers which could predict and detect possible relapse of breast cancer disease are required in the treatment of this disease. Moreover, predictive markers are necessary for the selection of patients who should receive a certain type of treatment [1]. Considering that current adjuvant systemic therapies are not specific for all types of breast cancer, more specific molecular markers for the therapy and follow-up of breast cancer patients are needed [2,3].

The cyclins represent a class of proteins that are associated with the cell cycle. Cyclin D1 is a protein involved in the G1 phase of the cell cycle by helping the phosphorylation of retinoblastoma protein (pRb) [4]. The hypophosphorylated form of pRb blocks the growth of the cell. Therefore, phosphorylation of pRb is crucial for the cell cycle transition into the S-phase [5]. Estrogens can induce proliferation by activating cyclin D1 that phosphorlates Rb [6]. Moreover, it has been shown...
that estrogen receptors can be activated only by cyclin D1 independently from estrogen binding [7]. Also, the human epidermal growth factor receptor 2 (HER2) in breast cancer can be bound to the cyclin D1-cdk4/6 complex [8].

Even though it has been confirmed that cyclin D1 induces cell cycle progression and growth of breast cancer, the activation of cyclin D1 is associated with longer survival and a low rate of relapse of breast cancer patients. Moreover, 15-20% of breast cancer tumour tissue has amplified the cyclin D1 gene [9]. The increased expression of the cyclin D1 protein has been detected in 30-83% of breast cancer tissues by immunohistochemistry [10]. Importantly, breast cancer patients with overexpression of cyclin D1 show complete response to hormonal therapy [11].

Prognostic and predictive value of cyclin D1 in tumour tissue in breast cancer patients is contradictory due to different methods of detection [10-12]. Considering that cyclin D1 had a prognostic and clinical value for breast cancer patients, the adequate measurement of cyclin D1 is necessary. In a published study, it has been shown that immunohistochemistry is often an unreliable method due to the occurrence of equivocal results [13]. Consequently, we evaluated and analyzed the value of cyclin D1 expression by using Western blot technique and immunohistochemistry (IHC) in breast cancer patients.

Methods

The ethical committee of our hospital gave approval for publication of this study.

Patients

All patients provided signed informed consent before the study entry. The expression of cyclin D1 in the tumour and adjacent tissue samples of 80 early breast cancer patients by Western blot analysis and immunohistochemistry were investigated. Children were not included in the study. The study was performed at the Institute of Oncology and Radiology of Serbia.

Tissue samples

Homogenized fresh malignant and peritumoral breast tissue samples obtained immediately after surgery were treated with lysis buffer. Normal peritumoral tissue differs morphologically from tumour tissue under the microscope. The tissue sample was lysed for 30 min on ice in 200μl RIPA buffer containing Tris (pH 7.5 50mM), NaCl (150mM), NP-40 (1%), Na deoxycholate (0.5%), SDS (0.1%) and it was centrifuged. After centrifugation, the supernatant fluids were measured by Bradford assay [14]. Tissue lysates equivalent to 50 μg protein were mixed with loading buffer [15].

Western blot analysis

50μg of proteins per sample was diluted in loading buffer (Tris pH 6.8) and then denaturized by cooking at 100°C for 5 min and afterwards kept on ice for 10 min. The protein lysates were electrophoresed on 8% SDS-polyacrylamide (29:1 acrylamide/ bisacrylamide) gel at 100V and then electrotransferred (Trans-Blot Cell; Bio-Rad, Hercules, CA, USA) to the nitrocellulose membrane (Bio-Rad Laboratories, Hercules, USA) using 100V voltage for one h. The nitrocellulose membrane was blocked for 1 h with 5% non-fat dried milk in 1xTBST buffer (20mM Tris-HCl, pH 7.6, 137mM NaCl, 0.1% Tween 20).

The primary rabbit anti-human cyclin D1 antibody (Sigma-Aldrich, UK) was diluted 1:200 in TBST and the blots were incubated overnight at -4°C. The blots were washed 3 times in 1xTBST buffer and incubated with peroxidase-conjugated secondary antibody (Amersham, Arlington Heights, IL, USA) for 1 h at room temperature. After incubation, the blots were washed 3 times in 1xTBST buffer and protein bands were detected using enhanced chemiluminescence (ELC) detection system (Amersham, Arlington Heights, IL, USA). The expression of β actin was used for endogenous control for Western blot reaction and immunohistochemistry. The blots were analyzed with Gel Doc BioRad system using Multi-Analyyst 1.1 software.

Quantification of Cyclin D1 expression

Blots were scanned by the image system (Kodak Image 1D 3.6.) using the auto density characteristics on a scale ranging from 0 (clear) to 255 (opaque). The pixel density was used to count the integrated density of the specific band. Values of integrated density were presented in volume units of pixel intensity per mm². The integrated density of each band is presented as the mean of three different calculations of the same blot for each sample evaluated in triplicate.

Pathological assessment of primary tumours

This investigation involved 80 breast cancer patients with age range from 36 to 79 years (median 59) who underwent surgery as primary treatment. Tumour size (T) was stratified by two pathologists as T1, T2 or T3. Regional lymph node involvement (N) was classified as N₀ (lymph node negative) or N₁ (lymph node positive). The imaging methods were performed to rule out the presence of distant metastases (Mo). Breast cancer types were classified according to the WHO classification. Immunostaining was carried out on formalin-fixed paraffin-embedded 4μm tissue sections using the primary mouse monoclonal antibodies for estrogen receptor (ER), progesterone receptor (PR), HER2 receptor, Cytokeratin 5/6 (CK5/6) and epidermal growth factor receptor (EGFR/ HER1), respectively. Antibodies used for IHC staining were: anti-human ERα (clone SP1, 1:200 dilution; LabVision), anti-human PR (clone PgR 636, 1:500 dilution; Novocastra), anti-human HER2 (clone CB11, 1:800 dilution; Dako), anti-human Cytokeratin 5/6 (CK5/6, clone D5/16 B4, DAKO, 1:25, cytoplasmic) and EGFR/ HER1 (Rabbit MAb; Cell Signalling Technology; 4267, 1:50). Staining was visualized by the Envision method (Dako-
Cyclin D1 expression in breast cancer

Patients’ clinical and pathological characteristics were estimated and presented in Table 1. In our study the expression of cyclin D1 detected by Western blotting was found in 75% of all investigated patients with breast carcinoma (60 out of 80). The expression of cyclin D1 was detected by immunohistochemistry in 67.5% of patients (54 out of 80). The expression of cyclin D1 in tumour and adjacent tissue of breast cancer patients and the expression of β actin, as control, used in this study are shown in Figure 1A. Representative blots of cyclin D1 expression in breast cancer patients showed that cyclin D1 had a more intense band in tumour tissue when compared to normal adjacent tissue (Figure 1A). More detailed analyses of the mean values with standard deviation (mean±SD) of cyclin D1 expression showed a significant increase of cyclin D1 in tumour tissue compared to adjacent tissue (p=0.03, Mann–Whitney U test) (Figure 1A,B).

Cyclin D1 expression decreased significantly with each advanced clinical stage of disease (p=0.04, p<0.001, p=0.03). Analysis of the expression of cyclin D1 showed a significant increase in patients with clinical stage I compared to patients with clinical stage II (p=0.04, Mann–Whitney U test).

Figure 1. A: Representative blots showing cyclin D1 expression in tumour and adjacent tissue and the expression of β actin as control. B: The mean values with standard deviation (mean±SD) of cyclin D1 expression show a significant increase of cyclin D1 in tumour tissue compared to adjacent tissue. Values of integrated density were reported in volume units of pixel intensity per mm² (**p=0.03).
Patients with clinical stage I had a significantly higher expression compared to patients with clinical stage III (p<0.001, Mann–Whitney U test). Analysis of the expression of cyclin D1 showed a significant increase in patients with clinical stage II compared to patients with clinical stage III (p=0.04, Mann–Whitney U test) (Figure 2A). T2 tumours compared to T1 tumours had higher expression of cyclin D1 (T2 vs T1, 1.24±0.04 vs 1.53±0.06, p=0.001, Mann–Whitney U test). Also, T3 group compared to T2 and T1 tumours had higher cyclin D1 expression (T3 vs T1 p<0.0001; T3 vs T2 p=0.04, Mann–Whitney U test) (Figure 2B). Moreover, patients with N+ compared to patients with N0 showed significantly decreased cyclin D1 expression (p=0.002, Mann–Whitney U test) (Figure 2C).

Figure 2. Cyclin D1 expression is significantly decreased depending on the clinical stage. A: disease (**p<0.001) as well as on tumour size. B: (**p<0.0001, Mann-Whitney U test). C: Patients with N+ have significantly decreased cyclin D1 expression compared to patients with N0 (**p=0.002). D: Patients with ER+ have significantly higher cyclin D1 expression compared to patients with ER- (**p<0.0001, Mann–Whitney U test). The results are presented as mean values with standard deviation (mean±SD), and Mann–Whitney U test). The results are presented as mean values with standard deviation (mean±SD).

Figure 3. A: There was no statistically significant difference between cyclin D1 expression in HER-2 receptor positive breast cancer patients compared to negative ones in the tumor tissues (p=0.31, Mann–Whitney U test). B: The patients with Luminal A cancers had significantly higher cyclin D1 tumour expression (**p=0.015) compared to patients with basal-like cancers. The results are presented as mean values with standard deviation.
Cyclin D1 expression in tumour tissue with respect to receptor status

Cyclin D1 expression with respect to expression of ER, PR and HER2 in tumour tissue samples was analyzed. Cyclin D1 expression was significantly higher in breast cancer patients with ER+ compared to patients with ER- (1.13±0.04 vs 1.44±0.05, p=0.0001, Mann–Whitney U test) (Figure 2D). However, we found no significant difference in cyclin D1 expression between HER2+ (+2,+3) and HER2- (0,+1) patients (1.26±0.09 vs 1.36±0.05, p=0.39, Mann–Whitney U test) (Figure 3A).

Also, in this study we showed that patients with basal-like cancers had significantly lower cyclin D1 expression (1.12±0.05 vs. 1.44±0.05, p<0.001, Mann–Whitney U test) compared to patients with luminal A tumours (Figure 3B).

Survival analysis

When we analyzed all patients, those with the higher expression of cyclin D1 in tumour tissue had a significantly longer RFS than those with the low expression of cyclin D1 (p=0.003, Log-rank test) (Figure 4A). Moreover, in the group of ER-positive patients, patients with the higher expression of cyclin D1 in tumour tissue had a significantly longer RFS than those with the low expression of cyclin D1 (p=0.001, Log-rank test) (Figure 4B). Also, when patients with negative HER2 receptors were analyzed, those with the higher expression of cyclin D1 in tumour tissue had a significantly longer RFS than those with the low expression of cyclin D1 (p=0.003, Log-rank test) (Figure 4C).

Furthermore, when we analyzed all patients, there was no significant difference in OS between the group with the high expression of cyclin D1 and the group with the low expression of cyclin D1 (p=0.056, Log-rank test) (Figure 5A). In the group of all investigated patients, the survival rate at 5 years was 87.7% (75.3-100%). In the group of HER2+ patients, there was no difference in the patient prognosis between the group with the high expression of cyclin D1 and the group with the low expression of cyclin D1 (p=0.003, Log-rank test) (Figure 4C).

Figure 4. A: No significant difference was found in OS between patients with high cyclin D1 and the low cyclin D1 expression. (Log-rank test, p=0.056). B: ER+ patients with high expression of cyclin D1 showed a significantly longer OS than those with low expression of cyclin D1 (p=0.003, Log-rank test). C: A representative photomicrograph of the breast cancer patients displaying a carcinoma with strong nuclear expression of cyclin D1 (HE staining, ×400). D: A Representative photomicrograph of breast cancer patient with low expression of cyclin D1 (HE staining, ×400).
expression of cyclin D1 (p=0.55, Log-rank test). In the group of HER2-positive patients, the survival rate at 5 years was 93.7% (87.0-100%). Also, in the group of HER2-negative patients there was no significant difference in prognosis between the group with the high expression of cyclin D1 and the group with the low expression of cyclin D1 (p=0.055, Log-rank test). In the group of HER2-negative patients the survival rate at 5 years was 86.5% (72.7-100%). In the group of ER+ patients, those with the high expression of cyclin D1 showed a significantly longer OS than patients with the low expression of cyclin D1 (p=0.003, Log-rank test) (Figure 5B). In the group of hormone-positive patients, the survival rate at 5 years was 83.1% (67.0-100%). We show photomicrographs of tumour samples of breast cancer patients displaying a carcinoma with strong nuclear expression of cyclin D1 and of a tumour sample of patients with the low expression of cyclin D1 (Figures 5 C,D).

**Discussion**

It has been shown that the overexpression of cyclin D1 was detected in approximately 35-81% of breast cancer tissues, by using immunohistochemistry, with cyclin D1 gene amplification in 20% of invasive breast cancer tissues [12,18,19]. In our study we found the high expression of cyclin D1 in 67.5% of patients by immunohistochemistry, and in 75% of patients by Western blotting. In most of the published studies immunohistochemistry has been used as the detection method [10,13,20], while in our study we used two methods, Western blot and immunohistochemistry. Since cyclin D1 expression by immunohistochemistry is often an unreliable method due to the occurrence of equivocal results [15], the Western blotting as the detection method, can be an adequate method for quantification. In addition, we showed that breast cancer tissue has a higher expression of cyclin D1 compared to adjacent tissue of the investigated patients. Although the functional effects of cyclin D1 in cancers are not completely investigated, our results suggest that cyclin D1 has an important role in breast cancer carcinogenesis.

Decreased cyclin D1 expression associated with an increase in the clinical stage, represents a novel result, considering that there are no studies investigating the clinical stage in relation to cyclin D1 expression. Furthermore, cyclin D1 decreases with the increase of tumour size. Also, we showed lower cyclin D1 expression in patients with positive lymph node involvement compared to patients with negative lymph node involvement. Other studies had controversial results regarding the association of lymph node involvement and cyclin D1 tumour expression [10, 18, 21]. These results suggest that activation of cyclin D1 could be one of the crucial steps in the formation of breast cancer.

In *in vivo* and *in vitro* studies it has been shown that hormones, such as estrogens and progesterones enhance breast cell proliferation via cyclin D1 activation [6,22]. Patients with hormone receptor positivity had a higher expression of cyclin D1 which is in accord with previously published studies showing the association of cyclin D1 expression with ER status [6,10,18,23]. Our results implicate the association of cyclin D1 expression and a favourable subtype of breast tumours of smaller size and hormone-positive receptors.

Cyclin D1 represents one of the targets of HER2 signalling pathway. Lenferink et al have shown that HER2 induces transcription of cyclin D1 gene via the Ras/MAPK pathway [24]. Considering these data, we investigated the association of cyclin D1 and HER2, and showed that there is no association between these factors in the group of investigated patients. Lee et al have shown the association of cyclin D1 and HER2 expression, while several other studies did not show any correlation between these markers in breast cancer patients [10,12,18]. Modern oncology needs to detect new molecular markers which might define a high-risk group of breast cancer patients. It has been published that basal-like breast cancers often show high proliferation, short survival and do not have CCND1 gene amplification [25]. In our study we showed that basal-like tumours had a low expression of cyclin D1 in tumour tissue compared to Luminal A tumours. These results implicate that the lack of activation and expression of cyclin D1 might enhance the progression of basal-like cancers.

It has been reported that breast cancer patients with overexpression of cyclin D1 show complete response to hormonal therapy [26]. There are controversial results regarding cyclin D1 expression as a prognostic or predictive marker [27,28]. In the present study, we showed that cyclin D1 expression is associated with longer RFS in the entire group of patients, as well as in the group of ER-positive and in the group of HER2-negative breast cancer patients. Some previous studies have reported that cyclin D1 is connected with shorter RFS in breast cancer, while other studies have shown that cyclin D1 is associated with longer OS [27-29]. Bilalovic et al have shown that cyclin D1 overexpression is associated with longer survival in breast cancer patients [21]. The present study has shown no significant relationship between the expression of cyclin D1 and the survival outcome in breast cancer patients, although in the group of hormone-
positive breast cancer patients, the high expression of cyclin D1 showed better OS than the low expression of cyclin D1. These results suggest that the activation of cyclin D1 might induce programmed cell death and interact with signalling pathways of cell growth by blocking them. Cyclin D1 in different types of cells can have different functional effects based on its level of activation [30,31]. Moreover, the effects of cyclin D1 on growth inhibition of cancer cells can also explain our results, showing tumour size-dependent decrease of cyclin D1 expression [32,33].

Conclusions

Our results, showing the association of cyclin D1 with clinical stage, tumour size and nodes suggest that cyclin D1 has an important role in tumour progression. Reported correlation of cyclin D1 expression detected by Western blotting with RFS and OS of hormone-positive patients indicate that cyclin D1 could give valuable staging and prognostic information for breast cancer management. Quantification of cyclin D1 expression by Western blotting is important considering that it has different effects on cancer cells depending on its level.

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Conflict of interests

The authors declare no conflict of interests.

References


