



The Interplay of Proteins Encoded by CDKN1A, CDKN1B, CDKN2A and TP53 in the Prognosis of Breast Cancer

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Abstract

The use of palbociclib has increased the spotlight on cell cycle regulators, especially on the proteins encoded by CDKN2A (p16), CDKN1A (p21), CDKN1B (p27), cyclin D1 and TP53 (p53). Cell cycle regulators and cyclin-dependent kinases show significant promise as new breast cancer biomarkers. Expression patterns of small sets of proteins in breast carcinoma were reported, and their overall role in breast cancer progression was investigated, with mixed results. The goal of our study was to simultaneously evaluate multiple cell cycle regulators in breast carcinoma. The protein expression was assessed on Tissue Microarrays (TMA) and the intensity and percentage of positive tumor cells were quantified. Statistical analysis was performed to establish patterns of protein associations, as well as correlation with prognostic factors, including metastases/recurrence and overall survival. Non-triple negative carcinoma showed significantly increased expression of cyclin D1, marginally increased expression of p21, 27, and decreased expression of p16, p53. This is in contrast to triple negative carcinoma which expressed high levels of p16 and p53. Statistically significant dual proteins associations were demonstrated, including direct correlations of expression of cyclin D1 with p21 or 27, and indirect correlations with p16 and p53. Cyclin D1 (p=0.0001) and p21 (p=0.0049) expression imparted a favorable outcome on development of metastases and/or survival, while p16 (p=0.0048) had a negative impact, particularly on non-triple negative carcinoma. The remainder of the tested proteins showed no prognostic significance. Given these proteins expression patterns, associations and prognostic impact, the results render cyclin D1, p21 and p16 as potential biomarkers for risk stratification in breast carcinoma.

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Introduction

Breast carcinoma is the most common cancer accounting for approximately 30% of new cancer diagnoses and 14% of cancer deaths in women according to the 2018 statistics of the American Cancer Society [1]. While survival has improved with early diagnosis and advancement in treatment, breast cancer is still the second most common cause of death in women [1]. The recent FDA approvals of palbociclib, ribociclib, and abemaciclib, selective inhibitors of Cyclin Dependent Kinase 4 (CDK4) and CDK6, for treatment of hormone positive advanced breast cancer has brought renewed focus on cell cycle regulators, especially CDK Inhibitors (CDKI). A critical function of CDKs is phosphorylation of Retinoblastoma protein (Rb) leading to progression from G1 to S phase of the cell cycle, while CDK inhibitors are believed to prevent this progression [2]. Conversely, inactivation of CDKI leads to unopposed CDK4/6-Cyclin D1 complex formation and progression from G1 to S phase of the cell cycle [2,3]. The CDK inhibitor proteins can be divided into 2 families: 1) the INK4 family and 2) the CIP/KIP family. The INK4 family comprises of p16^{INK4A}, p15^{INKB}, p18^{INKC} and p19^{INKD-4} these bind to CDK4/6 and inhibit its kinase activity, leading to cell cycle arrest in G1 phase. The CIP/KIP family on the other hand consists of p21^{CIP1}, p27^{KIP1} and p57^{KIP2}, these bind to a broader spectrum of cyclin/CDK complexed already formed (including cyclin D/CDK4/6 complex) and are therefore able to inhibit cell cycle progression [4]. p21 was shown to allow for repair of damaged DNA, [5] while p27, expressed in high concentrations in quiescent cells, decreases during entry into the cell cycle and promotes tumorigenesis [6,7].

In Estrogen Receptor (ER) positive breast cancer, the prognostic significance of cyclin D1 over expression is still unclear. Some studies show cyclin D1 expression is associated with a poor prognosis and anti estrogen resistance, while others claim it is associated with improved outcomes [8-12]. Among CDKIs, cyclin-dependent kinase inhibitor 2A (CDKN2A) p16 expression was

shown to play a significant role in advanced tumor stage, higher grade, increased tumor proliferation, hormone receptor negativity, metastasis, and decreased overall survival [4,13-17].

Similarly, there is strong evidence correlating the reduced or absent p21 expression and progression of colorectal carcinoma, Hodgkin lymphoma, and breast carcinoma, which tends to be expressed more often in HER2 negative than in HER2 positive tumors [18-22]. A p27 decreased expression has been seen in the transition from ductal carcinoma in situ to invasive ductal carcinoma [6]. In addition, a study of African American women with breast cancer showed a significant decrease in p27 expression coupled with c-Myc over expression and decreased overall survival in patients with ER negative, PR negative, triple negative, and grade 3 breast cancers by Immunohistochemistry (IHC) [23].

The emerging role and early successes of CDK4/6 inhibitors to treat advanced breast cancer has highlighted the importance of finding specific biomarkers that could guide patient selection and treatment. Additionally, these early successes have led to growing interest in applying these drugs to early stage breast cancer [24]. Given that inhibitors of CDK4/CDK6 affect the progression from G1/S or G2/M phase of the cell cycle, it stands to reason to expect the expressions of cell cycle protein regulators like p16, p21 and p27 to achieve and maintain tumor cells growth arrest. In the present study, we sought a comprehensive approach and simultaneously evaluated multiple cell cycle regulator proteins, including p16, p21, p27, cyclin D1 and p53, in invasive breast carcinoma. Their immunohistochemical expression and correlation with clinicopathologic features such as distant metastasis or survival were investigated.

Materials and Methods

Patient demographics

The study analyzed invasive breast carcinoma from patients diagnosed and treated at our institution between 2005 and 2013. Consult cases and cases reviewed for second opinions were excluded. Only cases with adequate follow-up information were included. Clinical-pathologic and demographic information including distant metastasis and survival, were collected via patient electronic medical records and the Cancer Registry.

Immunohistochemistry and scoring

Tissue microarrays were constructed (1.0 mm cores) using Beecher ATA27 Tissue microarrayer. Each case has triplicate 1 mm cores for the TMA. Immunohistochemistry for Cyclin D1 (clone EP12, Dako), p16 (clone INK4a, Roche), p21/WAF1 (clone DCS-60.2, Cell Marque,) and p27/KiP1 (clone SX53G8, Cell Marque), and p53 (clone DO-7, Dako) were performed.

Two board certified pathologists independently evaluated the immunohistochemically stained slides, for percentage of positive cells and intensity of staining, using scales of 0% to 100% and 0-3, respectively. The percentage and intensity scores were converted to Allred score and H score. Allred scoring system uses the sum total of proportion score and intensity score (range 0-8), while H score uses the multiplication of percentage reactivity with intensity score (range 0-300) [25,26]. Cases with at least one scorable TMA core were considered evaluable. Average of the scores per case was used when two or more cores are adequate.

Statistical analysis

Association of each biomarker expression with binary outcomes

Table 1: Patients demographics.

Characteristic	Number patients (%)
Age	
<50	61 (28.1)
>50	156 (71.8)
Ethnicity	
White	101 (46.5)
African-American	107 (49.3)
Other	10 (4.6)
Body Mass Index (BMI)	
<35	171 (78.8)
>=35	46 (21.1)
Histologic type	
Ductal	186 (85.7)
Lobular	19 (8.7)
Other	12 (5.5)
Histologic grade	
I	28 (12.9)
II	65 (29.9)
III	95 (43.7)
n/a	29 (13.3)
Hormonal status	
Luminal A	135 (62.2)
Luminal B	10 (4.6)
HER2	13 (5.9)
Triple negative	52 (23.9)
Unknown	7 (3.2)
Pathologic tumor stage	
T1	83 (38.2)
T2	107 (49.3)
T3	23 (10.5)
T4	4 (1.8)
Pathologic nodal stage	
N0	83 (38.2)
N1	61 (28.1)
N2	31 (14.2)
N3	18 (8.2)
Unknown	24 (11)
Recurrence	
None	40 (18.4)
Loco-regional	137 (63.1)
Distant	61 (28.1)
Unknown	26 (11.9)
Survival	
Alive free/ unknown disease	60 (27.6)
Alive with disease	87 (40)
Died of disease	70 (32.2)

was inferred using single covariate logistic regression. As the expressions of several biomarkers are often strongly correlated, models with multiple covariates were avoided. A type I error threshold of 5% was used to infer statistical significance. As reviewers assigned the A score and H score, Cohen's kappa measure was calculated for each marker and in most cases, were statistically significantly different from 0. Thus only the average score was used in all analysis. Only statistical significant association with both Allred and H score scoring system are considered significant. Significant association with only one scoring system is not considered medically significant. Association of time to event outcomes, such as overall survival, was assessed using a Cox proportional hazards regression model.

Results

Characteristics of the study population

All 217 patients included in the study were women, age range 27 to 92 years old, diagnosed with invasive breast carcinoma, ductal and lobular types. The demographic and clinicopathologic information is summarized in (Table 1).

Immunohistochemical expression of markers

Individual staining characteristics were identified for the evaluated markers (Figure 1). Some proteins, cyclin D1, p16, p53,

Table 2: Distribution of protein expression according to tumor hormonal phenotype (*triple negative and non-triple negative phenotypes*).

Tumor phenotype (number cases)	Protein	Number of cases scored	Mean Allred score	Mean H score
Non-triple negative (159)	P16	141	0.62	8.42
	P21	141	3.82	35.51
	P27	141	3.18	41.82
	CyclinD1	141	5.28	139.87
	P53	142	1.60	26.11
Triple negative (51)	P16	47	4.18	100.57
	P21	48	3.249	25.46
	P27	47	1.90	20.66
	CyclinD1	47	2.90	29.66
	P53	48	4.27	124.59

Note: The Allred scores range from 0-8; the H scores range from 0-300

Table 3: Correlation of the different protein expression to distant metastasis (H Scoring System). Logistic regression of correlation between promitotic and antimitotic protein and breast cancer distant metastasis/recurrence for all cases (*triple negative and non-triple negative phenotypes*).

	Maximum likelihood estimates H score	Odds ratio (95% confidence) H score	P value H score
Cyclin D1	0.00820	1.008 (1.004-1.013)	0.0001'
P16	-0.00597	0.994 (0.990-0.998)	0.0048''
P21	0.00757	1.008 (0.999-1.016)	0.0049'
P27	0.00184	1.002 (0.996-1.008)	0.5432
P53	-0.00208	0.998 (0.995-1.001)	0.1840

Note: The probability model is distant metastasis = negative. The analysis of maximum likelihood estimates, odds ratio/ 95% confidence limits and correlation with distant metastasis *p* value are provided. Direct (') and indirect (') significant correlations are identified

Table 4: Logistic regression of correlation between promitotic and antimitotic protein and breast cancer distant metastasis/recurrence for *triple negative and non-triple negative phenotypes* (H Scoring System).

	Maximum likelihood estimates Non-triple negative	Maximum likelihood estimates Triple negative	P value Non-triple negative	P value Triple negative
Cyclin D1	0.00706	0.00908	0.0059'	0.2011
P16	-0.00974	-0.00279	0.0365''	0.3045
P21	0.0106	-0.00145	0.0933	0.8382
P27	-0.00115	0.0147	0.7444	0.1364
P53	-0.00097	0.00124	0.7359	0.5977

Note: The probability model is distant metastasis = negative. The analysis of maximum likelihood estimates and correlation with distant metastasis *p* value are provided for triple negative vs. non-triple negative tumors. Direct (') and indirect (') significant correlations are identified

showed strong nuclear staining in the majority of tumor cells. p21 and p27 were detected in a wider range of percent positive cells with lower cytoplasmic (p21) or nuclear (p27) intensities. The observed heterogeneity of patterns highlighted the need for standardization in reporting the immunohistochemical staining expression, best achieved by using both the H and Allred scores. The proteins differential expression in non-triple negative and triple-negative tumors was evaluated (Table 2). Non-triple negative carcinoma showed high levels of cyclin D1, along with a slight increased positivity for p27. P21 was only minimally increased. Conversely, the expression of p16 and p53 was higher in the triple negative carcinoma.

To assess the potential protein associations as they pertain to roles in the cell cycle regulation, statistical analysis was performed for correlations between the reactivity of the antibodies. Regardless of scoring system used, cyclin D1 showed direct correlations with p21 ($p < 0.0001$), p27 ($p = 0.0035$), as well as a statistically significant inverse correlations with p16 ($p < 0.0001$), p53 ($p = 0.0002$). p27 inversely correlated with p53 ($p = 0.0011$), but showed no significant correlation, positive or negative, with p21 or p16. p21 showed no significant correlation with any protein, except cyclin D1. Finally, there is a strong correlation between expression of p16 and p53 ($p < 0.0001$), proteins predominantly expressed in the triple-negative tumors, along with inverse correlations between p16 and cyclin D1 ($p < 0.0001$) or p21 ($p = 0.0156$).

Prognostical significant of protein expression

The impact of the proteins expression on prognostic factors such as development of distant metastases and overall survival was assessed, and the results were concordant in majority of instances, regardless of the scoring system used. Increased expression of cyclin D1 significantly correlated ($p = 0.0001$) with decreased risk of distant metastasis (Table 3). For cyclin D1, this association was also seen in non-triple negative cases ($p = 0.0059$), but not triple negative cases (Table 4). Conversely, there was an inverse significant correlation between the expression of p16 and distant metastasis ($p = 0.0048$) (i.e. increasing expression of p16 is associated with higher possibility of distant metastasis), also noted in non-triple negative cases only ($p = 0.0365$) (Table 4). p21 expression showed a direct significant association with distant metastasis in the entire cohort ($p = 0.0049$), and was borderline in non-triple negative tumors ($p = 0.0933$) (Table 3 and 4). Finally, in triple negative cases, there was no association between the expressions of any of the antibodies with distant metastasis, regardless of the scoring system used (Table 4).

Cyclin D1 expression was the only marker with a significant association with longer survival ($p = 0.0190$) (Table 5). The association of p16 to survival depended on the scoring system, showing no significant association with H score ($p = 0.1323$), but an adverse effect on survival when Allred scoring is used ($p = 0.0375$), data not shown.

Table 5: PHREG Procedure of correlation between promitotic and antimitotic proteins with survival (H Scoring System).

H Score	Maximum likelihood estimates	Hazard ratio (95% confidence)	Overall survival P value	Comment
Cyclin D1	-0.00407	0.996 (0.993-0.999)	0.0190 **	Longer survival.
P16	0.00196	1.002 (0.999-1.005)	0.1323	No correlation to survival
P21	0.00329	1.003 (0.998-1.008)	0.1798	No correlation to survival
P27	-0.00100	0.999 (0.994-1.004)	0.6933	No correlation to survival
P53	0.00158	1.002 (0.999-1.004)	0.1774	No correlation to survival

Note: Negative significant association** means a positive outcome; higher protein expression leads to lower hazard ratio, therefore longer overall survival. Positive significant association* means a negative outcome; higher protein expression leads to higher hazard ratio, therefore lower overall survival

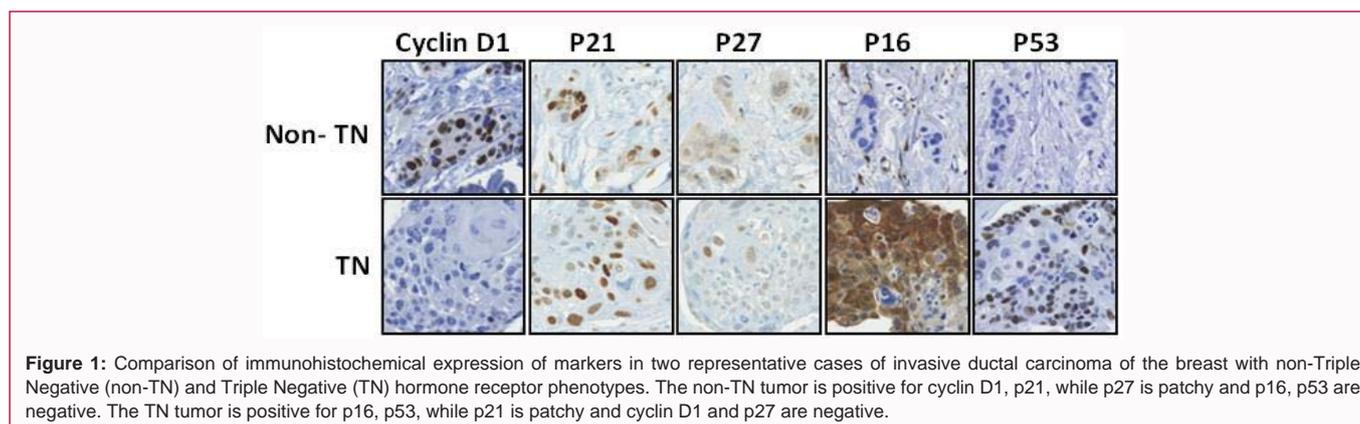


Figure 1: Comparison of immunohistochemical expression of markers in two representative cases of invasive ductal carcinoma of the breast with non-Triple Negative (non-TN) and Triple Negative (TN) hormone receptor phenotypes. The non-TN tumor is positive for cyclin D1, p21, while p27 is patchy and p16, p53 are negative. The TN tumor is positive for p16, p53, while p21 is patchy and cyclin D1 and p27 are negative.

Survival analyses show African Americans (AA) have a higher hazard ratio, and increased risk of death compared to Caucasians ($p=0.0106$). The hazard ratio for survival or positive outcome is almost double for Caucasians compared to AA. Body Mass Index (BMI) on the other hand, appears to have no significant association to survival ($p=0.8885$).

Discussion

Non-triple negative breast cancer is much more commonly diagnosed compared to triple negative cases. While endocrine therapy is effective in the initial treatment of non-triple negative breast cancer, a percentage of these patients will relapse, sometimes with distant metastasis. The success of CDK4/6 inhibitors in the treatment of advanced breast cancer has prompted interest in their use for early-stage disease [24]. Thus, identifying biomarkers that may predict these responses and guide treatment selection becomes imperative.

This is one of the few studies to concomitantly analyze the expression patterns of multiple proteins involved in cell cycle regulation and their prognostic relevance to breast cancer progression. It is also unique in using the H scores to quantitate the reactivity of the proteins, rather than pre-set cut-offs for positivity.

Patterns of protein associations were inferred from the data. In non-triple negative breast carcinoma, high expression of cell cycle inducing protein cyclin D1 was noted, consistent with prior reports, [11,27] along with reduced expression of cell cycle inhibitors p16 and p53. Cyclin D1 expression was associated with p21 and p27, however no correlation between p21 and p27 expression was observed. Co-expression the p21 and cyclin D1 genes, has been previously shown in breast as well as ovarian cancers, leading to inhibition of growth and differentiation, through arrest of the G1 to S cell cycle progression. Alternatively, cyclin D1 may cooperate with p21 to regulate TGF β -mediated breast cancer cell migration and tumor local invasion [28].

The association of cyclin D1 and higher expression of p27 has been previously reported, [22] and was linked to enhanced apoptosis and inhibition of growth.

The clinical prognostic significance was largely driven only by a limited number of markers. Regarding metastases development, cyclin D1 and p21 expression was associated with a favorable outcome, while p16 had an adverse effect (Table 3). These outcomes were specific to non-triple negative carcinomas for cyclin D1, p16 and borderline for p21 (Table 4). Cyclin D1 was associated with increased survival. While no correlation of p16 expression with survival was noted when the H score was used for quantitation ($p=0.1323$), its association with poor prognosis was significant with Allred score ($p=0.0375$) and data not shown (Table 5). Predominantly concordant results were obtained using both H and Allred scores, with only rare discrepancies noted. The ASCO/CAP guidelines for reporting breast biomarkers allow for use of H scoring and Allred scoring, and the scores can be used interchangeably [29]. The choice to use a particular system is left up to the individual institution, with some favoring the Allred score due to better sensitivity and specificity, while others using the H score due to its theoretical higher level of prognostic information [30,31]. In our study, the rare result differences underscore the potential recommendation of both scoring methods being used in subsequent attempts at markers standardization.

We found cyclin D1 is an independent prognostic factor of an overall favorable outcome. This is important, because the existing data is still controversial; showing its expression it can be related to either good or poor outcomes. This is in contrast to CCND1 gene amplification, which has been consistently shown to correlate with early relapse and poor prognosis [8,9,12,32-34]. Improved time to recurrence in patients with cyclin D1 overexpression was also noted in a prior study [34]. In other reports, while cyclin D1 overexpression correlated with increased disease-specific and overall survival, it failed to demonstrate a significant difference in disease free survival

[10,11,35]. Moreover, a significantly longer survival after recurrence was also noted, hypothesizing a more indolent disease progression after metastasis in patients with high cyclin D1, possibly induced by tamoxifen resistance [34,35]. Consistent with the notion that the main function of cyclin D1 is to activate CDK4/6, its oncogenic activity is dependent on CDK4/6-associated kinase activity and CDK4/6 inhibitors may be more effective in tumors with overexpression of cyclin D1.

Increased p16 expression is associated with higher incidence of distant metastasis, also seen in non-triple negative cases. While more prevalent in triple negative cases, p16 did not show a prognostical significance in that cohort. However, the association of p16 with survival is still unclear, given the adverse effect on survival was noted only when the Allred score was used. These results mirror prior opposing findings, that range from studies showing high p16 expression as an indicator of poor survival, failing to demonstrate a correlation between the p16 expression and survival, to showing significant association of high p16 expression with both increased breast cancer-specific survival and disease-free survival [10,11,13,17]. While p16 functions through inhibition of CDK4/CDK6 complexes, the data suggests additional factors may contribute to the overall prognostic impact of p16 [2,36]. Indeed, while a prior study showed both cyclin D1 and p16 expression correlating with better prognosis, it also reported a lack of correlation between cyclin D1 and CDK4, pointing towards possible independent mechanisms of action [11]. In our cohort, cyclin D1 and p16 show inverse correlation patterns of expression, and appear to impart opposite prognostic significance (Tables 3-5). Thus, non-triple negative carcinoma with high expression of cyclin D1 and low expression of p16 may represent a good target for treatment with CDKI.

p21 expression correlated directly with cyclin D1, indirectly with p16, and showed no significant association with p27 or p53. Prognostically, it had an independent favorable influence on metastasis development and no impact on survival. As stated above, the synergistic functions of cyclin D1 and p21 may be due to roles in cell cycle progression or through a TGF β -mediated mechanism. Finally, we found no significant correlation of p27 with prognosis. Similarly, others could not establish p27 as an independent prognostic factor, while in other reports p27 alone was considered a strong indicator of poor prognosis [37-42].

In summary, several proteins involved in cell cycle regulation showed independent prognostic significance in non-triple negative carcinoma. Based on our results, tumor expression of cyclin D1, p21, along with decreased expression p16 and p53 may be associated with best prognosis. Given the clinical applicability of CDK4/6 inhibitors on breast cancer patients, these may serve as markers of additional risk stratification and may prove beneficial in therapy selection.

Ethics Approval and Consent to Participate

The study was performed under VCUHS institutional IRB approval. No consent for participation was needed for this study.

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