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Cross Sectional Study

Association of Cyclin D1 Expression and Histopathological Grading of Invasive Ductal Carcinoma Breast Cancer: An Observational Cross-Sectional Study in Surabaya

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ABSTRACT

Breast cancer is ranked as the fifth major contributor to global cancer-related fatalities. Cyclin D1 is frequently overexpressed in cases of invasive ductal breast cancer and plays a specific role during the advanced stages of tumor evolution. However, the utilization of cyclin D1 overexpression as a predictive tool remains a subject of debate. This study aims to determine the relationship between cyclin D1 expression and the histopathological grading of invasive ductal carcinoma in breast cancer. The results can serve as scientific evidence that cyclin D1 can aid in predicting the prognosis of patients, making it a valuable tool in health facilities. In this cross-sectional study, employing flow cytometric techniques, cyclin D1 expression was assessed in 30 patients categorized as having grade 1, grade 2, or grade 3 invasive ductal breast cancer based on histopathological diagnoses. The analysis indicated a negligible difference in cyclin D1 expression levels between patients with invasive ductal breast cancer grades 1 and 2 ($P = 0.283$). In contrast, a statistically significant disparity in cyclin D1 expression was observed between patients diagnosed with grades 1 and 3 of invasive ductal breast cancer ($P = 0.026$), as well as between grades 2 and 3 ($P = 0.026$). Our findings underscore a moderate yet significant correlation between cyclin D1 expression and breast cancer grading ($R: 0.508$; $P < 0.05$). A moderate correlation between cyclin D1 expression and the histopathological grade of breast cancer was substantiated. These observations lend credence to the utilization of cyclin D1 as a marker indicative of an unfavorable prognosis.

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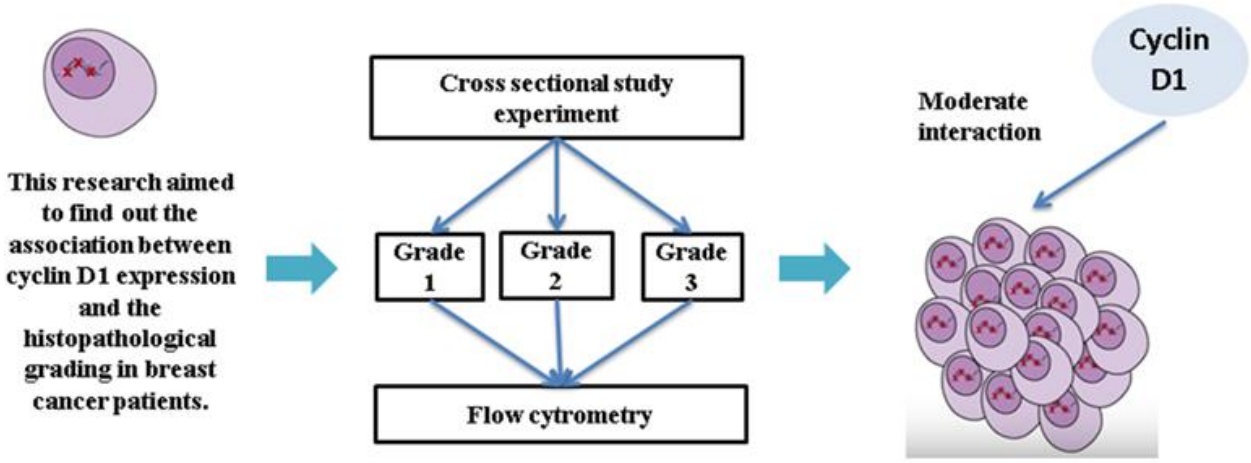
GRAPHICALABSTRACT



Introduction

In 2020, breast cancer stood out as the most frequently identified cancer type, securing fifth place among the primary contributors to global mortality [1-15]. In 2022, approximately 287,850 new cases of invasive breast cancer and 51,400 cases of ductal carcinoma *in situ* are expected to be diagnosed among women in the US, and 43,250 women are expected to die from breast cancer [16]. An estimated 297,790 new cases of invasive breast cancer are projected to be diagnosed in women in 2023 [17]. Notably, its hallmark encompasses invasive cancer and significant complications related to the spread of cancer cells [18, 19]. Preeminently prevalent among invasive breast cancer forms, Invasive Ductal Carcinoma (IDC) governs a substantial 55% of all breast cancer occurrences. This variant entails the malignant proliferation of ductal cells and invasion into the surrounding tissues [20-22]. The unfavorable prognostic outlook affiliated with advanced-stage breast cancer can be ascribed to disease advancement and the ensuing metastatic progression after surgical intervention [22]. To effectively manage and treat breast cancer, a deeper understanding of the molecular mechanisms underlying its development is necessary [23, 24]. IDC can be categorized into various histological subtypes based on factors such as the cell type (for instance, apocrine carcinoma), the quantity, type, and position of secretion (as seen in mucinous carcinoma), the structural characteristics (including papillary, tubular, and micropapillary carcinoma), and the immunohistochemical profile (as evidenced in neuroendocrine carcinoma) [21].

Early detection of breast cancer has a fairly good prognosis. A 5-year survival rate of 100% occurs in stage 0 and I cancers. The 5-year survival rates for stage II and stage III breast cancer are approximately 93% and 72%, respectively. When the disease spreads systemically, the prognosis significantly worsens. Only 22% of stage IV breast cancer patients can survive the next five years [25]. Breast cancer prognosis is crucial for providing patients with information about the future course of their disease, enabling early treatment of breast cancer. It also serves to include and stratify patients in experimental studies and assists policymakers in comparing mortality rates among hospitals and institutions [26]. Continuous efforts are underway to identify new and efficient biomarkers for timely prognosis of breast cancer [27].

Breast cancer treatment intricately hinges on the evaluation of predictive markers and pathological insights, forming the foundation for patient-

centered decisions and therapeutic strategies. Particularly within the context of early-stage breast cancer, where tailored systemic interventions mandate personalized considerations, three cardinal prognostic factors take precedence in routine clinical practice: lymph node status, tumor dimensions, and histological grade [28-30]. Notably, the Histological Grade (HG) of the tumor emerges as a paramount anatomical attribute. Within the spectrum of breast cancer, the Nottingham Grading System has emerged as a prevalent tool for HG determination, representing a refined iteration of the Scarff-Bloom-Richardson (SBR) classification schema. Conforming to global recommendations since 1991, this classification framework encompasses the subsequent delineations: HG-1 denotes well-differentiated tumors, characterized as favorable (scores 3 to 5), HG-2 signifies moderately differentiated tumors (scores 6 to 7), and HG-3 denotes poorly differentiated tumors (scores 8 to 9). The corresponding score allocations are lucidly, as presented in Table 1 [31].

Cyclin D1 emerges as the product engendered by the CCND1 (PRAD1) gene, situated at the chromosomal locus 11q13, and showcases amplification in roughly 15% of breast cancer cases [32-35]. Intriguingly, despite gene amplification, an excess of cyclin D1 manifests at both the mRNA and protein levels, comprising over 50% of breast cancer cases [36-38], thus asserting its status as one of the frequently overexpressed proteins in the context of breast cancer [39]. Various studies also discussed this issue. The predictive and prognostic utility of cyclin D1 overexpression in the realm of breast cancer remains a contentious matter, wherein a majority of studies propose that such overexpression aligns with a favorable prognostic outlook. However, a subset of researchers has advanced the viewpoint that cyclin D1 overexpression could herald an unfavorable prognosis [40]. Previous research has not only focused on breast cancer. Promiscuous colon cancer cell growth via autophagy is linked to ERβ-mediated degradation of cyclin D1 [34]. This study aims to determine the association between cyclin D1 expression and the histopathological grade of invasive ductal carcinoma in breast cancer. We hypothesize that cyclin D1 expression can support the prediction of patient prognosis and can be applied in healthcare facilities.

Materials and Methods

Study design

A cross-sectional study was conducted, and ethical clearance was obtained from the ethics committee of Dr. Soetomo in Surabaya. Strict adherence to the informed consent protocol was observed before sample acquisition. Paraffin-embedded blocks were meticulously collected from a cohort of 30 patients recently diagnosed with invasive ductal breast cancer between January and December 2022 at Dr. Soetomo Public Hospital, Surabaya. All specimens preserved within Formalin-Fixed Paraffin-Embedded (FFPE) tissues underwent scrutiny during the initial diagnostic phase.

Table 1: The Nottingham grading system score

Criteria	Description	Score
Tubular grade	Tubular formation is present in:	
	More than 25%	1
	10 to 75% of the tumor	2
	Less than 10% of the tumor	3
Nuclear grade	Mild nuclear atypia; regular and uniform small cores	1
	Moderate nuclear atypia; moderate size and variability	2
	Intense nuclear atypia; striking variability and the presence of nucleoli	3
Mitotic index*	0 to mitoses per large magnification field	1
*For Nikon microscope (0.44 mm field diameter and 40 × magnification)	6 to 10 mitoses per large magnification field	2
	More than 11 mitoses per large magnification field	3

The subsequent laboratory scrutiny took place within the Department of Anatomy and Clinical Pathology at the Faculty of Medicine, Airlangga University, in collaboration with Dr. Soetomo Public Hospital, Surabaya.

Data collection

The grading criteria adopted for assessment were predicated on The Nottingham Grading System, which constitutes a refined rendition of the Scarff-Bloom-Richardson (SBR) classification system [41]. The quantification of cyclin D1 expressions unfolded through the employment of BD FACSLIRIC, using monoclonal antibodies anti-CD45 coupled with peridinin chlorophyll protein (PerCP) and anti-cyclin D1 fused with phycoerythrin (PE), both sourced from Santa Cruz Biotechnology Inc.

To liberate tissue sections obtained from paraffin-embedded blocks, FFPE tissue sections are required, characterized by a cover thickness of 10 µm and an area of 40 to 100 mm^2. After that, the sections were immediately transferred into sterile microcentrifuge tubes, each with a volumetric capacity of 1.5 mL. Next, three xylene washes were administered to the FFPE tissue. This was performed by adding 1 mL of xylene to each tube, followed by vortex agitation for 10 seconds. The supernatant was extracted, and the washing process was repeated three times. This washing method was performed in stages, starting with 100%, 85%, and 70% ethanol. The procedure was carried out in two rounds, with 1 mL added to each tube. The tube was incubated at room temperature for 5 minutes, followed by centrifugation at 20,000 g for 2 minutes until pellets formed. The supernatant was removed via pipette, and the ethanol rinse was repeated twice [40].

Next, tissue homogenization was carried out using a ratio of 600 µl of PRO-PREP™ solution per 10 mg of tissue, which was facilitated by the homogenizer. The culmination of this sequence requires inducing cell lysis, achieved by incubating the homogenized tissue in PRO-PREP™ solution at -20 °C for 20-30 minutes. After this, centrifugation at 22,000 g for 5 min was performed, allowing extraction of the supernatant, then stored at −20 °C or used immediately [42]. Regarding cell suspensions derived from tissue samples, a 2 mL contingent of these suspensions was subjected to cytometric examination. A mixture of 50 µL of homogenized cell suspension and phosphate-buffered saline in a 1:1 ratio was combined with 2.5 µL of PerCP-labeled anti-CD45 and 2.5 µL of PE-labeled anti-cyclin D1 in a starter tube. Additionally, the second tube contained 2.5 µL of anti-cyclin D1 (preconditioned with 1 mL of lysis solution, 250 µL of cytofix/cytoperm, and 1 mL of perm wash reagent from Becton Dickinson®). The gating strategy was applied to measure cyclin D1 expression in cell suspensions [43]. Median Fluorescent Intensity (MFI) was used as the metric to scrutinize cyclin D1 expression.

Statistical analysis

To ascertain disparities in cyclin D1 expression across varying breast cancer grades, the Kruskal-Wallis test was applied, with subsequent significant pairwise evaluations carried out using the Mann-Whitney test. Moreover, the correlation between cyclin expression and breast cancer grading was probed through Spearman's rho correlation test. All computational procedures were executed employing SPSS version 25 (SPSS, Chicago, IL., USA).

Results and Discussion

A moderate correlation between cyclin D1 expression and the histopathological grade of breast cancer was substantiated (Figure 1). The analysis indicated a negligible difference in cyclin D1 expression levels between patients with invasive ductal breast cancer grades 1 and 2 ($P = 0.283$).

In contrast, a statistically significant disparity in cyclin D1 expression was observed between patients diagnosed with grades 1 and 3 of invasive ductal breast cancer ($P = 0.026$), as well as between grades 2 and 3 ($P = 0.026$). Our findings underscore a moderate yet significant correlation between cyclin D1 expression and breast cancer grading ($R: 0.508$; $P < 0.05$) (Figure 2). In the review of this study, cell suspensions extracted from tissue samples belonging to a

cohort of 30 individuals recently diagnosed with histologically affirmed invasive ductal carcinoma (IDC) of the breast by the department of anatomical pathology underwent analysis. Our principal objective rested on delving into the spectrum of cyclin D1 expression within these samples. Within this study population, the composition comprised 29 women and one man, ranging in age from 31 to 77 years. In alignment with the SBR classification system, instances encompassing grade I histopathology amounted to 8 cases (26%), grade II featured in 11 cases (37%), and grade III pathology were recorded in 11 cases (37%).

The results of flow cytometry analysis to determine cyclin D1 levels showed that in grade I breast cancer, the lowest cyclin level was 135 MFI, and the highest was 345 MFI. For grade II, the lowest cyclin D1 level observed was 235 MFI, and the highest was 337 MFI. In grade III, the lowest cyclin D1 level was 247 MFI, while the highest level was 468 MFI (Table 2, Figure 3).

The controversy surrounding the role of cyclin D1 overexpression in breast cancer, both as a predictive and prognostic determinant, continues to be part of the scientific discourse. Multiple investigations have advanced the notion that cyclin D1 protein expression assumes significance as a prognostic indicator within breast carcinoma, with overexpression aligning with more favorable prognoses, especially within the subset of estrogen receptor (ER) positive patients [44-47]. Conversely, certain researchers have reported a predictive capacity of cyclin D1 overexpression in heralding an adverse prognosis [48].

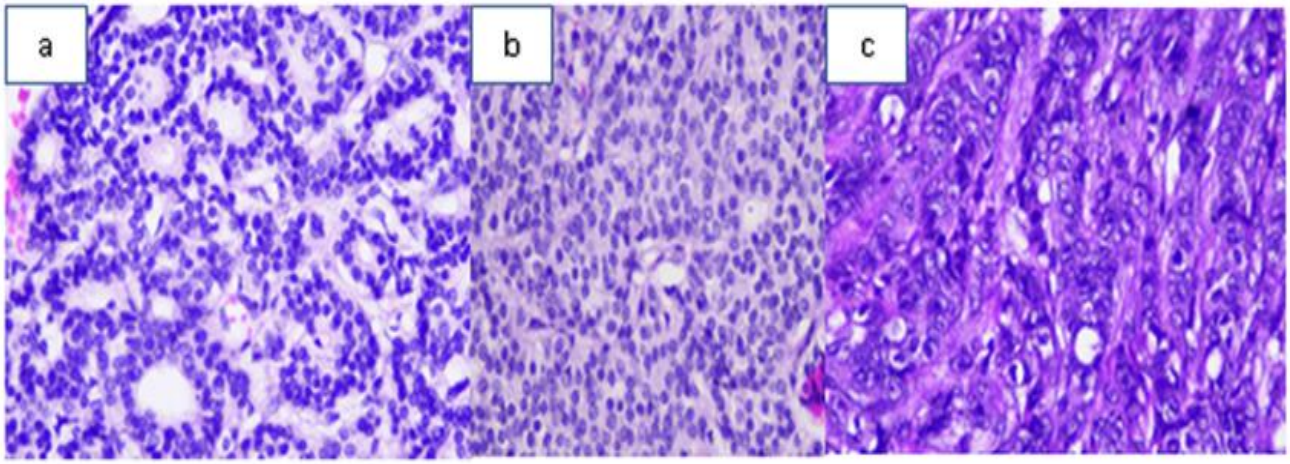


Figure 1: (a) Grade 1: tubular formation >75%, mild pleomorphic, (b) Grade 2: tubular formation 10-75%, (c) Grade 3: tubular formation <10%, severe pleomorphic, and mitotic easy to find

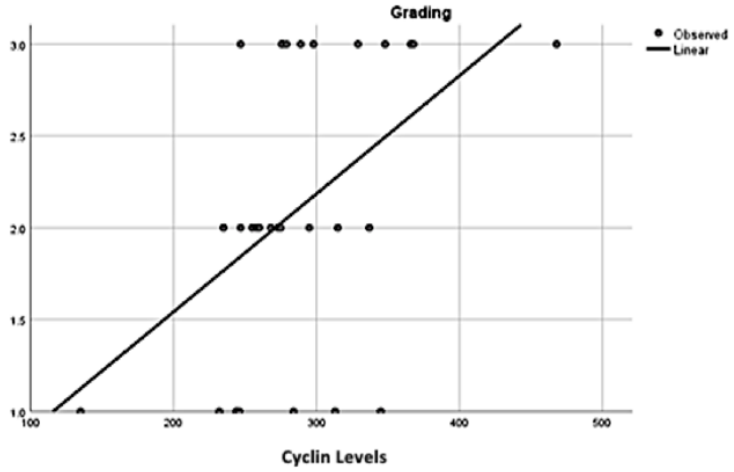


Figure 2: Correlation between cyclin D1 level and histopathological grade

Table 2: Cyclin D1 levels in histopathological grading of ductal invasive breast cancer examined by flow cytometry

No	Cyclin D1		
	A.Grade I (MFI)	B.Grade II (MFI)(B)	C.Grade III (MFI)
1	345	295	348
2	313	315	276
3	284	235	366
4	244	337	329
5	232	255	468
6	244	260	368
7	246	273	276
8	135	268	289
9		247	247
10		258	298
11		275	279

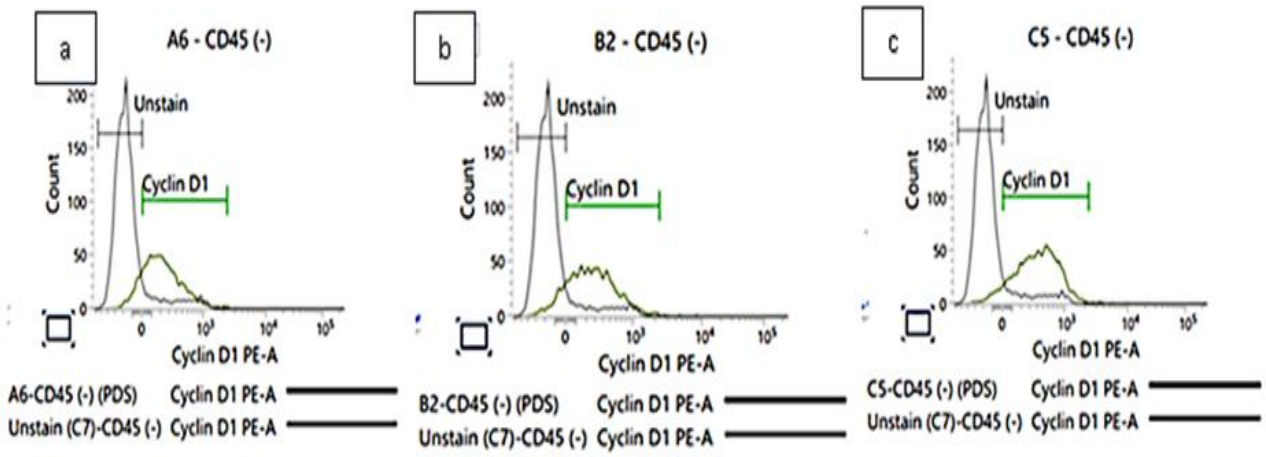


Figure 3: Flow cytometry analysis for the expression of cyclin D1 in an invasive ductal carcinoma breast cancer patients: (a) Grade 1(244 mfi), (b) Grade 2 (315 mfi), and (c) Grade 3 (468 mfi)

Mechanistically, cyclin D1 drives cellular proliferation by coupling with cyclin-dependent kinase 4/6 (CDK4/6), thereby initiating the phosphorylation cascade targeting retinoblastoma protein (Rb) as well as other substrates [40, 44]. Essential for orchestrating cell cycle dynamics, cyclin D1 plays a pivotal role in controlling the transition from the G1 to the S phase [39]. Hence, it is reasonable to assume that augmented cyclin D1 expression might be intertwined with expanded tumor dimensions and escalated proliferation rates [40]. Our observations resoundingly resonate with this premise, as they underscore a discernible positive correlation linking breast cancer grade and the levels of cyclin D1 within the ambit of this study. This observation is supported by a P value of 0.004 ($P < 0.05$).

Cyclin D1 overexpression in breast cancer is associated with less aggressive tumoral characteristics. The cyclin D1 gene, designated CCND1 or PRAD1, is a well-established oncogene with overexpression commonly found in various types of human cancer. However, the exact mechanism through which cyclin D1 exerts its neoplastic effects remains unclear. The complex and still poorly understood variation in cyclin D1 levels through the cell cycle is critical for continued cell proliferation [49]. Another study found an inverse association between cyclin D1 overexpression and tumor grade, as well as a positive association with the estrogen receptor and progesterone receptor in invasive ductal carcinoma. This suggests that cyclin D1 can directly or indirectly result in tumor cell maturation and differentiation [50]. Although the

absence of a standard approach to detect CCND1 amplification is acknowledged as a limitation, CCND1 amplification has the potential to serve as a predictive biomarker in breast cancer patients [51]. Following the identification of cyclin D1 as one of the frequently overexpressed oncogenes in breast cancer [52], the complex intersection between cyclin D1 overexpression and breast cancer outcome trajectories still fuels debate [53]. On the other hand, certain studies highlight promising aspects, associating cyclin D1 protein expression with a good prognosis-a correlation potentially supported by its positive association with ER expression and its antagonistic association with Rb mutations [53]. However, contrasting narratives have emerged from other investigations, failing to validate this relationship [34, 53]. Accumulating evidence lends credence to the hypothesis that cyclin D1 occupies an important echelon as a downstream target of the modulating influence of estrogen, thereby regulating estrogen-induced mitogenesis in the cellular milieu of breast cancer. This strengthens the functional bridge between ER expression and cyclin D1 manifestation [54].

These insights collectively imply that increased cyclin D1 expression through activated cell cycle pathways, in turn, indicates a worse prognosis for ER-positive breast cancer whereas its association with cell cycle activation and prognosis is less apparent in the case of ER-negative tumors [55]. Amidst these limitations, an interesting dichotomy emerges, in which amplification of CCND1 gene seems to be associated with unfavorable disease outcomes in breast cancer patients [56]. Besides the complexity of interpretation, it has been suggested that, under certain circumstances, cyclin D1 overexpression could potentially result in a less favorable clinical trajectory by conferring resistance to endocrine interventions [56]. In a previous investigation that included breast cancer cases without specific selection criteria, a significant discovery was made namely, the prognostic influence of cyclin D1 expression depended on the presence of the estrogen receptor [57].

A series of investigations have initiated the exploration of the interconnection between cyclin D1 expression and various clinicopathological aspects inherent in breast cancer [58]. The important observation regarding high cyclin D1 expression has unfavorable prognostic implications for metastasis-free survival in patients with ER-positive tumors who have not undergone chemotherapy [59]. However, in the primary ductal adenocarcinoma domain, cyclin D1 appears in an overexpressed form in a spectrum covering 30–60% of cases, indicating its early involvement in the disease trajectory. This fact is further confirmed by the increased expression observed in ductal hyperplasia and ductal carcinoma *in situ* [60].

To sum up, it appears that increased cyclin D1 expression has a major influence on breast cancer mortality in ER-positive cases but remains insignificant in the ER-negative scenario. Notably, in ER-positive cases, increased cyclin D1 expression is associated with signs of increased proliferation, as evidenced by cyclins A and B. This interaction is absent in ER-negative tumors [35, 36]. In addition, investigations examining cyclin D1 mRNA expression described that increased cyclin D1 mRNA levels were linked to relapse and death in ER-positive cases but failed to demonstrate a similar association in the ER-negative spectrum of disease [59]. These insights collectively imply that escalated cyclin D1 expression threads through an activated cell cycle trajectory, thereby orchestrating a graver prognosis for ER-positive breast cancer, whereas its association with cell cycle activation and the prognosis is less discernible within ER-negative tumorspheres [35, 45]. To comprehensively address these questions, a more nuanced investigation of cyclin D1 expression and its interplay with patient outcomes within the context of prospective randomized clinical trials is imperative. A patient may experience multiple primary malignancies [61]. Women also need support for their treatment [62].

Conclusion

Several studies in the last decade have identified cyclin D1's involvement in the progression of human breast cancer. However, its role as a prognostic marker is still highly controversial. The results of these studies support the

conception of cyclin D1 as an unfavorable prognostic indicator among breast cancer patients, attributing this assertion to the direct correlation between cyclin D1 overexpression and the histopathological grade of breast cancer. Nevertheless, recent research on the different roles of cyclin D1 in differentiation, chromosome stability, and transcriptional regulation clarifies that their role in breast cancer is far more complex than previously imagined. Further research is likely to result in a deeper understanding of the role of cyclins in breast cancer pathophysiology, with the potential for clinical benefits through the identification of new prognostic markers and therapeutic targets for innovative interventions. Further research is recommended to utilize a larger sample size and involve multiple institutions.

Limitation

This research has not been able to access potential biases or confounding factors, which would help readers assess the reliability of the results. This study also used a small sample size, so future research needs to study a larger number. The research was conducted at a single institution.

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No potential conflict of interest was reported by the authors.

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Authors' Contributions

All authors contributed to data analysis, drafting, and revising of the paper and agreed to be responsible for all the aspects of this work.

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