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Association of CDK4 Expression with Histopathological Grade and Metastasis in Luminal Breast Cancer: A Cross-Sectional Study

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ABSTRACT

Background: The luminal (hormone receptor–positive/HER2–negative) subtype constitutes the majority of breast cancer cases. Despite a generally favorable prognosis, a significant proportion of patients experience metastasis. Cyclin-dependent kinase 4 (CDK4) is a key regulator of the cell cycle, and its dysregulation is a known driver of uncontrolled proliferation in luminal breast cancer. However, data on its association with adverse pathological features in the Indonesian population are limited. This study aimed to investigate the association between CDK4 expression, histopathological grade, and metastatic status in patients with luminal subtype breast cancer in Makassar, Indonesia.

Methods: This cross-sectional study included 74 patients with luminal subtype breast cancer. CDK4 expression in formalin-fixed, paraffin-embedded tumor tissues was assessed via immunohistochemistry and categorized as high or low. The association between CDK4 expression, histopathological grade, and metastatic status was evaluated using the χ^2 test. Binary logistic regression was performed to calculate the odds ratio (OR) for metastasis.

Results: Of the 74 patients, 29 (39.2%) had metastatic disease. High CDK4 expression was significantly associated with high-grade tumors ($P=0.02$) and with the presence of metastasis ($P=0.04$). Patients with high CDK4 expression had 1.86 times higher odds of having metastasis compared with those with low CDK4 expression (OR, 1.86; 95% CI, 1.03–3.38).

Conclusion: Overexpression of CDK4 in luminal subtype breast cancer is significantly associated with higher histopathological grade and an increased likelihood of distant metastasis. This suggests CDK4 is a marker of more aggressive tumor biology and a potential prognostic marker in this patient population, warranting further investigation in longitudinal studies.

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INTRODUCTION

Breast cancer represents a significant global health burden. According to GLOBOCAN 2022 data, it is the most frequently diagnosed cancer among women and the leading cause of female cancer mortality worldwide.¹ This global trend is mirrored locally in Indonesia. A study at Wahidin Sudirohusodo Hospital in Makassar identified breast

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cancer as the most common malignancy, accounting for 12.9% of all cancer cases, with a peak incidence in the 40– to 49–year age group.² This underscores the urgent need for research focused on improving risk stratification and management for patients with breast cancer in this region.

Modern breast cancer management relies on a personalized approach guided by molecular subtyping, primarily determined through immunohistochemistry (IHC). The luminal subtypes (hormone receptor–positive/HER2–negative), classified as luminal A and luminal B, are the most prevalent, comprising up to 70% of all cases.³ While generally associated with a better prognosis than other subtypes, luminal breast cancer most likely leads to metastatic disease, which remains the primary cause of mortality.

The molecular driver of proliferation in luminal breast cancer is often linked to the cyclin D–CDK4/6–retinoblastoma (Rb) pathway. Cyclin-dependent kinase 4 (CDK4), along with cyclin D, phosphorylates the Rb protein, thereby releasing the E2F transcription factor and enabling the cell to transition from the G1 (growth) to the S (synthesis) phase of the cell cycle.⁴ Dysregulation of this pathway, commonly through overexpression of cyclin D1 or amplification of the *CDK4* gene, leads to uncontrolled cell division and is a hallmark of estrogen receptor (ER)–positive breast cancer.^{5,6} This biological rationale has led to the successful development of CDK4/6 inhibitors, which have revolutionized the treatment of metastatic luminal breast cancer.

Despite the established role of the CDK4 pathway, its specific utility as a prognostic biomarker for predicting aggressive features like high grade and metastasis is still an area of active investigation, particularly in diverse populations. While studies in Western populations have linked CDK4 expression to prognosis, there is a paucity of data from Southeast Asia, especially Indonesia. Therefore, this study was conducted to address this knowledge gap by examining the association between CDK4 protein expression and both histopathological grade and metastatic status in patients with luminal subtype breast cancer in Makassar, Indonesia.

METHODS

Study design and setting

This cross-sectional study was conducted from December 2022 to April 2023 at Wahidin Sudirohusodo Hospital and Hasanuddin University Hospital, 2 major referral centers in Makassar, Indonesia. All laboratory analyses were performed at the Department of Anatomic Pathology, Faculty of Medicine, Hasanuddin University.

Study population and sampling

A consecutive sampling method was used to recruit patients, resulting in a final sample of 74 women. The inclusion criteria were a diagnosis of luminal breast cancer (ER⁺ and/or progesterone receptor [PR⁺], HER2⁻); age between 17 and 65 years; a body mass index (BMI) within the normal range for Asian populations (18.5–22.9 kg/m²); and provision of informed consent. Patients were excluded if they had other concurrent malignancies, if their clinicopathological data were incomplete, or if their formalin-fixed, paraffin-embedded (FFPE) tissue blocks were unsuitable for immunohistochemical analysis due to poor fixation or extensive necrosis.

Data collection and research procedure

Clinicopathological data, including patient age, histopathological grade (using the Nottingham Grading System), molecular subtypes, and metastatic status (confirmed by imaging or biopsy), were retrieved from patient medical records. All breast cancer patients included in the study provided informed consent, which included an explanation of the benefits and procedures of the research. Medical history taking was conducted to record patient information and examination results according to the research form prepared. Data in this study were divided based on histopathological grading (I, II, or III) and metastasis (presence or absence). Breast tissue samples were collected from patients under sterile conditions and placed in bottles containing 10% buffered formalin solution. All samples were examined via IHC using the CDK4 (DCS–31) monoclonal antibody to assess the nuclear expression of the CDK4 protein within the tumor cells.

Molecular subtypes

Molecular subtypes were determined based on immunohistochemical results for ER, PR, HER2, and the Ki-67 proliferation index. Each staining batch included external positive and negative tissue controls to ensure staining validity. The subtypes were defined as luminal A–like and luminal B–like according to a previous study.^{7–9}

ER and PR expression was considered positive if distinct nuclear staining was detected in 1% or more of tumor cells, using antibodies from GenomeME Lab Inc (Cat No. IHC423-100 for ER and Cat No. IHC751-100 for PR), respectively. The assessment of HER2 status was conducted in accordance with the latest American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines using the HER2/neu antibody (GenomeME Lab Inc, Cat No. IHC042-100). Cases were considered positive for HER2 if



they exhibited an IHC score of 3⁺ (strong, complete, circumferential membrane staining in >10% of tumor cells) and negative for scores of 0 or 1⁺. All cases showing an equivocal (IHC 2⁺) score were reflexively evaluated with in situ hybridization (ISH) to determine the definitive *HER2* gene amplification status.

To determine the Ki-67 proliferation index, tissue slides were treated for 25 minutes with the Ki-67 antibody (clone MIB-1) diluted 1:500. The slides were visualized with diaminobenzidine (DAB) chromogen and counterstained with hematoxylin. The index was calculated as the percentage of positively stained cells among all invasive cells counted. Based on a 20% cutoff point, the results were then categorized as either low Ki-67 or high Ki-67.

Immunohistochemistry for CDK4

Four-micrometer (4- μ m) sections were cut from FFPE tissue blocks. The sections were deparaffinized and rehydrated. Antigen retrieval was performed using a heat-induced epitope retrieval method with citrate buffer (pH 6.0). Slides were then incubated with a concentrated primary monoclonal antibody against CDK4 (Clone: DCS-31; Cat No. CMC47829000; Cell Marque, Rocklin, USA). The antibody was optimized in-house through serial dilutions using control tissue to achieve the best signal-to-noise ratio, and a final dilution of 1:100. A polymer-based detection system was applied, and the reaction was visualized using DAB chromogen, followed by counterstaining with Mayer hematoxylin. Human tonsil tissue was used as a positive control for CDK4 expression,¹⁰ and negative controls were prepared by omitting the primary antibody.

These controls were included in each staining run to ensure specificity, consistency, and reliability of the immunostaining process.

Scoring of CDK4 expression

Two independent pathologists, blinded to the clinical data, evaluated CDK4 nuclear staining. The initial interobserver agreement was substantial, with a Cohen κ coefficient of 0.88. Any cases with discordant initial scores were jointly reevaluated by both pathologists using a multihead microscope to reach a final consensus score. A semiquantitative, binary scoring method was used. Positive expression (high expression or overexpression) was defined as tumor cells exhibiting moderate to strong (2⁺ to 3⁺) nuclear staining in 25% or more of the tumor area. All

other staining patterns were categorized as negative expression (low expression). This threshold was adapted from previously published scoring systems for cell cycle and nuclear proteins in breast cancer that have shown prognostic relevance.^{11,12}

CDK4 expression was assessed semi-quantitatively by evaluating the staining intensity and percentage of stained carcinoma nuclear cell and comparing the results to those of all carcinoma cells. Immunostaining intensity was scored as follows (Figure 1): 0 (negative), 1 (weak), 2 (moderate), and 3 (strong) according to the percentage of area. CDK4 expression was considered overexpressed if staining expression (2⁺ to 3⁺) was found in 25% or more of tumor cells in cancer specimens. CDK4 expression was considered not overexpressed if staining expression (0 to 1⁺) was found in less than 25% of cancer cells in tumor specimens.

Statistical analysis

Data were analyzed using SPSS version 24.0 (IBM Corp). For the purpose of analysis, histopathological grade was dichotomized into low grade (combining grades I and II) and high grade (grade III). This grouping was implemented for 2 key reasons. First, from a statistical standpoint, it ensures more robust analysis by preventing small expected cell counts in the χ^2 test, which could otherwise compromise statistical power given the study's sample size. Second, this approach is clinically and biologically meaningful, as Grade III tumors represent a distinct, highly aggressive phenotype, making the comparison against lower-grade tumors a relevant measure of aggressive biology. The association between CDK4 expression (low vs high) and categorical variables (histopathological grade and metastatic status [no vs yes]) was assessed using the χ^2 test or Fisher exact test where appropriate. The association between CDK4 expression and the Ki-67 proliferation index (categorized as low [$<20\%$] vs high [$\geq 20\%$]) was also assessed using the χ^2 test. The odds ratio (OR) with a 95% CI was calculated using binary logistic regression to estimate the risk of metastasis. A *P* value of <0.05 was considered statistically significant.

To assess whether CDK4 expression was an independent predictor of metastasis, a multivariable logistic regression analysis was performed. Variables with a *P* value of 0.20 or less in the initial univariable analysis were selected for inclusion in the multivariable model to control for potential confounding.

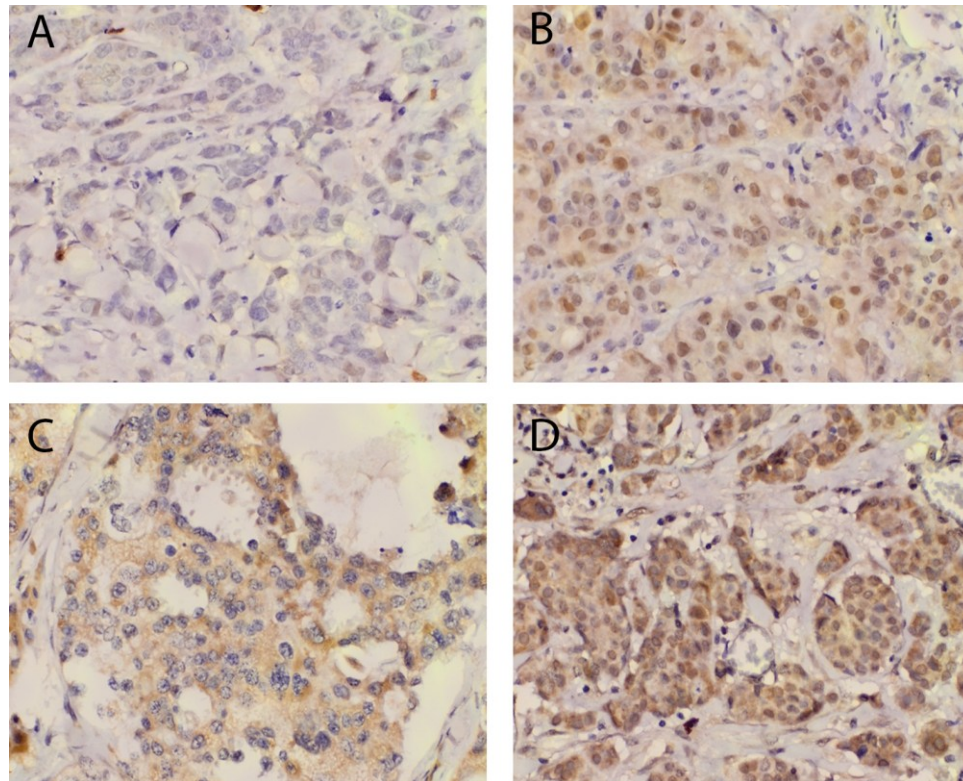


Figure 1. Representative Images of CDK4 Immunohistochemical Staining. A, Low expression, showing negative (0) nuclear staining. B, Low expression, with weak (1+) nuclear staining. C, High expression, demonstrating moderate (2+) nuclear staining. D, High expression, with strong (3+) and diffuse nuclear staining in the majority of tumor cells. All images are shown at $\times 400$ magnification.

The final model included CDK4 expression status, histopathological grade (high vs low/moderate), molecular subtype (luminal B vs luminal A), and patient age (≥ 50 years vs < 50 years). The adjusted odds ratio (aOR) with a 95% CI was calculated.

RESULTS

The demographic and clinicopathological characteristics of the 74 study participants are detailed in Table 1. In summary, this study was predominantly composed of patients aged 50 years and older. The more proliferative luminal B subtype was more common than luminal A, and more than half of the tumor samples showed high CDK4 expression. The most common histological subtype was invasive ductal carcinoma (no special type), and nearly 40% of patients presented with metastatic disease, with bone being the most frequent site of metastasis.

Association between CDK4 expression and histopathological grade

Tumors were grouped into low-grade vs high-grade categories. A significant association with CDK4 expression was observed ($P = 0.04$). High CDK4 expression was present in 69.0% of high-grade

tumors, compared with only 42.2% in the low-grade group (Table 2).

Association between CDK4 expression and metastatic status

CDK4 expression was significantly associated with the presence of distant metastasis in the univariable analysis ($P = 0.04$). To determine if this association was independent of other

clinicopathological factors, a multivariable logistic regression analysis was conducted. The analysis

included histopathological grade, molecular subtype, and age as covariates. After adjusting for these variables, high CDK4 expression remained a significant independent predictor of metastasis (aOR, 2.15; 95% CI, 1.18–4.02; $P = 0.03$). Additionally, high-grade tumors and the luminal B subtype were also independently associated with an increased likelihood of metastasis (Table 3).

Association between CDK4 expression and Ki-67 proliferation index

To further validate the role of CDK4 in cell proliferation, we analyzed its association with the Ki-67 proliferation index. A strong and statistically significant positive association was found between CDK4 expression and the Ki-67 index ($P < 0.001$).



Among tumors with high CDK4 expression, 76.9% (30 of 39) also exhibited a high Ki-67 index. In contrast, only 28.6% (10 of 35) of tumors with low CDK4 expression had a high Ki-67 index (Table 4).

Table 1. Demographic and Clinicopathological Characteristics of Study Participants

| Characteristic | No. (%) (N = 74) |
|--------------------------------|------------------|
| Age, y | |
| <50 | 35 (47.3) |
| ≥50 | 39 (52.7) |
| Menopausal Status | |
| Pre-menopause | 38 (51.3) |
| Menopause | 36 (48.7) |
| Grading | |
| Low grade | 15 (20.3) |
| Moderate grade | 30 (40.5) |
| High grade | 29 (39.2) |
| Subtype | |
| Luminal A | 25 (33.8) |
| Luminal B | 49 (66.2) |
| CDK4 Expression | |
| Positive | 39 (52.7) |
| Negative | 35 (47.3) |
| Metastasis | |
| Yes | 29 (39.2) |
| No | 45 (60.8) |
| Histological Type | |
| Invasive ductal carcinoma, NST | 58 (78.4) |
| Invasive lobular carcinoma | 9 (12.2) |
| Special histological types | 7 (9.4) |
| Metastasis Location | |
| Lung | 8 (27.6) |
| Bone | 10 (34.5) |
| Liver | 3 (10.4) |
| Brain | 1 (3.4) |
| Multiple | 7 (24.1) |

NST, No Special Type

Table 2. Association Between CDK4 Expression and Histopathological Grade

| CDK4 expression | Low grade, No. (%) | High grade, No. (%) | P value ^a |
|-----------------|--------------------|---------------------|----------------------|
| Positive | 19 (42.2) | 20 (69.0) | 0.04 |
| Negative | 26 (57.8) | 9 (31.0) | |

^aCalculated using the χ^2 test.

This study provides critical evidence regarding the role of CDK4 in the biology of luminal subtype breast cancer within an Indonesian patient population. Our principal findings—that high CDK4 expression is significantly associated with high histopathological grade and metastatic status—reaffirm the central role of the cyclin D–CDK4 pathway in driving an

aggressive phenotype.¹³ This analysis, conducted on a specific cohort in Makassar, not only validates findings from other populations but also provides clinically relevant contextual insights.

The strong association between high CDK4 expression and high-grade tumors (69.0% in the high-grade group) is a biologically plausible finding. As a cyclin-dependent kinase, CDK4 functions as a primary engine driving the cell to bypass the G1 restriction point and enter the S (synthesis) phase of the cell cycle.^{14,15} Its overexpression, a frequent characteristic of ER-positive breast cancer, leads to the persistent phosphorylation and inactivation of the Rb protein.^{16,17} This, in turn, liberates the E2F transcription factor to activate genes required for DNA replication, thereby accelerating cell proliferation. This uncontrolled proliferative activity is directly reflected in histopathological assessment, where a high mitotic count is a key component of the Nottingham Grading System, ultimately leading to a high-grade designation.^{18,19} Our findings are consistent with previous studies that have consistently shown a positive correlation between CDK4 expression, the Ki-67 index, and tumor grade, particularly in the more proliferative luminal B subtype.²⁰

Furthermore, to strengthen the role of CDK4 as a key driver of proliferation, our analysis demonstrated a strong positive correlation between high CDK4 expression and a high Ki-67 index. Given that Ki-67 is a well-established marker of actively dividing cells, this finding provides important internal validation for our results. It suggests that the association between high CDK4 expression and aggressive tumor features such as high histological grade and metastasis is fundamentally linked to its biological role in promoting a highly proliferative tumor environment.

An intriguing observation from our data is the high proportion of CDK4 expression (60%) even in low-grade tumors. While seemingly counterintuitive, this may suggest several hypotheses. First, CDK4 overexpression could be an early molecular event in tumorigenesis, essential for initiating tumor growth.

However, progression to a high-grade phenotype may require additional genetic or epigenetic “hits,” such as mutations in tumor suppressor genes like *TP53*. Second, this could reflect the well-known intratumoral heterogeneity of breast cancer. Third, and most clinically relevant, this group of low-grade, high-CDK4 tumors might represent a subpopulation at higher risk for future recurrence or progression, a concept that warrants validation through longitudinal studies.

**Table 3.** Univariable and Multivariable Logistic Regression Analysis for Predictors of Metastasis

| Variable | Univariable analysis | | Multivariable analysis | |
|-------------------------|----------------------|----------------|------------------------|----------------|
| | OR (95% CI) | <i>P</i> value | aOR (95% CI) | <i>P</i> value |
| CDK4 expression | | | | |
| Low | 1 [Reference] | NA | 1 [Reference] | NA |
| High | 1.86 (1.03–3.38) | 0.04 | 2.15 (1.18–4.02) | 0.03 |
| Histopathological grade | | | | |
| Low/moderate | 1 [Reference] | NA | 1 [Reference] | NA |
| High | 2.45 (1.31–4.58) | 0.02 | 2.21 (1.15–4.25) | 0.02 |
| Molecular subtype | | | | |
| Luminal A | 1 [Reference] | NA | 1 [Reference] | NA |
| Luminal B | 2.05 (1.09–3.85) | 0.03 | 1.98 (1.04–3.76) | 0.04 |
| Age, y | | | | |
| <50 | 1 [Reference] | NA | 1 [Reference] | NA |
| ≥50 | 1.32 (0.68–2.56) | 0.19 | 1.25 (0.61–2.49) | 0.25 |

aOR, adjusted odds ratio; NA, not applicable; OR, odds ratio.

Table 4. Association Between CDK4 Expression and Ki-67 Proliferation Index

| CDK4 expression | Low Ki-67 Index (<20%), No. (%) | High Ki-67 Index (≥20%), No. (%) |
|-------------------|---------------------------------|----------------------------------|
| High ^a | 9 (23.1) | 30 (76.9) |
| Low ^a | 25 (71.4) | 10 (28.6) |

^a*P* value <0.001

The significant association between CDK4 and metastasis (OR, 1.86) suggests its role extends beyond merely driving cell division in the primary tumor. The metastatic process is a complex, multistep cascade, and CDK4 likely contributes at several stages. Emerging evidence indicates crosstalk between cell cycle machinery and pathways that regulate cell migration and invasion. CDK4 activity can influence cellular metabolism and potentially interact with signaling pathways that promote epithelial-mesenchymal transition, a process by which cancer cells acquire motile and invasive properties.^{21–25} Furthermore, CDK4 overexpression may help cancer cells evade stress-induced cellular senescence, conferring a survival advantage on circulating tumor cells and enabling them to establish colonies at distant sites.^{22,26,27} Therefore, high CDK4 expression may reflect not only a rapidly growing tumor but also one that is biologically primed for dissemination.

While our study is limited to centers in Makassar, these hospitals serve as major referral hubs for the broader Eastern Indonesian region, suggesting a degree of heterogeneity in our cohort. Given the highly conserved nature of the CDK4 pathway, it is plausible that these findings are generalizable to other Southeast Asian populations, though this warrants validation in multiregional studies. Importantly, in the clinical context of Makassar and similar settings where access to genomic prognostic tests and targeted therapies like CDK4/6 inhibitors is constrained by cost and reimbursement issues, our findings have practical potential by offering a cost-effective method for enhanced risk stratification.

IHC for CDK4 is a relatively affordable and widely available technique. Our results suggest that assessing CDK4 expression could serve as an ancillary, cost-effective tool to identify patients with higher-risk tumor biology. For instance, a patient with a luminal A tumor (traditionally considered low risk) who presents with high CDK4 expression could be identified as being at higher risk, potentially influencing decisions regarding adjuvant therapy. Moreover, the high prevalence of CDK4 expression in this cohort reinforces the biological rationale for the use of CDK4/6 inhibitors—a standard of care in international guidelines such as National Comprehensive Cancer Network guidelines²⁸ and European Society for Medical Oncology guidelines²⁹—in the local patient population.

The primary strength of this study lies in its focus on a Southeast Asian (specifically Indonesian) population, which is often underrepresented in global breast cancer research. This study provides valuable local data that can inform clinical practice. Crucially, our multivariable logistic regression analysis confirms that the association between high CDK4 expression and metastasis is not merely a reflection of its link to proliferation. After adjusting for powerful prognostic indicators such as histopathological grade, molecular subtype, and patient age, high CDK4 expression remained an independent and significant predictor of metastatic disease. This finding strengthens the argument that CDK4's role in promoting metastasis may involve mechanisms beyond simply accelerating cell division, potentially including the regulation of cell motility and invasion pathways.

However, several limitations must be acknowledged. First, the cross-sectional design allows for the identification of associations but precludes the establishment of causality or the tracking of changes over time. Therefore, while our data show a strong association between CDK4



overexpression and metastasis, it cannot be concluded that high CDK4 expression is an independent predictor of future metastatic relapse. Based on these findings and limitations, a further study needs to be conducted as a prospective cohort study. Following nonmetastatic patients from their initial diagnosis will allow us to confirm whether high CDK4 expression is an independent predictor of future metastatic relapse. Second, while our results are statistically significant, the sample size ($n = 74$) is modest, which limits the precision of our estimates and the statistical power for subgroup analyses. Validation in a larger, multicenter cohort would strengthen the findings and enhance their generalizability. Third, the interpretation of IHC staining, despite being standardized with 2 blinded observers, has inherent subjectivity. Finally, while our multivariable model was constructed based on the events per variable (EPV) rule, our final model's EPV of approximately 7 falls slightly below the most conservative threshold of 10. Nevertheless, this provides a more robust estimate than a univariable analysis, and future validation in a larger, multicenter cohort is warranted to confirm these findings and enhance their generalizability.

Future studies should also correlate CDK4 protein expression (via IHC) with *CDK4* gene amplification status (via fluorescence in situ hybridization or next-generation sequencing) to dissect the molecular mechanisms in greater detail. Finally, evaluating the predictive role of CDK4 in the response to CDK4/6 inhibitor therapy within this population would be a critical step toward true precision medicine.

CONCLUSION

In this study, CDK4 overexpression was found to be a significant indicator of aggressive tumor biology, marked by higher histopathological grade and a greater likelihood of metastasis at the time of diagnosis. Our findings support the potential utility of CDK4 as an accessible biomarker for risk stratification in this specific population and underscore the critical role of the CDK4 pathway in driving metastatic luminal breast cancer. Definitive validation of CDK4 as a true prognostic marker for predicting long-term patient outcomes requires future prospective, longitudinal studies.

CONFLICTS OF INTEREST

The authors have no competing interests.

ETHICAL CONSIDERATIONS

The study was approved by the Research Ethics Committee of the Faculty of Medicine Universitas Hasanuddin, Makassar, Indonesia (No.

975/UN4.6.4.5.31/PP36/2024) on November 20, 2024. All participant data were anonymized, confidentiality was maintained, and informed consent was obtained from all individual participants.

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DATA AVAILABILITY

The data used in this study are accessible upon justifiable request.

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AI DISCLOSURE

Artificial intelligence tools were used only for language editing. The authors take full responsibility for the content of the manuscript.

AUTHOR CONTRIBUTIONS

PRI: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing.

BJN: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Software, Validation, Visualization, Writing – original draft.

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