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CDK4 and Ki67 as an important prognostic biomarkers in Breast Cancer

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Abstract
Background:

Breast cancer is a global health concern, affecting many women. Various treatment approaches to breast cancer treatment have been developed, including CDK4/6 inhibitors which can block CDK4/6 and have shown great effectiveness. CDK4 is a specific CDK involved in the G1 phase of the cell cycle. This study aims to investigate the correlation between CDK4 and Ki67 (a marker of cell growth) in breast cancer. By this study, researchers hope to gain new insights into breast cancer and develop effective treatment strategies.

Materials and Methods: CDK4 and Ki67 immunostain was performed on 73 paraffin blocks of patients with breast cancer who had not undergone chemotherapy. Mann-Whitney tests were used to analyze the data, which were then summarized in tables using SPSS 27 software.

Results: There were 62 samples (84.9%) stained with CDK4 and 11 samples (15.1%) unstained with CDK4. There were 35 samples (47.9%) with high Ki67 and 38 samples (52.1%) with low Ki67. Mann Whitney test showed a significant association between CDK4 and Ki67 (p=0.009).

Conclusion: CDK4 increases when Ki67 increases, so these biomarkers can be used to predict a better prognosis and as targeted treatment strategies for breast cancer therapy in the future.

Keywords: CDK4, Ki67, Breast cancer

Introduction

Breast cancer or invasive breast carcinoma is one of the most common cancers in women and it causes morbidity worldwide, so its treatment has received enormous attention. Today researchers are developing CDK4/6 inhibitor approaches (Palleschi M, et al., 2020). Currently,

the FDA (Food and Drug Administration) has approved three CDK4/6 inhibitor drugs for breast cancer patients including palbociclib, ribociclib, and abemaciclib (Pavlovic D et al., 2023).

Based on a 2020 report from The Global Cancer Observatory, the total number of cancer cases worldwide increased significantly, reaching 19.3 million new cases and resulting in 10 million deaths (Sung H et al., 2021). One of the most common cancers that affect women globally is breast cancer with an incidence of 68.8 per 100,000 people and a fatality rate of 22.4 per 100,000 (Andinata B et al., 2023). This data shows us how serious this problem is, both at the national and global levels.

The increasing number of breast cancer encourages researchers to research breast cancer both to determine the causes and risk factors as well as for its therapy. In the last few decades, research on CDK4/6 inhibitors has grown rapidly for the treatment of breast cancer as a therapeutic target (Miskad UA et al., 2021; Sinclair WD and Cui X, 2022; Baker SJ and Reddy EP, 2012). This is interesting for further research, especially about CDK4 and Ki67 as proteins that are expressed in proliferating cells (Miller I et al., 2018).

Cyclin-dependent kinases (CDKs) are a family of serine/threonine kinase enzymes that play a crucial role in regulating development and cell cycle (Juric et al., 2019), and they are involved in cancer pathogenesis. A complex set of genetic and epigenetic mechanisms regulates the production of CDK proteins. However, this regulatory system was dysregulated during the development and progression of cancer. Abnormal CDK activation leads to uncontrolled cancer cell proliferation and induction of cancer stem cell features. CDK can predict the prognosis and future responsiveness to cancer therapy targeting CDKs. Some cancer treatments have been developed to target the abnormal cell cycle by either reducing the production of CDK proteins or inhibiting their function (Ghafouri-Fard S et al., 2022 and Fassl A et al., 2022).

As members of the cyclin-dependent kinase family, CDK4 (Cyclin-dependent kinase 4) is a key regulator of the G1 phase of the cell cycle, with their activity restricted to this phase. The activity of these kinases is critically dependent on interactions with D-type cyclins, specifically D1, D2, and D3 (Baker SJ and Reddy EP, 2012). The binding of cyclins to CDK4 is a more controlled process, regulated by multiple mechanisms. CDK4 inhibitors, such as INK4, which consists of p16INK4A, p15INK4B, p18INK4C, and p19INK4D, are proteins that attach to CDK4 and CDK6, acting as the sole inhibitors of CDK4. INK4 proteins may alter D-cyclin binding to CDK4. Furthermore, INK4 proteins can bind to the catalytic domains of CDK4, potentially inhibiting kinase action (Fassl Anne et al., 2022 and Asghar U et al., 2015).

Proliferation is an important biological parameter in cancer cell development. It refers to the rate at which cells divide and multiply (Uxa S et al., 2021). Ki67, a protein marker suggestive of cell proliferation, plays a crucial role in tumor growth (Uxa S et al., 2021; Sun X and Kaufman PD, 2018; Jing Y et al., 2018). Ki67 appears in developing cells from the first phase to the M stage of the cell cycle, but not in the G0 phase (resting phase) (Sun X and Kaufman PD, 2018 and Jing Y et al., 2018). The subcellular location of Ki67 during mitosis is tightly regulated by the balance between CDK/cyclin-dependent phosphorylation and PP1-mediated dephosphorylation. This intricate regulatory mechanism ensures that Ki67 participates in both the initiation and termination phases of mitosis. In humans, the MK167 gene, a major target of the transcription factor E2F, encodes the Ki67 protein. In its development, Ki67 became a standard in cancer diagnosis and prognosis (Uxa S et al., 2021).

Currently, research into breast cancer treatment using CDK4/6 inhibitors is growing faster and giving good results (Palleschi M, et al., 2020). The purpose of this study was to examine whether CDK4 correlated with Ki67 in invasive breast cancer.

Methods

Tissue samples

The tissue samples were paraffin blocks obtained from the Anatomical Pathology Laboratories of Dr. Wahidin Sudirohusodo Hospital and Hasanuddin University Hospital which were previously diagnosed as invasive breast carcinomas. This study was performed at the Laboratory of Anatomical Pathology of Hasanuddin University Hospital Makassar from August to October 2024

Immunohistochemistry (IHC) procedure and interpretation

Tissues were cut at 4 um thickness and deparaffinized and rehydrated according to the protocol. The standard avidin-biotin-peroxidase complex (ABC) technique was used for immunohistochemistry staining. The heating process used a retrieval solution (Scytek laboratory, USA) at 95^0C for 25 minutes. Immunohistochemical staining using CDK4 (DCS-31) *Mouse Monoclonal Antibody* (Cell Marque, Sigma-Aldrich company, United States) at a dilution of 1:50 was incubated for 1 hour at room temperature. Protein expression was then detected using UltraTek Complete HRP Anti_Polyvalent (DAB) Staining System kit (Scytek laboratories, USA).

The CDK4 expression levels were assessed by labeling these proteins in the cytoplasm and nucleus, and both. This was achieved through immunohistochemical methods and

visualized using a light microscope. CDK4 is strongly expressed when stained in the cytoplasm, and nucleus, and both with high intensity with brown color (score +3), moderately expressed, and both with medium intensity with yellow color (score +2), weakly expressed when yellow pale stained in the cytoplasm, nucleus and both (score +1) and score 0 if basophilic (blue) in the cytoplasm, nucleus and both. CDK4 used cut-off 1% stained in the cytoplasm and nucleus to diagnose negative or positive with weak until strongly expressed stained. The Ki67 staining in the nucleus and proliferation index score is low if stained in nuclear <20% and high if stained in nuclear ≥20% (Rakha EA et al., 2019).

Statistical analysis

SPSS 27 for Windows was used to conduct statistical analysis, and the data was processed using Mann-Whitney tests. A p-value of less than 0.05 indicates statistical significance.

Ethical considerations

The Research Ethics Commission of the Faculty of Medicine, Hasanuddin University has approved this study (Registration number: 680/UN4.6.4.5.31/PP36/2024).

Results

Table 1. Characteristics of the Samples

		n	%
Age	< 50 y.o	36	49.3
	≥50 y.o	37	50.7
Grade	Grade 1	5	6.8
	Grade 2	43	58.9
	Grade 3	25	34.2
CDK4	Stained	62	84.9
	Unstained	11	15.1
KI67	Low	38	52.1
	High	35	47.9
Total		73	100.0

Table 1 shows the sample characteristics from 73 breast cancer patients. The data was categorized based on variables such as age, tumor grade, proliferation index (Ki67), and CDK4. Most patients were ≥50 years old (50.7%), and this indicates that breast cancer is more common in this age group. Tumor grading showed that most tumors were categorized as grade

2 (58.9%), followed by grade 3 (34.2%) and grade 1 (6.8%). CDK4 stained in the nucleus and cytoplasm were 62 samples (84.9%), and not stained were 11 samples (15.1%). Based on the Ki67 index, 52.1% of patients had a low Ki67 proliferation index.

Table 2. Correlation between CDK4 expression with Ki67 in Breast cancer

		CDK4					P value
		Mean	SD	Median	Min	Maks	
KI67	Low	4.00	4.00	2.00	0.00	12.00	0.009**
	High	6.57	4.25	6.00	0.00	12.00	

** Uji Mann Whitney

Using the Mann Whitney test, Table 2 shows that a higher CDK4 compared to the Ki67 low. The p=0.009 value indicates that the relationship between CDK4 and Ki67 is significant.

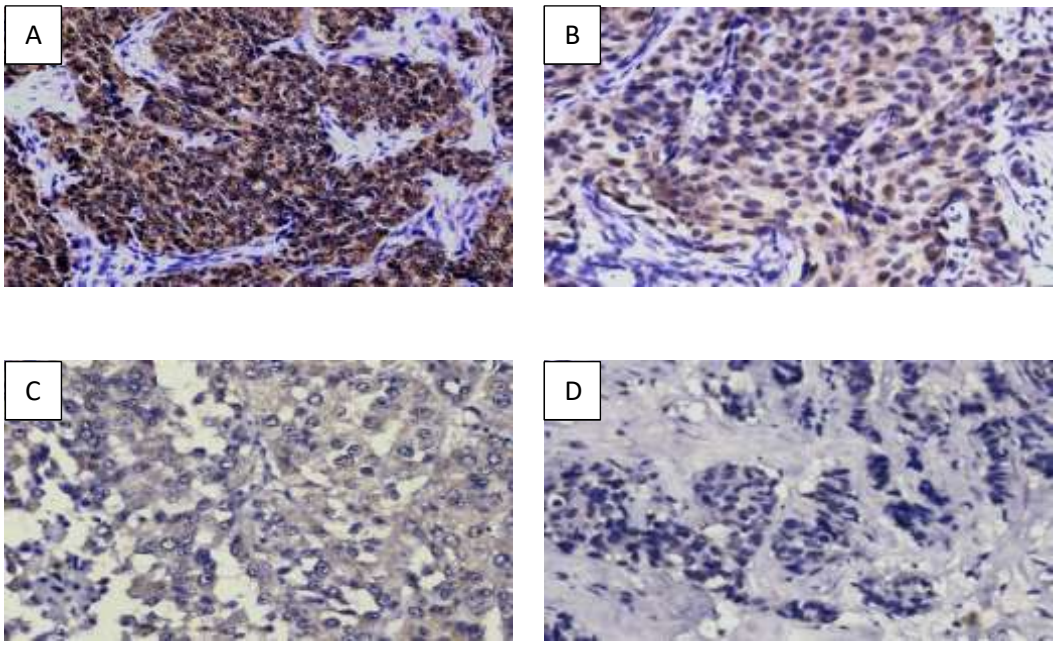

Figure 1. CDK4 Expression in Invasive Breast Cancer (Obj 40x).

A. Strong expression; B. Moderate expression; C. Weak expression; D. Unstained.

Figure 1 shows the intensity of CDK4 in breast cancer samples, CDK4 is colored in the nucleus and cytoplasm. In this study, there were 24 samples with weak intensity, 16 samples with moderate intensity, 22 samples with strong intensity, and only 11 samples were negative (unstained).

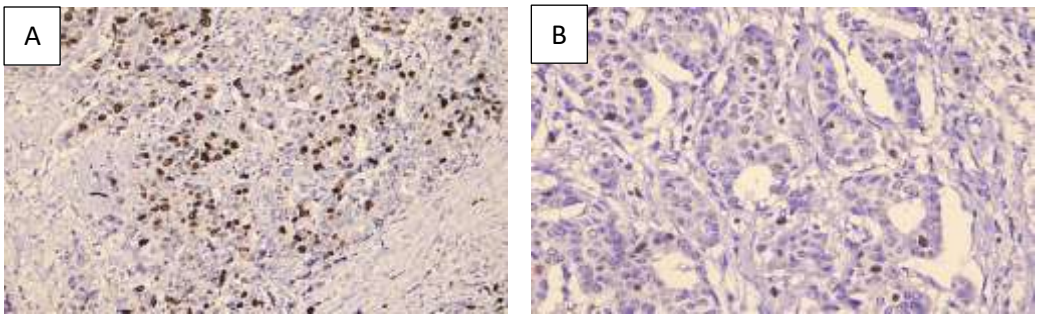

Figure 2. Ki67 Expression in Invasive Breast Cancer (Obj 40x).

A. High expression (≥20%); B. Low expression (<20%).

Figure 2 shows an expression of Ki67 in breast cancer samples, Ki67 is colored in the nucleus. In this study, there were 38 samples (52.1 %) with low expression and 35 samples (47.9 %) with high expression.

Discussion

This study provides an overview of the characteristics of breast cancer patients. The sample has a near-equal distribution between patients less than 50 years old (49.3%) and patients more than 50 years old or older (50.7%). However, a significant portion of cases occurs in older women, particularly those over 50 years old, where the incidence rates increase sharply with age (Hendrick RE et al., 2021). this distribution increased due to hormonal exposure, genetic mutations, changes in breast tissue composition, declining immune function, and various lifestyle factors (Łukasiewicz S et al., 2021).

Based on grade breast cancer, the majority of the patients are classified in grade 2 (58.9%) and grade 3 (34.2%), and a small proportion are grade 1 (6.8%). The prevalence of high-grade breast cancer is attributed to factors such as poor differentiation, increased proliferation rates (Orrantia-Borunda E et al., 2022 and Menon G et al., 2025), genetic instability, late diagnosis, and treatment resistance. These characteristics contribute to the overall aggressiveness of high-grade tumors, leading to worse outcomes for patients diagnosed with this type of breast cancer.

Ki67 shows how fast a tumor cell develops, so if Ki67 is associated with CDK4, it is expected to be an additional biomarker in predictive and therapeutic breast cancer patients. In the Ki67 examination, there is no standardized to determine the cut-off, so in our study, we use a limit of 20% to distinguish low and high Ki67 (Rakha EA et al., 2019) and some studies such as those conducted by Nischal Koirala et al (2022) used a cut-off of 14% for Ki67. (Koirala N et al., 2022). For CDK4 screening we took a 1% cut-off to determine positive and negative.

This study assessed the relationship between CDK4 and Ki67 which showed highly significant results (p=0.009) between the expression of CDK4 with Ki67, mean of CDK4 in the Ki67 high increased (6.57) compared to the Ki67 low group (4.00). This indicates that patients with breast cancer who have a high level of cell proliferation (Ki67 high) tend to have higher CDK4 expression as well. Ki67 and CDK4 show the progressivity of a cancer. Ki67 has a role in the cell cycle in almost all phases of the cell cycle except the G0 phase (Sun X and Kaufman PD, 2018 and Jing Y et al., 2018). While CDK4 plays a role in the G1 phase only where CDK4 causes cell proliferation by encouraging cells to enter the S phase (Asghar U et al., 2015; Baker Stacey J et al., 2022; Ziegler DV et al., 2023).

The formation of a complex between Cyclin D1 and CDK4 leads to the phosphorylation of the retinoblastoma protein (RB). This phosphorylation event releases the transcription factor E2F from its inhibitory association with RB. Consequently, E2F becomes active, promoting the transcription of genes required for DNA synthesis and the transition of the cell cycle from the G1 to the S phase (Zhang C et al., 2024). When CDK4 is active, it facilitates progression through the G1 phase, allowing cells to enter the S phase where Ki67 levels rise as cells prepare for division (Baker SJ and Reddy EP, 2012 and Uxa S et al., 2021). This pathway is closely associated with increased Ki67 expression, which is the marker of active cell proliferation (Zhang C et al., 2024; Witkiewicz AK et al., 2014**).**

CDK4 was significantly associated with unfavorable prognosis and aggressive tumor behavior in several studies. In nasopharyngeal carcinoma, high levels of CDK4 are associated with more advanced stages of cancer and poorer patient survival (Zhou Y et al., 2018). Similarly, in osteosarcoma, elevated levels of CDK4 are associated with the ability of the tumor to spread to other parts of the body and a worse patient outcome. However, one study for breast cancer found no significant difference in serum levels of CDK4 between patients of breast cancer and controls, or among different breast cancer subtypes, and these results are contradictory with another study (Al-Owaidi D et al., 2020).

Based on the results of this study which showed an increase in CDK4 expression with an increase in Ki67 expression in breast cancer samples, it can be concluded that these two biomarkers have a close relationship. These findings have significant clinical implications in the management of breast cancer patients. First, the combination of these two biomarkers may provide more accurate information regarding disease prognosis. Patients with high expression of CDK4 and Ki67 tend to have a worse prognosis and require more aggressive therapy.

Secondly, the results of this study support the development of more targeted treatment strategies. By identifying patients with high expression of CDK4, therapies that inhibit CDK4 activity, such as CDK4/6 inhibitors, can be prescribed.

While the results of this study are significant, it should be noted that some limitations need to be addressed. Firstly, this study was conducted on a limited tissue sample, so further studies in larger populations are needed to confirm these results. Secondly, the molecular mechanisms underlying the relationship between CDK4 and Ki67 still need to be studied further.

Suggestions for future research can analyze CDK4 expression in various subtypes of breast cancer so that the effectiveness of combination therapies targeting CDK4 and other signaling pathways can be evaluated. And to develop a more accurate risk prediction model based on the combination of biomarkers that have been studied.

Conclusion

This study demonstrates the potential of CDK4 as a promising biomarker to predict prognosis and personalized treatment strategies to improve quality of life and patient survival in breast cancer patients.

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Conflict of Interest: All authors state that they have no conflicting interests in this research

Author's Contribution: All authors played a role in the study's conceptualization, development of research methods, data curation, gathering necessary resources, drafting the initial manuscript, and reviewing and editing the manuscript. All authors have approved the final version of the manuscript.

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