Increased KI67 Immunostaining is Associated with Breast Cancer Aggressiveness

Dhafer A. Alghezi[1,*], Rasha Aljawher[2] and Hameed Naeem Mosa[2]

[1]*Microbiology Department, College of Medicine, University of Thi-Qar, Thi-Qar, 64001, Iraq*

[2]*Department of Histopathology and Forensic Medicine, College of Medicine, University of Thi-Qar, Thi-Qar, 64001, Iraq*

Abstract: *Background*: Breast cancer remains one of the most prevalent tumors among females worldwide, and current prognostic methods are limited in their ability to accurately predict tumor aggressiveness and long-term outcomes. KI67 is a nuclear protein marking active cell proliferation and is associated with tumor differentiation, growth, and breast cancer subtypes.

Objective: This study aims to assess KI67 in benign and malignant breast tissues and explore its potential association with clinical outcomes.

Methodology: Immunohistochemistry was used to assess KI67 staining in benign (n=57) and malignant (n=430) breast tissue samples. KI67 expression was then correlated with clinic opathological data, such as grade and stage, and breast cancer molecular subtypes.

Results: Increased KI67 was significantly found in breast carcinoma compared to benign tissues (P <0.0001). A positive association was observed between KI67 immunostaining and tumor grade (P <0.0001), tumor size (P0.007), and molecular subtype (P 0.002).No significant relationship was recognized between KI67 immunostaining and lymph node involvement.

Conclusion: KI67 might be involved in the aggressiveness of malignancy. Further research is needed to determine the significance of KI67 inbreast carcinoma and its potential as a biomarker for breast cancer progression.

Keywords: Breast cancer, Grades, IHC, KI67, Tumor size.

INTRODUCTION

Breast cancer (BC) remains a major global public health challenge, ranking as the most prevalent non-cutaneous malignancy and the second leading cause of cancer-related mortality among women worldwide [1,2]. In 2022, BC accounted for an estimated 2.3 million newly diagnosed cases and approximately 666,000 deaths globally, representing about 23.8% of all female cancer diagnoses and 15.4% of cancer-related fatalities [3]. This disease constitutes the most frequently diagnosed malignancy in Iraq, with 8,299 newly reported cases, comprising 21.2% of all cancer cases [4].

There are different histopathological diagnostic methods utilized in the detection of breast carcinoma, such as Nottingham grading and the Tumor-Node-Metastasis (TNM) staging systems [5]; however, they remain insufficient due to certain limitations. Firstly, the Nottingham system assesses tumor aggressiveness by examining three histological parameters: tubule formation, nuclear pleomorphism, and mitotic index. Based on cumulative scoring, tumors are classified from grade 1 (well-differentiated) to grade 3 (poorly differentiated), with higher grades denoting greater malignancy potential [6]. In addition, the TNM staging system provides an anatomical assessment of disease spread, considering the primary tumor's size and extent (T), regional lymph node involvement (N), and the presence of distant metastases (M). This system is applied both pre-therapeutically and post-surgically to guide treatment decisions and prognostication [7]. Nevertheless, both approaches exhibit notable limitations, as they are insufficiently predictive of tumor aggressiveness and lack accuracy in forecasting post-treatment outcomes such as recurrence or long-term remission [8,9].

In addition to these histopathological methods, prognostic biomarkers, including estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2),have been employed to enhance breast cancer diagnosis and guide therapeutic stratification [10]. Nonetheless, these biomarkers exhibit inherent limitations, as they fail to reliably forecast therapeutic response or recurrence risk. For instance, a subset of ER-positive tumors may acquire resistance to endocrine therapies over time, while HER2-positive tumors can experience relapse despite the administration of targeted agents.

*Address correspondence to this author at the Microbiology Department, College of Medicine, University of Thi-Qar, Thi-Qar, 64001, Iraq;
Tel: +9647825347701; E-mail: Dr.daf79@utq.edu.iq

ISSN: 1929-2260 / E-ISSN: 1929-2279/25

Moreover, triple-negative breast cancer (TNBC), defined by the lack of ER, PR, and HER2 expression, presents significant clinical challenges due to its restricted therapeutic options and intrinsically unfavorable prognosis. Therefore, there is a pressing demand for the identification of novel biomarkers with enhanced predictive accuracy for tumor behavior, therapeutic efficacy, and recurrence potential, thereby advancing the paradigm of personalized and precision oncology [12].

Due to these challenges, it has been observed that the identification of prognostic biomarkers is essential to mitigate such limitations, among which Ki-67 has gained considerable attention. Ki-67 is a nuclear protein expressed during all active phases of the cell cycle (G1, S, G2, and mitosis), but absent in quiescent (G0) cells, making it a reliable marker for evaluating tumor proliferative activity [11]. It has also been found to be closely associated with tumor differentiation and growth as well as breast cancer molecular subtypes [12,13].

Immunohistochemistry (IHC)-based assessment of Ki-67 has been increasingly utilized to stratify patients into prognostic groups and inform therapeutic decisions, particularly for luminal subtypes where treatment selection between endocrine therapy alone or combined chemo-endocrine therapy may depend on proliferation indices [14,15]. Recent studies have demonstrated that higher Ki-67 expression correlates with poor histological grade, advanced tumor stage, and inferior survival outcomes [13,15,16]. However, despite its potential clinical utility, challenges persist regarding the standardization of IHC scoring protocols, cut-off thresholds for high versus low Ki-67 expression, and its reproducibility across different laboratories and populations.

Although extensive international data support the prognostic value of Ki-67, Iraqi studies remain scarce, small, and methodologically inconsistent. Most available studies have either small sample sizes, heterogeneous methodologies, or lack a comprehensive correlation of Ki-67 with established clinicopathological parameters and molecular subtypes.This study addresses that gap by providing the largest standardized evaluation of Ki-67 in an Iraqi cohort, thereby strengthening local evidence and contributing to global reproducibility efforts.Moreover, no consensus exists in Iraq regarding the optimal Ki-67 cut-off value for prognostic and predictive purposes, further emphasizing the need for population-specific research. The present investigation aims to assess the immunohistochemical expression of Ki-67 in breast cancer instances within the Iraqi population and to analyze its association with crucial clinicopathological characteristics, including tumor grade, stage, receptor status (ER, PR, HER2), and molecular subtypes. By addressing this deficiency, the study aspires to elucidate the prognostic significance of Ki-67.

MATERIAL AND METHODS

Patients and Control Samples

This retrospective cohort analysis was approved by the Ethics Committee of the College of Medicine at the University of Thi-Qar and Thi-Qar Health Directorate – Ministry of Health, Iraq (Approval No. 2021159, dated 7/12/2022). A comprehensive examination was conducted on 487 archival formalin-fixed, paraffin-embedded (FFPE) specimens of breast tissue, which included 430 breast carcinomas and 57 benign breast tissue samples serving as controls, all surgically resected between April 2021 and December 2025. These samples were obtained from Al Hussein and Al NasiryjaTeaching Hospitals in Thi-Qar. Demographic and clinical data, encompassing age, histological grading (utilizing the Nottingham grading system), staging (TNM classification), and therapeutic history, were meticulously extracted from histopathological documentation. Patients who had undergone chemotherapy or hormonal therapy were systematically excluded from the study. Negative controls, characterized by the absence of the primary antibody, were employed to ensure the specificity of the immunohistochemical staining process. The clinical data of the breast tissues are detailed in Table **1**.

Immunohistochemistry Protocol for KI67 Immunostaining

Immunohistochemical (IHC) analysis of KI67 protein expression was performed on paraffin-embedded breast tissue sections using the Novolink™ Polymer Detection System (RE7140K, Leica Biosystems, UK), following the methodology described by Alghezi *et. al.* [17].

Breast tissue was initially fixed in formalin, embedded in paraffin, and sectioned at a thickness of 4–5 µm onto positively charged slides. The slides were incubated overnight at 37 °C to enhance tissue adherence. Several pretreatment steps were then applied to these sections. Tissue sections underwent deparaffinization using two changes of Histoclear (H5-

Table 1: The Benign and Malignant Breast Information According to Histopathological Data

Breast histopathological data		Number	Percentage %	P value
Sample size	Benign	56	11.5%	<0.0001
	Malignant	430	88.5%	
Benign (Age range)	<40	20	35.1%	<0.0001
	40-65	21	36.8%	
	>65	16	28.1%	
Cancer (Age range)	<40	66	21.82%	
	40-65	301	69.09%	
	>65	63	9.09%	
Grade	Grade 1	15	3.5%	0.001
	Grade 2	108	25.1%	
	Grade 3	307	71.4	
Stage T	T1	33	7.68%	<0.0001
	T2	196	45.58%	
	T3	80	18.6%	
	T4	38	8.84%	
	N/A	83	19.3%	
Stage N	N0	86	20%	0.0002
	N1	46	10.7%	
	N2	56	13.02%	
	N3	44	10.23%	
	N/A	198	46.05%	
Stage M	M0	20	4.65%	N/A
	M1	0	0%	
	MX	410	95.35%	
Breast cancer subtypes	HER2 positive	92	21.4%	0.022
	Luminal A	`129	30%	
	Luminal B	92	21.4%	
	Triple negative	117	27.2%	

200, National Diagnostics, UK) at 37 °C for 5 minutes each, followed by rehydration through a graded ethanol series (100%, 95%, and 70%; France Alcools, France) for 2 minutes each, and final rinsing in distilled water. Antigen retrieval was then performed in citrate buffer (pH 6.0), heated to 95 °C using the PT Link device (PT200, Agilent Technologies, Denmark), and cooled to 60 °C to ensure optimal epitope exposure. Sections were subsequently washed twice with phosphate-buffered saline (PBS) for 5 minutes each.

Drops of hydrogen peroxide (H_2O_2) were added to these tissue sections and then incubated for 10 minutes at room temperature, followed by an additional PBS wash for10 minutes. To enhance antigen accessibility, Proteinase K was applied for 5 minutes, followed by two PBS washes. At this stage, all pretreatment steps were complete, and the slides were ready for immunostaining.

Tissue sections were incubated overnight at 4 °C with the primary anti-KI67 antibody (cat. no. 1234456; Leica Biosystems, UK). The following day, the sections were washed three times in PBS (10 minutes each), then incubated with the secondary antibody for 30 minutes at 37 °C, followed by two additional PBS washes for 5 minutes each. Signal detection was performed using 3,3′-diaminobenzidine (DAB) chromogen for 5 minutes, resulting in a visible brown precipitate at antigen sites. The reaction was terminated with distilled water.Counterstaining with hematoxylin stain was then carried out on these

sections for a minute at 37 °C to enhance nuclear contrast. Finally, sections were dehydrated, cleared, and mounted with DPX to preserve morphology and staining quality.Slides were examined under a microscope to assess the localization and expression of KI67 proteins. This methodology ensured high specificity and reliable detection, providing valuable insights into the proliferative characteristics of the analyzed breast tissues.

IHC Quantification for KI67

A semi-quantitative scoring system was used to evaluate KI67 immunostaining in breast tissue specimens. This method assessed protein expression by analyzing the percentage of positively stained nuclei and the staining intensity. For each case, five randomly selected microscopic fields were examined to determine KI67 expression levels.

Only distinct nuclear staining in invasive carcinoma cells was considered for evaluation. Ki-67 expression was evaluated using a semiquantitative approach and classified into four categories based on the proportion of immunopositive nuclei: nil (0%, absence of detectable staining), low (≤10%), borderline (10–20%), and high (>20%). This classification system was applied in accordance with previously published Iraqi data and established regional practice [18,19].

The scoring was performed semiquantitatively and categorized as follows: nil (no detectable immunostaining), low (≤10% immunopositive nuclei), borderline (10-20 % immunopositive nuclei), and high (>20% immunoreactive cells [18]. Assessment was conducted across the entire tumor area represented within each tissue section to ensure accuracy and consistency [20].

Statistical Analysis

All statistical procedures were carried out using GraphPad Prism software (version 8.4.2, Windows platform; GraphPad Software, La Jolla, CA, USA; www.graphpad.com). The Shapiro–Wilk test was employed to examine the data for distributional normality. Variables demonstrating normal distribution ($p > 0.05$) were compared between groups using an independent (unpaired) t-test. For datasets that deviated from normality ($p \leq 0.05$), the Mann–Whitney U test was utilized. Associations between categorical variables were assessed via the Chi-square (χ^2) test. A p-value less than 0.05 was interpreted as statistically significant.

RESULTS

Study Cohort and Clinicopathological Characteristics

This study encompassed a total of 487 breast tissue specimens, comprising 430 malignant breast carcinoma cases (88.5%) and 57 benign breast tissue samples (11.5%), demonstrating a statistically significant distribution between the two groups (*P*<0.0001). For benign lesions, the patients were stratified into three age categories: <40 years (n = 20; 35.1%), 40–60 years (n = 21; 36.8%), and >60 years (n = 16; 28.1%). Similarly, malignant breast carcinoma cases were categorized according to the same age brackets: <40 years (n = 66; 21.82%), 40–60 years (n = 301; 69.09%), and >60 years (n = 63; 9.09%). Statistical analysis revealed a significant age-associated difference between benign and malignant cohorts (*P* 0.009).

Histopathological grading of malignant cases was performed according to the Nottingham Grading System. Among the 430 carcinomas, 15 cases (3.5%) were classified as grade 1, 108 cases (25.1%) as grade 2, and 307 cases (71.4%) as grade 3. The distribution across these grades was statistically significant (*P* 0.001). Tumor size was available for approximately 80% of carcinoma cases and categorized as follows: T1 (n = 33; 7.68%), T2 (n = 196; 45.58%), T3 (n = 80; 18.6%), and T4 (n = 38; 8.84%). A significant difference in distribution by tumor size was observed (*P*< 0.0001). Regarding lymph node involvement, malignant specimens were stratified as N0 (n = 86; 20%), N1 (n = 46; 10.7%), N2 (n = 56; 13.02%), and N3 (n = 44; 10.23%). The nodal status distribution also demonstrated statistical significance (*P* 0.001).

Molecular subtyping was performed based on archival immunohistochemical data for ER, PR, and HER2 status. The tumors were classified as HER2-positive (n = 92; 21.4%), luminal A (n = 129; 30%), luminal B (n = 92; 21.4%), and triple-negative breast cancer (TNBC) (n = 117; 27.2%). The differences among these molecular subtypes were statistically significant (*P* 0.002), Table **1**.

KI67 Immunostaining of Breast Tissue Samples

IHC was conducted on both cancerous and non-cancerous breast tissues to assess the immunostaining of KI67. This study indicated the presence of nuclear expression of KI67 in both groups, with staining

intensities varying from robust to faint. In benign breast tissues, diverse nuclear staining of KI76 was recorded, with staining intensity fluctuating from absent (Figure **1A**, red arrow) to strong (Figure **1B**, red arrow). Breast cancer tissues demonstrated nuclear KI67 immunostaining with significant variability in signal intensity, encompassing strong (Figure **1C**, red arrow), moderate (Figure **1D**, red arrow), weak (Figure **1E**, red arrow), and negative immunostaining. The negative control, in which the primary antibody was omitted, exhibited no KI67 staining (Figure **1F**, arrow).

Quantification of KI67 Immunostaining in Breast Tissue

Quantitative examination of IHC data indicated a statistically significant augmentation in nuclear KI67 immunoreactivity within breast cancer specimens when compared with benign breast tissues (P< 0.0001; Table **2** and Figure **2A**). This high nuclear KI67 staining demonstrated a positive correlation with tumor grade as determined by ANOVA analysis (P<0.0001; Table **2** and Figure **2B**). Using a multi-Tukey test, elevated KI67 immunostaining was identified in patients with grade 3 to those with grade 1 (P< 0.0001) or grade 2 (P 0.041). In addition, increased KI67 staining was also observed in grade 2 compared to grade 1 (P0.0012). An increase in nuclear KI67 was also noted in patients with T3-4 compared to those with T1-2 (P0.007; Table **2**, Figure **2C**). A significant correlation between Ki-67 immunostaining and breast cancer subtypes was established (P0.002; Table **2** and Figure **2D**). This notable outcome of KI67 staining was exclusively observed when comparing luminal A patients to those with HER2 positivity (P 0.023) or luminal B (P0.002).

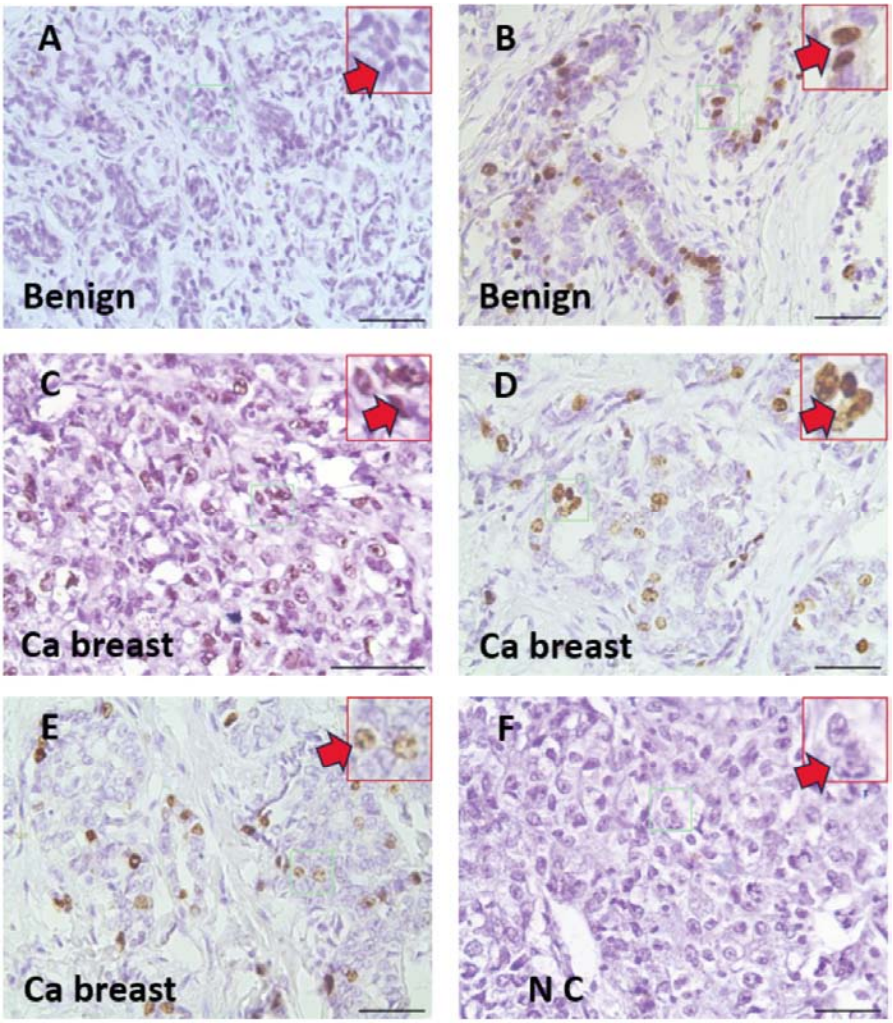

Figure 1: Illustrates the KI67 immunostaining in benign and malignant breast tissues. (**A**) Absence of KI67 immunostaining was noted in benign tissues (Red arrow). (**B**) Strong nuclear KI67 immunostaining was detected in benign tissues (Red arrow). (**C**) Breast cancer tissue had strong nuclear Ki-67 immunostaining (Red arrow). (**D**) Moderate nuclear Ki-67 immunostaining was observed in breast cancer tissues (Red arrow). (**E**) Faint nuclear KI67 staining was identified in malignant breast tissue (Red arrow). (**F**) No background staining was detected in breast tissue, affirming the specificity of the assay. Scale bars = 50 μm; insets illustrate 3x magnification.

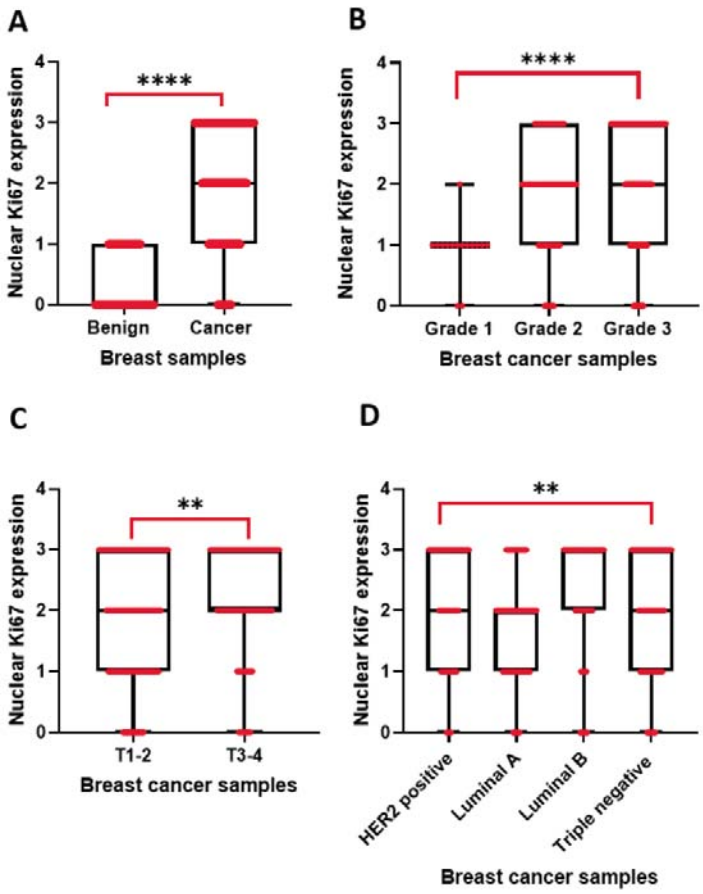

Figure 2: Illustrates the quantification of KI67 immunostaining in breast tissues. **A**) Increased nuclear staining of Ki-67 was observed in breast carcinoma as opposed to benign breast tissues (P <0.0001). **B**) increased KI67 immunostaining was associated significantly with increasing grade. (P <0.0001). **C**) A significant increase in KI67 immunostaining was observed in T3-4 stages compared to T1-2 stages (P 0.007). **D**) There was a significant association between Ki-67 immunostaining and breast cancer subtypes (P 0.002). Benign (n=56), breast cancer (n=430), Grade 1 (N=15), grade 2 (n=108), Grade 3 (n=307), T1-2 (n=227), T3-4 (n=120).

DISCUSSION

The current study represents one of the largest Iraqi datasets (n=430 breast cancers) with systematic Ki-67 scoring and correlation to grade, size, and molecular subtype. While international series have established Ki-67 as a proliferation marker, regional studies are few, often limited by small numbers or heterogeneous scoring. Our study adds novelty by delivering robust, standardized data in a Middle Eastern context, underscoring both similarities with global patterns and the urgent need for local validation of cut-offs and reproducibility standards.

This research investigated KI67immunostaining through IHC analysis in both malignant and non-malignant breast tissues, aiming to evaluate its potential association with key clinicopathological parameters, including tumor grade and stage. Moreover, the study sought to explore the prognostic significance of KI67 , assessing its utility as a predictive biomarker for disease outcome and its possible role in guiding therapeutic strategies for breast cancer management.

The demographic and pathological analyses of the breast tissue samples demonstrated significant associations between age, Nottingham grade, tumor size, and lymph node involvement with breast cancer diagnoses and molecular subtypes. The current study analyzed 487 breast tissue samples, which included 430 malignant breast carcinoma cases (88.5%) and 57 benign breast tissue specimens (11.5%). The statistically significant variation in distribution (P< 0.0001) underscores the predominance of malignant cases within the sample cohort.

The age distribution observed, with marked differences across various age categories in both benign and malignant specimens, highlights the age-dependent incidence of breast abnormalities. This study found that the majority of malignant cases were predominantly in the 40–60 years age group (69.09%), and this aligns with existing literature, which indicates

Table 2: KI67 Quantification in Breast Cancer Histopathological Data

Comparison	SampleNumber	Nuclear KI67 immunostaining			
		Result			P value
Benign vs. Ca breast	56	0.357±0.483	Increased significantly in Cancer		<0.0001
	430	2.00±0.987			
Grades	G1: (n: 15)	1.00±0.655	Increased significantly in high-grade	ANOVA test	<0.0001
				Grade 1 vs. Grade 2	0.0012
	G2: (n:108)	2.03±0.880			
				Grade 1 vs. Grade 3	<0.0001
	G3: (n:307)	2.04±1.01			
				Grade 2 vs. Grade 3	0.041
Stage (T)	T1-2: (n:227)	1.872±1.020	Increased significantly in high tumor size		0.007
	T3-4: (n:120)	2.175±0.9318			
Stage (N)	N0: (n:86)	1.98±1.10	No significant differences		0.8568
	N1-3 (n:146)	1.95±0.949			
Ca breast subtypes	HER2 positive (n:90)	2.16±0.970	Significant association between KI67 staining and breast cancer subtypes	Anova test	0.002
				HER2 positive vs. Luminal A	0.023
	Luminal A (n:129)	1.78± 0.877		HER2 positive vs. Luminal B	0.927
				HER2 positive vs. Triple negative	0.619
	Luminal B (n:90)	2.24±2.24		Luminal A vs. Luminal B	0.002
				Luminal A vs. Triple negative	0.297
	Triple negative (n:117)	1.99±1.01		Luminal B vs. Triple negative	0.243

that breast cancer incidence increases with age, particularly after 40 years [21,22]. Histopathological assessment according to the Nottingham Grading System indicated that 71.4% of malignant cases were classified as grade 3, signifying poorly differentiated tumors. This finding aligns with previous research indicating a higher prevalence of high-grade tumors among breast cancer patients [23].

The significant distribution across histological grades (*P* 0.001) further highlights the importance of histological grading in evaluating tumor aggressiveness and prognosis. Additionally, tumor size evaluation revealed that 45.58% of carcinoma cases were classified as T2, with a significant difference in distribution based on tumor size (*P*< 0.0001). Larger tumor sizes have been associated with increased lymph node involvement and poorer prognosis [24]. The current study's results support the critical role of tumor size as a prognostic indicator in breast cancer.

Lymph node involvement was found in 54% of malignant cases, with a statistically significant difference in the distribution of nodal status (*P*0.001). Positive lymph node status remains a well-documented prognostic factor, correlating with an elevated risk of recurrence and diminished survival rates [25]. Molecular subtyping revealed that 27.2% of cases were identified as triple-negative breast cancer (TNBC), 21.4% as HER2-positive, 21.4% as luminal B, and 30% as luminal A. The significant differences observed among these molecular subtypes (*P* 0.002) are consistent with findings from other studies that have elucidated the distinct biological behaviors and therapeutic responses associated with these subtypes [26].

The present study provides robust evidence that KI67 expression is differentially regulated between benign and malignant breast tissues and is significantly associated with key clinicopathological variables, including histological grade, tumor size, and molecular subtypes.These findings strengthen the argument for considering KI67 as a clinically relevant biomarker of tumor proliferation and aggressiveness. Increased KI67 immunostaining was observed in malignant breast tissues compared to benign tissues (*P*≤0.0001). This agrees with previous breast cancer published data [27].

The finding of the current study supports the significance of KI67 expression as a prognostic biomarker of breast cancer proliferation and growth [13].

This investigation demonstrated a significant correlation between increasedKI67 staining and an increased histological grade, with tumors classified as grade 3 exhibiting substantially greater immunostaining when compared with grade 1 and grade 2 tumors. This is consistent with the previous data [13,19,28-32]. Nonetheless, the definitive threshold values delineating "high" KI67 remain contentious. Although our results clearly advocate for a biological gradient of expression across varying tumor grades, the absence of an international consensus on standardized scoring thresholds hampers the biomarker's reproducibility across different laboratories [34,35]. Collectively, these observations suggest that KI67 may play a critical role in tumor differentiation.However, the precise cut-off values for “high” KI67 remain a matter of controversy. While our findings clearly support a biological gradient of expression across tumor grades, international consensus on standardized scoring thresholds remains elusive, thereby limiting the biomarker’s reproducibility across laboratories [34,35]. Taken together, KI67 may have an important role in tumor differentiation.

In terms of tumor size, this research revealed a distinct correlation between KI67 immunostaining and proliferative activity, with patients presenting advanced T-stage lesions (T3–T4) demonstrating significantly elevated KI67 immunostaining in comparison to those with smaller tumors (T1-2). This association supports the hypothesis that rapidly proliferating tumors are predisposed to attain larger volumes, indicative of a biologically aggressive phenotype. This data agrees with the previous reports [13]. However, it was inconsistent with other data [18,28]. The discrepancies in data may be because of variations in sample size, antigen retrieval protocols, scoring systems, and individual differences. This observation emphasizes the relationship between proliferative activity and tumor aggressiveness, positing that KI67 may function not solely as a biomarker of intrinsic biological behavior but also as a prognostic indicator of disease progression.

Our findings did not reveal a correlation between KI67 and nodal involvement, consistent with previous studies [18,28]. Conversely, another prior study presented results that contradicted our findings [36]. The variability may be attributed to small sample sizes, differing types of antibodies, antigen retrieval methodologies, and individual variation. The potential association with distant metastasis (M stage) could not be definitively established due to a substantial number of cases categorized as MX. Nevertheless, antecedent studies have associated elevated KI67 with enhanced metastatic potential, implying that proliferation may augment cellular motility and invasiveness through dysregulated signaling pathways, such as the PI3K/AKT and MAPK cascades [36].

The analysis of molecular subtypes further elucidated distinct patterns of KI67 immunostaining, revealing that luminal A tumors exhibited the lowest levels of proliferative activity, while HER2-positive, luminal B, and triple-negative breast cancer (TNBC) subtypes demonstrated significantly elevated levels. These outcomes are congruent with the biological characterization of luminal A cancers as indolent and responsive to endocrine therapies, whereas luminal B and HER2-driven cancers are recognized for their increased proliferation and reduced responsiveness to endocrine therapy alone [37,38].

Importantly, TNBC, a clinically challenging subtype, also demonstrated high KI67 expression in our cohort, consistent with previous observations that link this biomarker to the intrinsic aggressiveness of basal-like tumors [33,39]. This finding underscores the potential role of KI67 in guiding therapeutic decisions.

From a clinical perspective, the implications of our findings are significant. Increased KI67 expressionnot only signifies tumor aggressiveness but also possesses potential predictive value concerning therapeutic response. For instance, patients with luminal B or HER2-positive tumors exhibiting high KI67 expression are more likely to derive benefits from chemotherapy in conjunction with endocrine or targeted therapies [33,38] . Similarly, in TNBC, high proliferative activity has been associated with increased chemosensitivity, albeit with poor long-term survival due to the high risk of relapse [39,40]. Therefore, incorporation of KI67 into diagnostic algorithms could facilitate more nuanced treatment decisions, particularly in resource-limited settings where molecular profiling remains inaccessible.

The interpretation of Ki-67 is limited by the lack of a universally accepted cut-off value, and this remains one of the major barriers to global reproducibility.In this study, Ki-67 expression was categorized as nil (0%), low (≤10%), borderline (10–20%), and high (>20%), following previously published Iraqi data and regional practice [19]. This stratification ensured comparability with local studies while reflecting clinically meaningful

proliferative groups.Compared with prior Iraqi studies [19,41] that applied similar thresholds but were limited by smaller sample sizes, our study provides the largest standardized evaluation to date in this setting and corroborates their reported associations with grade and subtype. These results strengthen global reproducibility efforts by confirming consistent findings in a Middle Eastern cohort.At the international level, several alternative thresholds have been proposed. The St. Gallen International Consensus recommended 14% as a cut-off for endocrine therapy decision-making, while a large meta-analysis supported 20% as prognostically informative [33]. The International Ki-67 in Breast Cancer Working Group (IKWG) further suggested ≤5% and ≥30% as rule-out and rule-in thresholds for clinical utility [331,34,35]. To assess robustness, we re-examined our dataset using these alternative thresholds, and the associations of high Ki-67 with higher grade, larger tumor size, and aggressive subtypes remained consistent across all cut-off strategies.In comparison,

Despite the strengths of this study, certain limitations must be acknowledged. Notably, the absence of clinical survival data and follow-up information precludes a definitive assessment of Ki-67 as an independent prognostic biomarker within this cohort. While our findings demonstrate clear associations of Ki-67 with histological grade, tumor size, and molecular subtype, the prognostic value of this marker ultimately depends on its ability to predict recurrence and survival outcomes.International evidence has consistently shown that high Ki-67 expression is associated with worse disease-free and overall survival, particularly in luminal subtypes where it informs treatment selection between endocrine therapy alone or combined chemo-endocrine regimens [13,32,42]. In the Iraqi context, no large-scale studies have yet examined survival in relation to Ki-67, underscoring the importance of prospective, longitudinal investigations. Such studies would allow validation of Ki-67 as an independent prognostic marker in our population and provide evidence to guide its integration into clinical decision-making.

The second linitaion is that the dependence on archival tissue and immunohistochemistry introduces inherent variability related to fixation, antigen retrieval, and scoring methodology. Although our resultsdemonstrated statistically significant associations across multiple clinicopathological variables, the lack of a universally standardized scoring system remains a barrier to global reproducibility [31]. Third, the retrospective design precludes longitudinal analysis of patient outcomes, which would be essential to validate KI67 as an independent prognostic marker in this cohort. Finally, the limited assessment of distant metastasis status restricts our ability to fully explore the relationship between proliferative activity and metastatic dissemination.

CONCLUSION

This study provides the largest standardized evaluation of Ki-67 expression in an Iraqi breast cancer cohort and demonstrates significant associations with higher histological grade, larger tumor size, and aggressive molecular subtypes, though no relationship was observed with nodal status. These findings reinforce Ki-67 as an indicator of tumor proliferation and aggressiveness. Nevertheless, the absence of survival data and reproducibility testing constrains definitive conclusions regarding its independent prognostic value and immediate clinical application.

Future investigations should prioritize prospective validation incorporating survival outcomes, as well as the development and adoption of standardized cut-off thresholds in the Iraqi setting. Furthermore, multicenter studies across diverse populations and the formulation of unified scoring protocols will be essential to establish Ki-67 as a reliable and clinically applicable biomarker. Such efforts will contribute not only to improving prognostic assessment in local practice but also to enhancing global reproducibility and comparability of breast cancer biomarker research.

AUTHOR CONTRIBUTIONS

All authors designed the research outline, collected data. Dhafer didexperimental, statisticalanalysis, and manuscript preparation. All of us evaluated and edited the final document.

ACKNOWLEDGEMENTS

The authors express their gratitude to the Al Hussein teaching hospital staff for their assistance in sample collection.

CONFLICT OF INTEREST

The authors affirm the absence of any conflicts of interest.

REFERENCES

[1] Abdelaziz LA, Ebian HF, Harb OA, Nosery Y, Taha HF, Nawar N. Clinical significance of cytokeratin 19 and OCT4 as survival markers in non-metastatic and metastatic breast cancer patients. Contemp Oncol (Pozn) 2022; 26(1): 78-87. https://doi.org/10.5114/wo.2022.115678

[2] Smolarz B, Nowak AZ, Romanowicz H. Breast Cancer-Epidemiology, Classification, Pathogenesis and Treatment (Review of Literature). Cancers (Basel) 2022; 14(10). https://doi.org/10.3390/cancers14102569

[3] Zhang Y, Ji Y, Liu S, Li J, Wu J, Jin Q, *et al*. Global burden of female breast cancer: new estimates in 2022, temporal trend and future projections up to 2050 based on the latest release from GLOBOCAN. Journal of the National Cancer Center 2025; 5(3): 287-96. https://doi.org/10.1016/j.jncc.2025.02.002

[4] Salih HH, Abd SY, Al-Kaseer E, Al-Diwan J. Cancer in Iraq, general view of annual report 2022. Journal of Contemporary Medical Sciences 2025; 10(6). https://doi.org/10.22317/jcms.v10i6.1676

[5] Teichgraeber DC, Guirguis MS, Whitman GJ. Breast Cancer Staging: Updates in the AJCC Cancer Staging Manual, 8th Edition, and Current Challenges for Radiologists, From the AJR Special Series on Cancer Staging. AJR Am J Roentgenol 2021; 217(2): 278-90. https://doi.org/10.2214/AJR.20.25223

[6] van Dooijeweert C, van Diest PJ, Ellis IO. Grading of invasive breast carcinoma: the way forward. Virchows Arch 2022; 480(1): 33-43. https://doi.org/10.1007/s00428-021-03141-2

[7] Popa MT, Nodiţi A, Peleaşă TM, Stoleru S, Blidaru A. Breast Cancer: A Heterogeneous Pathology. Prognostic and Predictive Factors - A Narrative Review. Chirurgia (Bucur) 2025; 120(1): 32-47. https://doi.org/10.21614/chirurgia.3100

[8] Alghezi DA, P W, BeresfordM, R B, J M, D CA. Reduced β-catenin immunostaining in prostate cancer and negatively associated with poorly differentiated Gleason grades. Kerbala journal of pharmacy and pharmaceutical science 2021; 1(19): 179-95.

[9] Alalwany O, Alghezi DA, Aljawher RQ, Harb A. Increased CD3 immunostaining associated with high grade and tumor size in colorectal carcinoma. Egyptian Journal of Medical Microbiology 2025; 34(1): 205-12. https://doi.org/10.21608/ejmm.2024.331363.1364

[10] Harbeck N, Penault-Llorca F, Cortes J, Gnant M, Houssami N, Poortmans P, *et al*. Breast cancer. Nature Reviews Disease Primers 2019; 5(1): 66. https://doi.org/10.1038/s41572-019-0111-2

[11] Davey MG, Hynes SO, Kerin MJ, Miller N, Lowery AJ. Ki-67 as a Prognostic Biomarker in Invasive Breast Cancer. Cancers (Basel) 2021; 13(17). https://doi.org/10.3390/cancers13174455

[12] Wang W, Gong Y, Chen B, Guo H, Wang Q, Li J, *et al*. Quantitative immunohistochemistry analysis of breast Ki67 based on artificial intelligence. Open Life Sci 2024; 19(1): 20221013. https://doi.org/10.1515/biol-2022-1013

[13] Probert J, Dodwell D, Broggio J, Charman J, Dowsett M, Kerr A, *et al*. Ki67 and breast cancer mortality in women with invasive breast cancer. JNCI Cancer Spectr 2023; 7(5). https://doi.org/10.1093/jncics/pkad054

[14] Aleskandarany MA, Green AR, Benhasouna AA, Barros FF, Neal K, Reis-Filho JS, *et al*. Prognostic value of proliferation assay in the luminal, HER2-positive, and triple-negative biologic classes of breast cancer. Breast Cancer Research 2012; 14(1): R3. https://doi.org/10.1186/bcr3084

[15] Höller A, Nguyen-Sträuli BD, Frauchiger-Heuer H, Ring A. "Diagnostic and Prognostic Biomarkers of Luminal Breast Cancer: Where are We Now?" Breast Cancer (Dove Med Press) 2023; 15: 525-40. https://doi.org/10.2147/BCTT.S340741

[16] Sandilya U, K M. Expression of Ki-67 in Invasive Breast Carcinoma and Its Correlation With Different Clinicopathological Features. Cureus 2024; 16(9): e69820. https://doi.org/10.7759/cureus.69820

[17] Alghezi D, Aljawher R, Musawi S. Increased CD73 expression is associated with poorly differentiated Gle ason score and tumor size in prostate cancer. J Adv Biotechnol Exp Ther 2023; 6(1): 161. https://doi.org/10.5455/jabet.2023.d115

[18] Soliman NA, Yussif SM. Ki-67 as a prognostic marker according to breast cancer molecular subtype. Cancer Biology and Medicine 2016; 13(4): 496-504. https://doi.org/10.20892/j.issn.2095-3941.2016.0066

[19] Mahdi AS, Ibrahim HH, Mohammed AA. Ki-67 Expression as an Indicator of Invasiveness in Patients with Breast Cancer. Medical Journal of Babylon 2018; 15(4): 271-5. https://doi.org/10.4103/MJBL.MJBL_54_18

[20] Hoda SA, Brogi E, Koerner FC, Rosen PP. Rosen's breast pathology: Fourth edition2014. 1-1400 p.

[21] Verdial FC, Etzioni R, Duggan C, Anderson BO. Demographic changes in breast cancer incidence, stage at diagnosis and age associated with population-based mammographic screening. J Surg Oncol 2017; 115(5): 517-22. https://doi.org/10.1002/jso.24579

[22] Hendrick RE, Monticciolo DL, Biggs KW, Malak SF. Age distributions of breast cancer diagnosis and mortality by race and ethnicity in US women. Cancer 2021; 127(23): 4384-92. https://doi.org/10.1002/cncr.33846

[23] Kim JS, Lee JH, Yeon Y, An D, Kim SJ, Noh M-G, *et al*. Predicting Nottingham grade in breast cancer digital pathology using a foundation model. Breast Cancer Research 2025; 27(1): 58. https://doi.org/10.1186/s13058-025-02019-4

[24] Narod SA. Tumour size predicts long-term survival among women with lymph node-positive breast cancer. Curr Oncol 2012; 19(5): 249-53. https://doi.org/10.3747/co.19.1043

[25] Tonellotto F, Bergmann A, de Souza Abrahão K, de Aguiar SS, Bello MA, Thuler LCS. Impact of Number of Positive Lymph Nodes and Lymph Node Ratio on Survival of Women with Node-Positive Breast Cancer. Eur J Breast Health 2019; 15(2): 76-84. https://doi.org/10.5152/ejbh.2019.4414

[26] Wang J, Li B, Luo M, Huang J, Zhang K, Zheng S, *et al*. Progression from ductal carcinoma in situ to invasive breast cancer: molecular features and clinical significance. Signal Transduct Target Ther 2024; 9(1): 83. https://doi.org/10.1038/s41392-024-01779-3

[27] Feriancová M, Walter I, Singer CF, Gazdarica J, Pohlodek K. Expression of COX-2, p16, and Ki67 in the range from normal breast tissue to breast cancer. Neoplasma 2021; 68(2): 342-51. https://doi.org/10.4149/neo_2020_200731N798

[28] Tan P-H, Bay B-H, Yip G, Selvarajan S, Tan P, Wu J, *et al*. Immunohistochemical detection of Ki67 in breast cancer correlates with transcriptional regulation of genes related to apoptosis and cell death. Modern Pathology 2005; 18(3): 374-81. https://doi.org/10.1038/modpathol.3800254

[29] Yousef E, Furrer D, Laperriere D, Tahir R, Mader S, Diorio C, *et al*. MCM2: An alternative to Ki-67 for measuring breast cancer cell proliferation. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc 2017; 30. https://doi.org/10.1038/modpathol.2016.231

[30] Ma Q, Liu YB, She T, Liu XL. The Role of Ki-67 in HR+/HER2- Breast Cancer: A Real-World Study of 956 Patients. Breast Cancer (Dove Med Press) 2024; 16: 117-26. https://doi.org/10.2147/BCTT.S451617

[31] Dowsett M, Nielsen TO, A'Hern R, Bartlett J, Coombes RC, Cuzick J, *et al*. Assessment of Ki67 in breast cancer: recommendations from the International Ki67 in Breast Cancer working group. J Natl Cancer Inst 2011; 103(22): 1656-64. https://doi.org/10.1093/jnci/djr393

[32] Inwald EC, Klinkhammer-Schalke M, Hofstädter F, Zeman F, Koller M, Gerstenhauer M, *et al*. Ki-67 is a prognostic parameter in breast cancer patients: results of a large population-based cohort of a cancer registry. Breast Cancer Res Treat 2013; 139(2): 539-52. https://doi.org/10.1007/s10549-013-2560-8

[33] Petrelli F, Viale G, Cabiddu M, Barni S. Prognostic value of different cut-off levels of Ki-67 in breast cancer: a systematic review and meta-analysis of 64,196 patients. Breast Cancer Res Treat 2015; 153(3): 477-91. https://doi.org/10.1007/s10549-015-3559-0

[34] Nielsen TO, Leung SCY, Rimm DL, Dodson A, Acs B, Badve S, *et al*. Assessment of Ki67 in Breast Cancer: Updated Recommendations From the International Ki67 in Breast Cancer Working Group. J Natl Cancer Inst 2021; 113(7): 808-19. https://doi.org/10.1093/jnci/djaa201

[35] Polley MY, Leung SC, McShane LM, Gao D, Hugh JC, Mastropasqua MG, *et al*. An international Ki67 reproducibility study. J Natl Cancer Inst 2013; 105(24): 1897-906. https://doi.org/10.1093/jnci/djt306

[36] Sun J, Chen C, Wei W, Zheng H, Yuan J, Tu YI, *et al*. Associations and indications of Ki67 expression with clinicopathological parameters and molecular subtypes in invasive breast cancer: A population-based study. Oncol Lett 2015; 10(3): 1741-8. https://doi.org/10.3892/ol.2015.3461

[37] Goldhirsch A, Winer EP, Coates AS, Gelber RD, Piccart-Gebhart M, Thürlimann B, *et al*. Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013. Ann Oncol 2013; 24(9): 2206-23. https://doi.org/10.1093/annonc/mdt303

[38] Denkert C, Budczies J, von Minckwitz G, Wienert S, Loibl S, Klauschen F. Strategies for developing Ki67 as a useful biomarker in breast cancer. Breast 2015; 24 Suppl 2: S67-72. https://doi.org/10.1016/j.breast.2015.07.017

[39] Blows FM, Driver KE, Schmidt MK, Broeks A, van Leeuwen FE, Wesseling J, *et al*. Subtyping of breast cancer by immunohistochemistry to investigate a relationship between subtype and short and long term survival: a collaborative analysis of data for 10,159 cases from 12 studies. PLoS Med 2010; 7(5): e1000279. https://doi.org/10.1371/journal.pmed.1000279

[40] Denkert C, Loibl S, Müller BM, Eidtmann H, Schmitt WD, Eiermann W, *et al*. Ki67 levels as predictive and prognostic parameters in pretherapeutic breast cancer core biopsies: a translational investigation in the neoadjuvant GeparTrio trial. Ann Oncol 2013; 24(11): 2786-93. https://doi.org/10.1093/annonc/mdt350

[41] Jumaah AS, Shaker RA, Al-Ali Z, Altoriah KMJ, Al-Quzweni A, Jumaah SS, *et al*. Immunohistochemical Breast Cancer Profiling Among Iraqi Women: Molecular Subtype Classification, Clinicopathology Associations, and Treatment-Decision Making Tools: A Cross-Sectional Study. Health Sci Rep 2025; 8(5): e70553. https://doi.org/10.1002/hsr2.70553

[42] Dowsett M, Nielsen TO, A'Hern R, Bartlett J, Coombes RC, Cuzick J, *et al*. Assessment of Ki67 in Breast Cancer: Recommendations from the International Ki67 in Breast Cancer Working Group. JNCI: Journal of the National Cancer Institute 2011; 103(22): 1656-64. https://doi.org/10.1093/jnci/djr39

Received on 05-08-2025 Accepted on 02-09-2025 Published on 08-10-2025

https://doi.org/10.30683/1929-2279.2025.14.19