

ORIGINAL ARTICLE

Ki-67 as a prognostic marker according to breast cancer molecular subtype

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ABSTRACT

Objective: Ki-67 plays an important function in cell division, but its exact role is still unknown. Moreover, few works regarding its overall function were published. The present study evaluated the clinical significance of Ki-67 index as a prognostic marker and predictor of recurrence in different molecular subtypes of breast cancer. The relationship of Ki-67 index with different clinicopathological factors was also analyzed.

Methods: Ki-67 index was measured in 107 cases of primary breast cancer from 2010-2012. These patients were evaluated for estrogen receptor, progesterone receptor, and HER2. Ki-67 was divided according to percentage levels: < 15% and > 15%. Follow-up ranged from 32 months up to 6 years.

Results: Approximately 44, 23, 15, and 25 cases were grouped as luminal A, luminal B, HER2 subtype, and triple-negative (TN), respectively. No luminal A patients showed Ki-67 level higher than 15%, and their recurrence was 20%. In luminal B group, Ki-67 level higher than 15% was observed in 69% of patients, and recurrence was 39%. In HER2 subtype, Ki-67 was higher than 15% in 34% of cases, and recurrence was 40%. In triple-negative cases, Ki-67 was higher than 15% in 60% of cases, and recurrence was detected in 32% of patients. Patients with Ki-67 less than 15% displayed better overall survival than those with Ki-67 higher than 15% ($P = 0.01$). Patients with Ki-67 higher than 15% exhibited higher incidence of metastasis and recurrence than those with Ki-67 less than 15% ($P = 0.000$).

Conclusions: Ki-67 may be considered as a valuable biomarker in breast cancer patients.

KEYWORDS

Ki-67; prognostic; molecular subtypes; breast cancer

Introduction

Breast cancer is a heterogeneous disease with several biological subtypes¹. Conventional clinical factors, such as tumor grade, size, lymph node involvement, and surgical margin, are not sufficient as the only prognostic factors; therefore, breast cancer subtype should be considered in making treatment decisions².

Four main breast cancer subtypes have been identified according to estrogen receptor (ER), progesterone receptor (PR), and HER2. These subtypes include luminal types A and B, basal-like, and HER2-enriched subtype³. Luminal A is the most common breast cancer subtype and characterized by ER⁺ and/or PR⁺/HER2⁻ status, low-grade tumor, and good prognosis⁴⁻⁶. Luminal B subtype accounts for approximately

10% of all breast cancers and is distinguished by ER⁺ and/or PR/HER2⁻ status⁷. Luminal B-like (HER2 positive) is characterized by ER⁺, HER2 overexpression or amplification, and any Ki-67 or PR⁸.

Differentiation of luminal A from luminal B/HER2⁻ breast cancers results in important therapeutic implications. Hence, the Saint Gallen Guidelines recommended the assessment of the Ki-67 proliferation index⁹. Luminal B breast cancer should show a higher proliferation index than Luminal A; however, the Ki-67 cut-off point for differentiating these two categories has changed over time⁷. Breast cancer subtypes with negative ER, PR, and HER2 status are typically called “triple-negative” breast cancers and approximate the basal-like category. The basal-like subtype is common in premenopausal, young, and overweight patients. This subtype is also associated with high-grade tumors^{4,6,10}. HER2-enriched subtype (HER2⁺/ER⁻/PR⁻) is less common but is similarly characterized by high-grade tumors and poor outcomes⁴.

Uncontrolled proliferation is a distinct characteristic of malignancy and may be assessed through various methods,

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including counting mitotic figures in stained tissue sections, incorporation of labeled nucleotides into DNA, and flow cytometric evaluation of cell fraction in S phase¹¹. Dowsett et al.¹² reviewed that the most common measurement involves immunohistochemical assessment of Ki-67 antigen.

Ki-67 is present in all proliferating cells, and its role as a proliferation marker attracts considerable interest. Ki-67 is a nuclear nonhistone protein present in all active phases of cell cycle, except the G₀ phase¹³. Moreover, Ki-67 is among the 21 prospectively selected genes included in the Oncotype DXTM assay used to predict the risk of recurrence and extent of chemotherapy benefits in women with node-negative, ER⁺ breast cancers^{14,15}. The proliferation biomarker Ki-67 is also considered a prognostic factor for breast cancer and has been investigated in several studies^{16,17}.

In spite of consistent data on Ki-67 as a prognostic marker in early breast cancer, its role in breast cancer management remains uncertain. Potential uses of Ki-67 include prognosis of relative responsiveness, resistance to chemotherapy or endocrine therapy, estimation of residual risk in patients on standard therapy, and as a dynamic biomarker of treatment efficacy in samples obtained before, during, and after neoadjuvant therapy, particularly neoadjuvant endocrine therapy¹².

In the present study, we analyzed the relationship of Ki-67 index with clinicopathological factors in 107 cases of breast cancer, as well as with prognosis [disease-free survival (DFS) and overall survival (OS)], according to breast cancer subtypes, namely, luminal, HER2, and triple-negative.

Materials and methods

A total of 107 selected cases of invasive breast carcinoma were collected retrospectively from Mansoura University, Faculty of Medicine, Oncology Center, Egypt between January 2010 and December 2012. All cases underwent modified radical mastectomy operations and received postoperative hormonal, chemotherapy, or radiotherapy. Postoperative follow-up was performed periodically, and data were collected until August 2015. Follow-up period ranged within 32–68 months, with a median follow-up of 37 ± 20.51 months. This study was approved by the ethics committee of Mansoura University.

Hematoxylin and eosin-stained slides (cut from formalin-fixed, paraffin wax-embedded specimens) were retrieved from the archive of the oncology center and reviewed. Tumors were diagnosed according to the WHO classification 2012¹⁸. A total of 101 (94.4%) cases were diagnosed as invasive ductal carcinoma (IDC), not otherwise specified (NOS). Five (4.7%) cases were diagnosed as invasive lobular

carcinoma. One case was diagnosed as mucinous carcinoma (0.9%). Tumors were graded according to Nottingham Grading System¹⁹.

Tissue microarray construction

Manual tissue microarray (TMA) was assembled using a mechanical pencil tip^{20,21}. Cores from the surrounding normal breast tissue were also taken as an internal control.

Immunohistochemistry (IHC)

The constructed TMA blocks were recut at a thickness of 3–4 μm on coated slides, deparaffinized, and rehydrated in descending grades of alcohol into water. Antigen retrieval was conducted using citrate buffer at pH according to the type of primary antibody and via microwave heating for 10 min. Subsequently, the sections were incubated in 3% H₂O₂ blocking medium for 5 min, washed with distilled water, and incubated for 60 min at room temperature with mouse monoclonal primary antibodies against the following antigens: ER (1D5, 1:50; pH, 7.3; Dako, San Jose, USA), PR (PR 636, 1:50; pH, 7.3; Dako, San Jose, USA), HER2/neu (CB11, 1:50; pH, 7.3; Novocastra, Newcastle, U.K), and cell marque Ki-67 (sp6) rabbit monoclonal antibody (REF275R-18). Immunodetection was performed using Dako RealTM EnVisionTM system, peroxidase/DAB+, Rabbit/Mouse (Code: K5007, Dako, Glostrup, Denmark) with Dako automated immunostaining instruments. Staining was performed according to the manufacturer's instructions. Immunoreaction was visualized through adding DAB (Code: K5007) for 3 min. The slides were counterstained with Dako REAL hematoxylin (Code: S2020) for 1 min and cover slipped with mounting media. Internal positive controls were normal breast duct epithelia for ER and PR. Positive external controls were ER, PR, and HER2/neu-positive breast carcinomas for ER, PR, and HER2/neu, respectively. Negative controls were assessed via replacing primary antibody with PBS.

IHC evaluation

Tumors were considered positive for ER and PR when at least 1% of the tumor cells showed unequivocal nuclear staining according to ASCO/CAP guidelines²². HER2/neu was scored according to the pattern of membranous staining and percentage of stained malignant cells as follows: 0, no staining or faint incomplete staining in < 10% of cells; 1, faint incomplete staining in > 10% of cells; 2, weak to moderate complete staining in > 10% of cells; and 3, strong complete staining in > 10% of cells. Only score 3 was

considered positive²³. Different molecular subtypes were assessed after evaluating ER, PR, and HER2/neu based on IHC results.

Interpretation of Ki-67 staining and scoring

Ki-67 is a nuclear protein. Cytoplasmic staining and occasional membrane staining of Ki-67 can occur with MIB1 antibody and should be ignored when scoring Ki-67. Only nuclear staining (plus mitotic figures stained with Ki-67) should be incorporated into the Ki-67 score that is defined as the percentage of positively stained cells among the total number of malignant cells scored. Similar to other IHC stains, internal positive controls, such as mitotic figures, normal ducts, and lymphocytes, and endothelial and stromal cells (lesser extent), are helpful^{11,12}.

When staining is homogenous, at least three randomly selected, high-power ($\times 40$ objective) fields should be counted. However, biological heterogeneity of Ki-67 staining can occur across specimens; in this case, scoring should be from the tumor edge or hot spots. For the former, three fields should be scored at tumor periphery because the invasive edge is widely considered the most biologically active part and most probable to drive the disease outcome. Hot spots are areas where Ki-67 staining is particularly prevalent^{11,12}.

The fraction of proliferating cells was based on a count of at least 500 tumor cells. The Ki-67 values were expressed as the percentage of positive cells in each case. Cases with $> 15\%$ positive nuclei were classified as high Ki-67 expression, and those with $< 15\%$ were classified as low Ki-67 expression^{24,25}.

Statistical analysis

Data were tabulated, coded, and analyzed using SPSS version 17.0. Descriptive statistics was presented as mean \pm standard deviation and frequency (number-percent). Chi square test (χ^2 -value) was used for intergroup comparison of categorical data. Kaplan-Meier test was used to test the equality of survival distribution among Ki-67 categories. In addition, prognostic significance of the Ki-67 index in each molecular subtype was investigated.

The IHC expression of Ki-67 was correlated with clinical and histopathological features of breast carcinoma, including the patient's age, tumor size, histological type, tumor grade, nodal status, and patient outcome.

Results

This study was carried out retrospectively on 107 patients

with invasive breast carcinomas. The mean age of the patients was 54.6 ± 12 years, with an age range of 31–88 years. The different clinicopathological features of cases are shown in **Table 1**.

Table 1 shows that among the 107 cases, 101 cases were IDC NOS (94.4%), 5 cases were invasive lobular carcinoma (ILC) (4.9%) cases, and only 1 case was mucinous carcinoma (0.9%). Approximately 42% of the cases were grade 2, and 95% of the cases displayed tumor size of more than 2 cm. About 50% of the cases showed a mitotic count of 11–22/10 HPF. Approximately 75% of the patients exhibited pathologically positive lymph nodes, and 53% of the cases were in stage III. Additionally, 30% of the cases developed distant metastasis and recurrence, and 20% of the cases were dead. In terms of biological markers, the ER⁺ and PR⁺ rates were 57% and 59%, respectively. Approximately 19% of cases were HER2⁺ (score, 3+). Ki-67 nuclear positivity of more than 15% was detected in 34% of the cases (**Figure 1**). According to this immunophenotyping, the cases used in this study were classified as luminal A, luminal B, HER2, and triple-negative in 41%, 21%, 14%, and 23% of the cases, respectively.

Patient and tumor characteristics in relation to different molecular subtypes

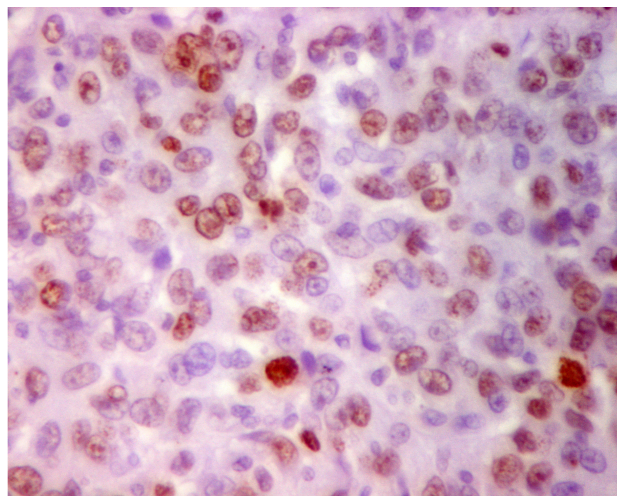
Table 2 reveals statistically significant association among molecular subtypes of cases with both tumor grade and Ki-67 positivity (*P* values of 0.005 and 0.00, respectively). Luminal A cases showed the highest proportion of grades 1 and 2 cases (45% and 50%, respectively). HER2 and triple-negative subtypes comprised a high proportion of grades 3 (53% and 32%, respectively) and 2 cases (20% and 44%, respectively). Ki-67 $< 15\%$ was present in 100% of luminal A, 31% of luminal B, 66% of HER2, and 40% of triple-negative cases. Ki-67 $> 15\%$ was present in 0% of luminal A, 69% of luminal B, 34% of HER2, and 60% of triple-negative cases. No statistically significant association was observed among molecular subtypes of the studied cases and age, mitotic count, tumor size, nodal status, stage, histological subtype, or patient outcome (death, recurrence, or metastasis).

Relationship of Ki-67 expression with clinicopathological characteristics of breast carcinoma

Table 3 shows that high Ki-67 expression ($> 15\%$) was present in 36 cases (33%). Furthermore, 39% and 44% of Ki-

Table 1 The clinicopathological features of the studied cases

Characteristics	n	%
Tumor grade		
G ₁	38	35.5
G ₂	45	42.1
G ₃	24	22.4
Mitotic count		
M1	48	44.9
M2	54	50.5
M3	5	4.7
Tumor size, cm		
<2	5	4.7
>2	102	95.3
Lymph node		
Negative	26	24.3
Positive	81	75.7
Tumor stage		
Stage I	2	1.9
Stage II	48	44.9
Stage III	57	53.3
Alive or dead		
Alive	84	79.0
Dead	23	21.0
Metastasis or recurrence		
Negative	75	70.0
Positive	32	30.0
Histological type		
IDC	101	94.4
ILC	5	4.7
Mucinous	1	0.9
ER		
Negative	50	46.7
Positive	57	53.3
PR		
Negative	48	44.9
Positive	59	55.1
HER2		
Negative	86	80.4
Positive	21	19.6
Ki-67		
<15	71	66.2
>15	36	33.8
Molecular type		
HER2	15	14.0
Lumial A	44	41.1
Lumial B	23	21.5
Triple -negative	25	23.4

**Figure 1** Nuclear positivity for Ki-67 in more than 15% of tumor cells with variable staining intensity (IHC, 400×).

67 > 15% positive cases were grades II and III, respectively, with statistically significant associations between Ki-67 > 15% expression and tumor grade ($P = 0.00$). A statistically significant association was also observed between mitotic count (M) and Ki-67 positivity, where 25%, 67%, and 8% of Ki-67 cases with > 15% positivity were M1, M2, and M3, respectively ($P = 0.01$). No statistically significant association was observed among age, tumor size, lymph node status, tumor stage, histological type, and Ki-67 positivity.

Relationship of Ki-67 expression with molecular subtypes, IHC characteristics of breast carcinoma, and patient outcomes

Table 4 shows that high Ki-67 expression (> 15%) was negatively associated with ER and PR, with P values of < 0.02 and 0.01, respectively. A statistically significant association was also found among Ki-67 expression and molecular subtypes, incidence of recurrence and metastasis ($P = 0.000$), and OS ($P = 0.01$).

Test of equality of survival distributions for different levels of Ki-67 in the studied cases

Figure 2 shows that patients with Ki-67 < 15% exhibited better OS than those with Ki-67 > 15% ($P = 0.012$). **Figure 3** illustrates that patients with Ki-67 > 15% were more likely to develop recurrence and distant metastasis than those with Ki-67 < 15% ($P = 0.000$).

Table 2 Patient and tumor characteristics in relation to different molecular subtypes, *n* (%).

Characteristics	Luminal A	Luminal B	HER2	Triple-negative	<i>P</i>
Age, years					0.9
<55	20 (46)	12 (52)	8 (53)	13 (52)	
>55	24 (54)	11 (48)	7 (47)	12 (48)	
Mitotic count					0.3
<11	24 (55)	12 (52)	4 (26)	8 (32)	
11-22	18 (41)	11 (48)	10 (67)	15 (60)	
>22	2 (4)	0 (0)	1 (7)	2 (8)	
Tumor grade					0.005 *
G ₁	20 (45)	8 (35)	4 (27)	6 (24)	
G ₂	22 (50)	9 (39)	3 (20)	11 (44)	
G ₃	2 (5)	6 (26)	8 (53)	8 (32)	
Tumor size, cm					0.7
<2	2 (5)	1 (4)	0 (0)	2 (8)	
>2	42 (95)	22 (96)	15 (100)	23 (92)	
Lymph node					0.8
Negative	12	6 (26)	3 (20)	5 (20)	
Positive	32 (73)	17 (74)	12 (80)	20 (80)	
Stage					0.6
I	0 (0)	1 (5)	0 (0)	1 (4)	
II	23 (52)	9 (39)	5 (33)	11 (44)	
III	21 (46)	13 (56)	10 (67)	13 (52)	
Histological type					0.8
IDC	40 (91)	22 (96)	15 (100)	24 (96)	
ILC	3 (7)	1 (4)	0 (0)	1 (4)	
Mucinous	1 (2)	0 (0)	0 (0)	0 (0)	
Ki-67					0.00 *
<15%	44 (100)	7 (31)	10 (66)	10 (40)	
>15%	0 (0)	16 (69)	5 (34)	15 (60)	
Alive or dead					0.6
Alive	37 (84)	17 (74)	11 (73)	19 (76)	
Dead	7 (16)	6 (26)	4 (17)	6 (24)	
Recurrence & metastasis					0.3
Negative	35 (80)	14 (61)	10 (60)	17 (68)	
Positive	9 (20)	9 (39)	5 (40)	8 (32)	

Test of equality of survival distributions for different levels of Ki-67 in each molecular subtype

with DFS in any molecular subtype. Additionally, in luminal A, estimation was limited to the longest survival time.

Table 5 reveals that Ki-67 index is not significantly correlated

Table 3 The relationship of Ki-67 expression with the clinicopathological characteristics of breast cancer, *n* (%).

Characteristics	Ki-67		<i>P</i>
	< 15%	> 15%	
Age, years			0.6
<55	34 (64)	14 (36)	
>55	37 (69)	17 (31)	
Tumor grade			0.00 *
G ₁	32 (45)	6 (17)	
G ₂	31 (44)	14 (39)	
G ₃	8 (11)	16 (44)	
Mitotic count			0.01 *
<11	39 (55)	9 (25)	
11-22	30 (42)	24 (67)	
>22	2 (3)	3 (8)	
Tumor size, cm			0.5
<2	3 (4)	2 (6)	
>2	68 (96)	34 (94)	
Lymph node			
Negative	20 (28)	6 (17)	0.1
Positive	51 (72)	30 (83)	
Stage			0.2
I	1 (1)	1 (3)	
II	36 (51)	12 (33)	
III	34 (48)	23 (64)	
Histological type			0.6
IDC	66 (93)	35 (97)	
ILC	4 (6)	1 (4)	
Mucinous	1 (1)	0 (0)	

Discussion

This study evaluated the clinical significance of Ki-67 index as a prognostic marker in relation to breast cancer molecular subtypes of 107 breast cancer cases. Moreover, the relationships between the Ki-67 index and clinicopathological factors reflecting prognosis were investigated.

The appropriate cut-off point is still a matter of debate among oncologists. Hence, the most suitable cut-off point for Ki-67 in clinical practice is widely investigated²⁶.

In our study, the cut-off point for Ki-67 status was more than 14% of positively stained cells, which approximated that of Fasching et al.²⁷ and was in accordance with the biological

Table 4 The relationship of Ki-67 expression with the molecular subtypes, immunohistochemical characteristics of breast cancer and patient outcome, *n* (%).

	Ki-67		<i>P</i>
	< 15%	> 15%	
ER			0.027 *
Negative	28 (39)	22 (61)	
Positive	43 (61)	14 (39)	
PR			0.014 *
Negative	26 (37)	22 (61)	
Positive	45 (63)	14 (39)	
HER2			0.4
Negative	58 (82)	28 (78)	
Positive	13 (18)	8 (22)	
Molecular subtypes			0.00 *
Luminal A	44 (100)	0 (0)	
Luminal B	7 (30)	16 (70)	
HER2	10 (67)	5 (33)	
Triple-negative	10 (40)	15 (60)	
Alive or dead			0.01 *
Alive	61 (86)	23 (64)	
Dead	10 (14)	13 (36)	
Recurrence & metastasis			0.00 *
Negative	59 (83)	16 (44)	
Positive	12 (17)	20 (56)	

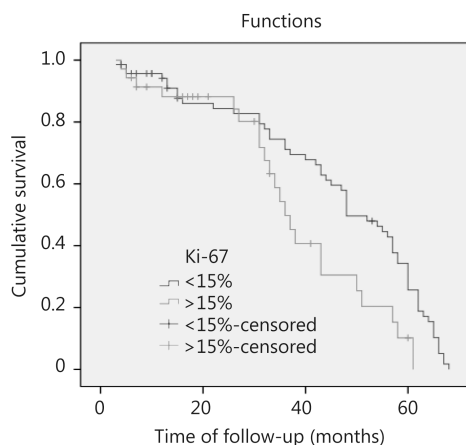


Figure 2 Survival curves of breast cancer patients. Patients with Ki-67 < 15% have better OS than those with Ki-67 > 15% (*P* = 0.012, HR 6.3)

analysis presented by Cheang et al.²⁸. This cut-off point was used because it was in the range reported by Yerushalmi

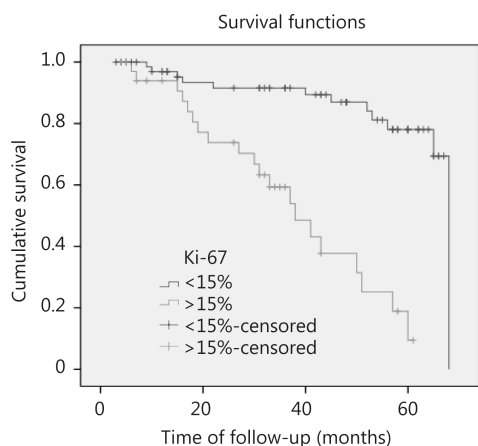


Figure 3 Disease free survival of breast cancer patients. Patients with Ki-67 > 15% are more likely to develop recurrence and distant metastasis than those with Ki-67 < 15% ($P = 0.000$, HR 30.47).

Table 5 Test of equality of survival distributions (DFS) for the different levels of Ki-67 in each molecular subtype

Molecular subtype	Chi-square	df	Sig.
Luminal A	Estimation is limited		
Luminal B	1.114	1	0.299
HER2 neu	0.021	1	0.885
Triple-negative	1.942	1	0.163

et al.¹⁶. Additionally, this cut-off point was correlated with the molecular subtypes of breast cancer, as reported by Cheang et al.²⁸. Furthermore, in our study, no variability was observed in the Ki-67 index. In addition, no significant prognostic difference existed between patients with Ki-67 < 14% and those with Ki-67 14%-20% because no results were obtained in the latter category. These results agreed with those concluded by Bustreo et al.⁷.

A high Ki-67 index ($\geq 15\%$) was significantly correlated with adverse prognostic factors. High Ki-67 index ($\geq 15\%$) was significantly correlated with ER⁻/PR⁻. These results were also in accordance with those of Inwald et al.²⁹. High Ki-67 index ($\geq 15\%$) is significantly correlated with high tumor grade^{29,30}. These results were in accordance with our results. In the present study, high Ki-67 index ($\geq 15\%$) was significantly correlated with high mitotic count, which was in agreement with the results of Nishimura et al.¹³ and Yerushalmi et al.¹⁶. Therefore, no significant association existed between high Ki-67 positivity and positive HER2/neu. This result may be explained by the considerably small number of HER2⁺ positive cases (only 21), in which eight cases showed Ki-67 $\geq 15\%$. Another possible explanation for this difference is attributed to the methods of interpretation.

We used manual interpretation of Ki-67, whereas most studies utilized image analysis, which is more accurate¹¹. Furthermore, the heterogeneity of Ki-67 tumor expression agreed with tissue microarray result and may explain our results regarding HER2/neu. This result was also reported by Yang et al.³¹. On the basis of the above results, we conclude that assessing Ki-67 on whole tumor is better than that of microarray. In triple-negative cases (25 cases), 15 were characterized by > 15% Ki-67 positivity (60%). Ricciardi et al.³² showed that 37.7% of triple-negative cases (45 case) are characterized by > 20% Ki-67 positivity.

The present analysis confirmed that Ki-67 expression is a predictive factor for DFS and OS, which was also proven by Albarracin and Dhamne³³ and Inwald et al.²⁹. Despite numerous investigations on the possible use of Ki-67 as a prognostic marker for breast cancer, the optimal cut-off point and scoring protocol have not yet been standardized. The present data included 107 tumors, but the Ki-67 index of luminal A type tumors was low at < 15% in 100% of the cases. This result was in agreement with that of Nishimura et al.¹³ and Yerushalmi et al.¹⁶. However, results regarding the other molecular types were different because of the small number of cases and different cut-off values for Ki-67 index used in each study.

A prognostic significance of the Ki-67 index in each molecular subtype was investigated. The Ki-67 index was not significantly correlated with DFS in any subtype. This result was in contrast to that reported by Nishimura et al.¹³, which confirmed the significant correlation of the Ki-67 index with DFS only in luminal A type tumors¹³. The difference can be explained by the limited number of luminal A cases ($n = 44$) in our study compared with that of Nishimura et al.¹³ ($n = 625$). All of our cases showed low Ki-67 expression. Thus, we cannot test the equality of survival distribution of different Ki-67 levels despite the data revealing that 37 patients, out of 44 luminal A patients, were still alive.

Conclusions

The present results indicated that Ki-67 level may be considered a valuable biomarker in breast cancer patients and be used in treatment and follow-up. Future work should focus on standardization of Ki-67 assessment and specification of its role in making treatment decisions.

Conflict of interest statement

No potential conflicts of interest are disclosed.

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