Journal of Molecular Biomarkers & Diagnosis
ISSN: 2155-9929

Ramkumar, et al., J Mol Biomark Diagn 2017, 8:3
DOI: 10.4172/2155-9929.1000336

Research Article Open Access

Assessment of Ki67 As a Prognostic Marker in Hormone Receptor Positive Breast Cancer: A Retrospective Study on An Indian Cohort

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Abstract

The prognostic utility of the proliferation marker Ki67 to decide breast cancer treatment has been widely investigated and continues to be a source of much controversy. In this study, we evaluated: i) the role of Ki67 as a prognostic and predictive tool in patients with hormone receptor positive (ER+/PR+) carcinoma of the breast and ii) analyzed its correlation with two commonly used clinicopathological parameters, viz node status and tumor grade to predict clinical outcomes. To determine the clinical utility of Ki67 in assessment of breast cancer prognosis, we examined its expression by immunohistochemistry (IHC) in a series of 160 hormone receptor positive patients in a retrospective cohort of Indian patients. Patients were stratified based on Ki67 expression and analyzed for 5-year distant metastasis free survival (DMFS). Amongst the baseline clinicopathologic variables, we found node status, tumor grade, and age correlated significantly with outcome. However no significant correlation was found between Ki67 based risk stratification and patient outcome. Interestingly, increased Ki67 expression was found to correlate significantly with higher tumor grade but not with worse DMFS. In our study, conducted in an Indian cohort comprising 160 patients, Ki67 was not found to be significantly prognostic or predictive in patients with hormone receptor positive breast cancer.

Keywords: Breast cancer; Ki67; Prognosis; Immunohistochemistry

Introduction

Breast Cancer is the most common cancer in women worldwide with over 1.7 million newly diagnosed cases every year. This number is increasing every year and predicted to reach a peak of 3.2 million per year by 2030 [1]. With 5 year survival rates ranging from over 90% in the USA to ~60% in India, breast cancer is a leading cause of cancer associated mortality in women, especially in low human development index (HDI) countries such as India [1]. Clinicopathological parameters such age, tumor size, node status, and tumor grade have traditionally been used to determine prognosis in patients with breast cancer. However, advances in science have demonstrated that breast cancer is a biologically, clinically, and molecularly heterogeneous entity [2]. which is now believed to represent a collection of distinct diseases that are best defined by molecular profiling. Further, the judicious use of biomarkers or combinations of biomarkers that act as surrogates for prognosis are now believed to be essential in guiding therapy and delivering personalized care to patients [3,4]. Uncontrolled proliferation is one of the key hallmarks of cancer [5] and it is routinely assessed as a marker of aggressive disease. Proliferation maybe measured by counting mitotic figures in H&E stained tissue sections, by incorporation of labeled nucleotides such as BrdU, and flow cytometric analysis of cells in various phases of the cell cycle. Newer studies have demonstrated that proliferation based gene signatures and biomarkers play a critical role in breast cancer prognosis and response to chemotherapy [6-8]. Higher expression of proliferation based signatures have been shown to be associated with greater predilection for metastasis [6].

One of the most common proliferation based markers used for predicting prognosis in breast cancer is the nuclear marker Ki67. Ki67 is a mitotic marker, expressed in the nucleolus of cells, and is known to be associated with Polymerase I-dependent ribosomal RNA synthesis [9,10]. However, it's exact function in the cell cycle has not been elucidated. Several studies with large numbers of patient samples have demonstrated a statistically significant association between Ki67 and distant recurrence-free survival in breast cancer [11], suggesting that the proportion of Ki67 expression in tumor cells would provide valuable prognostic and predictive insight into the intrinsic biology of the tumor and its response to chemotherapy. Importantly, in 2009 the St. Gallen international expert panel recommended the use of proliferation markers such as Ki67 along with traditional clinical parameters including stage, tumor grade and hormone Receptor status when making decisions about adjuvant chemotherapy in early stage breast cancer [12].

However, in subsequent meetings the St. Gallen panel noted that lack of standardization of Ki67 assessment by IHC along with the absence of an established cutoff for Ki67 expression based risk stratification has hampered its use in the clinical setting [13]. Additionally, many studies have shown that the role of Ki67 as a prognostic marker in early breast cancer is ambiguous [14]. The problems associated with application of Ki67 as a therapeutic indicator in breast cancer include: technical issues with IHC performance such as choice of antibody, optimization of IHC protocol, and establishment of definitive cutoffs for Ki67 based risk stratification [15], as well as the lack of clear guidelines for scoring Ki67 staining, and no consensus on the prognostic significance of Ki67 in breast cancer. An "International Ki67 in breast cancer" working group, comprised of experts in the field was convened in 2011 to analyze currently available data to substantiate the prognostic role of Ki67, and provide harmonized guidelines for Ki67 staining, which would lead to a clear definition of the utility of Ki67 in clinical practice [16]. The expert panel however did not provide any clear guidelines for the clinical use of Ki67 for risk stratification of breast cancer [17].

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Received December 28, 2016; **Accepted** February 26, 2017; **Published** March 02, 2017

Citation: Ramkumar C, Prakash C, Madhav L, Kumar A, Basavaraj C, et al. (2017) Assessment of Ki67 As a Prognostic Marker in Hormone Receptor Positive Breast Cancer: A Retrospective Study on An Indian Cohort. J Mol Biomark Diagn 8: 336. doi: 10.4172/2155-9929.1000336

In this study, we have used the technical guidelines provided by the "International Ki67 in breast cancer" working group to analyze 160 breast cancer samples retrospectively by IHC in order to test if Ki67 expression can be corroborated with DMFS in patients with hormone receptor positive breast cancer. We have chosen to focus specifically on hormone receptor positive disease because this is the most common type of breast cancer, and it is known to be associated with high rates of distant recurrence free survival [17]. Further, studies have shown that patients with hormone receptor positive disease can be spared chemotherapy, and respond well to hormone therapy alone [18]. Ki67 expression is routinely used to estimate risk of recurrence in this setting with a view to determine which patient would benefit from chemotherapy. In light of the debate surrounding the performance, assessment, and utility of Ki67 in predicting breast cancer outcomes, the aim of this study was to evaluate the accuracy of Ki67 as a prognostic marker in hormone receptor positive breast cancer in an Indian cohort.

Materials and Methods

Sample selection

All studies were performed with approval of the Institutional Review Board and Ethics Committees of the Hospitals participating in the study. Informed consent was waived according to Indian council of medical research (ICMR) guidelines since the study was retrospective, observational, non-interventional and anonymized. We selected women with Stage I, II, and III Invasive ductal carcinoma (IDC) or invasive lobular carcinoma (ILC) of the breast, age <74, ER+ or PR+, Her2+/-, with minimum 5-year follow up and known clinical outcome. The age limit was restricted to 74 because determination of the effect of comorbidities on patient health in older patients is difficult [19]. Using a patient population less than 74 enabled the elimination of ambivalence in determining patient prognosis correctly. Majority of the patients were in Stage II. All patient samples were stripped of personal identifiers. Information was collected on age and calendar year of diagnosis, surgery, tumor size, tumor grade, histologic type, and ER status, nodal status, radiation treatment, hormonal therapy or chemotherapy, and clinical follow-up, including local, loco regional, or distant recurrences, second primary malignancies, death or date of last visit. Paraffin embedded blocks of primary breast tumor from lumpectomy/breast conserving surgery/modified radical mastectomy samples diagnosed with invasive ductal carcinoma NST/NOS type or ILC, and which had been fixed and processed as per prescribed norms [16] were taken in for the study. Primary breast tumor FFPE blocks less than 15 years old (from either modified radical mastectomy or breast conserving surgery) were used. All samples had a cold ischemic time of less than 1 hour. The specimens were fixed by immersion in 20 times volume of 10% neutral buffered formalin. A minimum fixation time of 6 hours and a maximum fixation time of 72 hours was accepted. Standard norms for processing and embedding were followed, and samples were fixed in cassettes.

All samples meeting the following criteria on evaluation of H&E stained slides were accepted for analysis:

1). FFPE tumor blocks of patients diagnosed with invasive ductal carcinoma NST/NOS type

2). Blocks with minimum 30% tumor

3). Samples with minimum necrosis/hemorrhage/crush artifacts

4). Optimal fixation and processing of tissue with no artifacts

Special types of Ductal carcinoma, samples with extensive DCIS component and with minimal invasive component were excluded from the study to ensure the cohort has minimal heterogeneity.

ER/PR staining was repeated for all samples, and only those samples that were ER+ or PR+ were included.

IHC staining

IHC analysis was semi-automated and performed as described here. FFPE tissues are sectioned into 3 µ slices using a Leica microtome (#RM2125RTS). Poly L Lysine coated slides were used for taking sections. The sections were fixed on glass slides by placing them in a hot air oven (Apollo Scientific) at 55°C for 1 hour. The slides are then de-paraffinized with Xylene (Fisher Scientific) solution twice for 15 minutes each. Slides are rehydrated by washing twice with 100% alcohol for 5 minutes followed by 2 washes with 70% alcohol for 5 minutes, and finally with demineralized water (Nice Cat # D1505) for 5 minutes. Antigen retrieval is performed for Ki67 antibody using the Multiple Epitope Retrieval System for 15 minutes at medium setting. Following antigen retrieval, slides are cooled completely to room temperature in the same buffer. On attaining room temperature, the slides are washed in demineralized water for 5 minutes. After wiping extra moisture on the slide with a tissue, the tumor section is marked with a PAP pen. The rest of the steps are performed using the Novolink Polymer Secondary Kit (Leica, RE-7280K). Peroxidase block is added to each slide and incubated for 5 minutes. Slides are washed with wash buffer (10 mM TBS-Tween 20, pH 7.4) twice, for 5 minutes each. After washing, the protein block is added and slides are incubated for 5 minutes. Slides are then washed with wash buffer twice, for 5 minutes each. 50 µL primary antibody is then added on to the sections using the pre-diluted Ki67 antibody (Biogenix, Clone Mib1, Cat# AM297-5M) and slides are incubated for 1 hour in a humidifying chamber. After primary antibody incubation, slides are washed with wash buffer twice, for 5 minutes each. Post-primary solution is added to the slides, and incubated for 30 minutes, followed by 2 washes with wash buffer as described previously. Following this, slides are incubated with polymer for 30 minutes and then washed twice with wash buffer. Peroxidase activity is developed using DAB working solution for 5 minutes, following which the slides are rinsed with demineralized water for 2 minutes. Sections are counterstained with Hematoxylin (Fisher Scientific) for 8 minutes and rinsed in demineralized water for 8 minutes. The slides are subsequently dehydrated with 70%, 95% and 100% alcohol, each for 5 minutes. They are dried at room temperature and then incubated in Xylene for 5 minutes. Slides are dried and mounted with D.P.X. Mountant (NICE, Product # D30475).

IHC grading

Ki67 staining is mainly observed in the nucleus and occasionally in the cytoplasm and membrane. Only nuclear Ki67 staining is considered positive staining. The Ki67 score is obtained by calculating the average of the percentage of positively stained nuclei among the tumour cells in each of the fields across the entire tissue section. A minimum of 1000 malignant cells have to be counted to arrive at the percentage score. Ki67 staining less than 14% was considered low risk, and greater than 14% was considered high risk [14]. All slides were scored by 3 independent pathologists, and the average of their scores was considered as the final score.

Statistical analysis

Kaplan-Meier curves were plotted using GraphPad Prism. Cox regression analysis was performed using MedCalc software. Comparison between groups of patients was made using the two-sided *t*-test. P-values of <0.05 were considered statistically significant.

Results

Features of the study cohort

The clinicopathological features of the 160 sample cohort analyzed in this study are presented in Table 1. All patients received chemotherapy. Over 2/3 of the patients were under 60 and presented with T2 disease. Both node negative and node positive patients were well represented, with a slight preponderance of node positive cases. 2/3 of the cases were stage II by TNM staging, tumor grade 2 by the Bloom-Richardson-Elston method, and had Ki67 staining <14%. Ki67 staining was analyzed by IHC using the MiB 1 antibody as described in Materials and Methods. To demonstrate compliance with the technical recommendations of the "International Ki67 in breast cancer" working group in our system, representative images of Ki67 expression ranging from samples scored as negative to those that scored over 50% are displayed in Figure 1.

Clinicopathological risk stratification

Traditionally, prognostic assessment in breast cancer is performed using well-studied clinicopathological parameters including age, tumor size, node status, tumor grade and ER/PR status [20]. We studied some of these parameters as predictors of DMFS within 5 years. Studies have shown that post-menopausal women with hormone receptor positive disease have significantly better prognosis than younger, pre-menopausal women [21]. In accordance with earlier studies, we found that women under 60 had poorer survival rates (DMFS: 78%) than post-menopausal women over 60 (DMFS: 95%) (Figure 2a).

Patient Cohort: 160 ER+/PR+samples	
Age	**Number of patients**
<60	114
>60	46
Tumor Size	
T1	32
T2	120
T3	8
Node Status	
N0	68
N1	57
N2	35
Grade	
Well differentiated (Grade 1)	16
Moderately differentiated (Grade 2)	95
Poorly differentiated (Grade 3)	49
TNM Staging	
Stage I	23
Stage II	100
Stage III	37
Ki67 status	
Ki67-low (<14%)	93
Ki67-high (>14%)	67

Table 1: Clinicopathologic features and Ki67 status of the study cohort.

Figure 2b shows that in contrast with earlier reports [20], in our cohort, tumor size alone is not a significant predictor of DMFS. Patients with T1, T2, or T3 disease had comparable DMFS (T1: 81%, T2: 83%, T3: 75%) (Figure 2b).

Next, we tested the performance of Node status as a predictor of DMFS, we found that in keeping with findings from earlier studies [20-22], Node positive (consisting of both N1 and N2 disease) patients had a significantly higher rate of distant recurrence (DMFS: 75%) than node negative patients (DMFS 94%) (Figure 2c).

Histological tumor grade is a metric of the degree of tumor differentiation and is measured by the semi-quantitative evaluation of tubule formation, nuclear pleomorphism and mitotic count. Tumor grade was assessed by the modified Bloom-Richardson-Elston criteria and is known to be a critical predictor of prognosis [22,23]. Kaplan-Meier analysis of DMFS in patients stratified by tumor grade, showed that patients with moderate (Tumor grade 2) or poorly-differentiated (Tumor grade 3) disease had significantly worse DMFS (Tumor grade 2: 82%, Tumor grade 3: 79%) versus patients with well-differentiated disease (Tumor grade 1: 94%) (Figure 2d).

It is well established that patients with strong ER-positive disease respond better to hormone therapy and thereby have better outcome [24] compared to those with ER negative or weak ER positive disease. We stratified tumors based on perentage ER staining as strong ER positive (Allred Score 7-8) or moderate-weak ER positive (Allred Score 3-6) tumors [25]. We found no difference in DMFS (both at 82%) between these cohorts by Kaplan-Meier analysis (Figure 2e).

Ki67 based risk stratification

Finally, we tested the prognostic potential of the mitotic marker Ki67 in our cohort. We stratified patients using the criteria recommended by the 2011 St. Gallen's International Expert Consensus on primary therapy for early breast cancer, whereby patients with <14% Ki67 expression are called low risk, and those with >14% expression are called high risk [26]. Kaplan-Meier survival analysis showed no statistically significant difference in survival between Ki67-low (DMFS: 80%) and Ki67-high (DMFS 85%) patients (Figure 2f).

Determination of prognostic significance of all tested variables

To determine if the clinicopathologic variables under study correlated with disease outcome, we proceeded to perform univariate Cox regression analysis (Table 2). In our univariate analysis, we found that Age, Node status and Tumor grade were significant predictors of

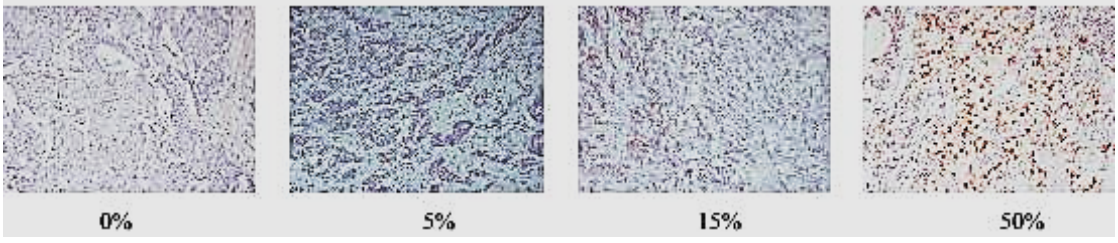

Figure 1: Ki67 expression in breast cancer sections. Figure 1 shows exemplary IHC images for Ki67 expression ranging from samples scored negative for Ki67 to those scored greater than 50% Ki67 positive.

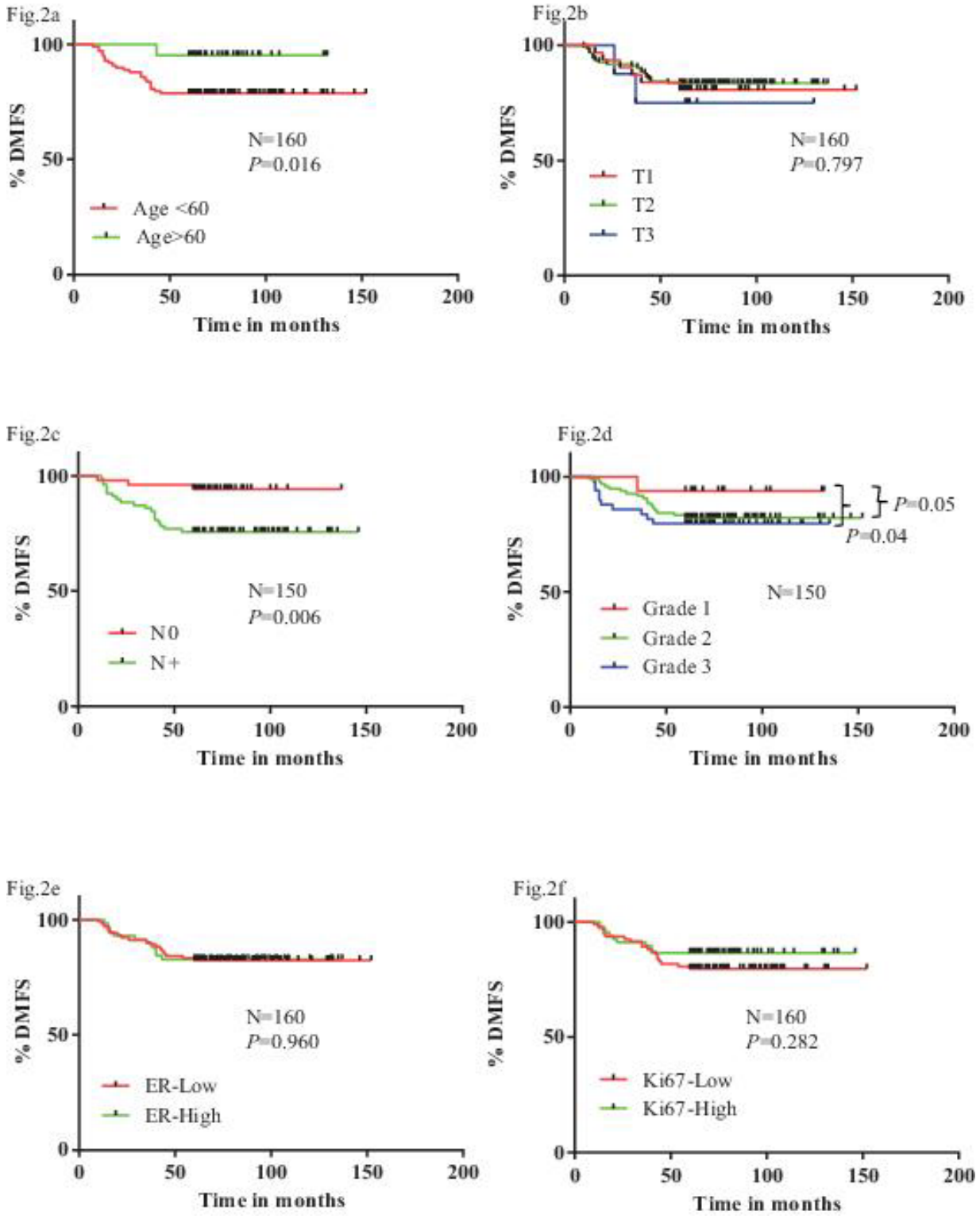

Figure 2: DMFS of hormone receptor positive breast tumors based on prognostic variables. Kaplan-Meier survival plots of DMFS in a cohort of hormone receptor positive Breast Cancer patients based on Age <60 or Age >60 (Figure 2a); Tumor size -T1: Less than 2 cm, T2: 2 cm u to 5 cm, or T3: >5 cm (Figure 2b); Node status- N0- node negative, N1- 1-3 positive nodes N2- 4-7 positive nodes (Figure 2c); Tumor Grade- Grade1, 2, 3 by modified Bloom-Richardson-Elston criteria (Figure 2d); ER expression- Tumors with Allred Score 7 or 8 are called ER high and those with Allred Score 3-6 are called ER low (Figure 2e); or Ki67 expression - <14% Ki67 are called Ki67 Low, >14% Ki67 are called Ki67 high (Figure 2f).

N: No. of patients, P-value: Log-rank test, DMFS: Distant metastasis-free survival.

Prognostic Factors	Hazard Ratio	P-Value	95%CI
Age (<60 vs >60)	0.202	0.0164	0.14-0.81
ER (ER-low vs ER-high)	0.9835	0.9601	0.45-21
Node status (NO vs N+)	4.640	0.0065	1.4-7.6
Tumor size (T1 vs T2)	0.8548	0.7352	0.32-2.2
Tumor size (T1 vs T3)	1.332	0.7247	0.24-7.7
Grade (G1 vs G2)	2.887	0.0499	1.0-8.3
Grade (G1vs G3)	4.074	0.0483	1.0-16.4
Ki67 (Ki67-low vs Ki67-high)	0.6499	0.2820	0.31-14

Table 2: Univariate Cox regression analysis of all studied variables.

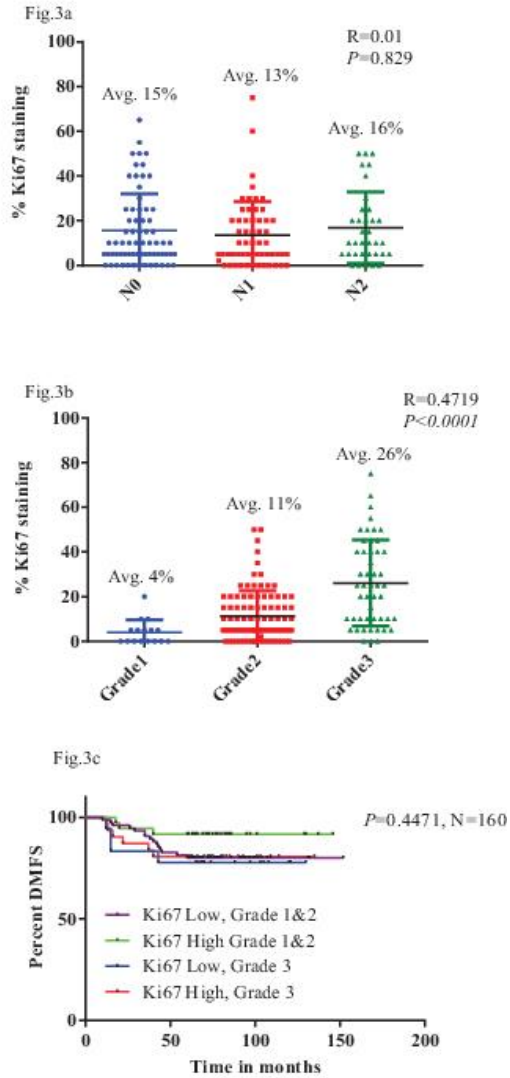

Figure 3: Association between Ki67 expression and prognostic indicators. 3a) 160 Patients were stratified based on Node status into N0, N1 and N2 on the X-Axis. Ki67 expression is plotted as % Ki67 staining on the Y-axis. Average Ki67 expression for each group stratified by node status is written above the graph where it is plotted. 3b) 160 Patients were stratified based on tumor grade into grade 1, 2 and 3 on the X-axis. Ki67 expression is plotted as % Ki67 staining on the Y-axis. Average Ki67 expression for each group stratified by tumor grade is written above the graph where it is plotted. R=Pearson's correlation, P-value: 2-tailed student's T-test. 3C) The Kaplan-Meier survival curve of 160 patients who were stratified based on Ki67 staining (low or high) as well as tumor grade (1 and 2/3) into the following groups is plotted: 1). Ki67 low, Grade 1, 2). Ki67 high, Grade 1, 3). Ki67 low Grade 2/3, 4). Ki67 high Grade 2/3. Time in months is plotted on the X-axis and distant metastasis-free survival is plotted on the Y-axis. *P-value- Log-rank test.*

Number of Patients				
Grade	Ki67-Low, DMFS >5 years	Ki67-Low, DMFS <5 years	Ki67-High, DMFS >5 years	Ki67-High, DMFS <5 years
Grade 1	15	1		
Grade 2	46	14	32	3
Grade 3	14	4	25	6

Table 3: Comparison of disease outcomes in association with Ki67 expression and tumor grade. Disease outcome is measured as DMFS. A DMFS of <5 years means patient had a recurrence in less than 5 years and is considered 'bad' as against DMFS >5 years which means patient is disease free for 5 years or more.

prognosis, however Ki67 was not. Further, to assess if Ki67 expression has any correlation with the most significant predictors of prognosis *viz* node status and tumor grade, we analyzed Ki67 expression in cohorts of patients based on node status (Figure 3a) or tumor grade (Figure 3b). Patients were stratified based on Node status into N0, N1 or N2 groups and analyzed for Ki67 expression. Average Ki67 expression did not differ between the 3 cohorts of patients stratified by Node status (Average Ki67 expression in N0-15%, N1-13% and N2-16%, Pearson's correlation R=0.017).

When we analyzed correlation between Ki67 expression and tumor grade, we found a significant direct correlation between them, i.e. Ki67 expression increases with increasing tumor grade. As shown in Figure 3b, tumor grade 1 disease showed lower average Ki67 expression (4%) compared with tumor grade 2 (11%) and tumor grade 3 (26%) disease (Pearson's correlation R=0.4791).

To test whether the association between Ki67 staining and tumor grade was a strong predictor of distant recurrence free survival within 5 years (Figure 3c and Table 3), we proceeded to examine the hypothesis that patients who were characterized as high risk by Ki67 staining and had poorly differentiated tumors (Grade 3) must have worse DMFS than patients called low risk by Ki67 and had well differentiated (Grade1) or moderately differentiated (Grade 2) disease. We found that ~15% patients (14/95) with tumor grade 2 disease, and ~9% patients (4/49) with tumor grade 3 disease who had low expression of Ki67 (were stratified as low risk based on Ki67 expression) suffered a distant recurrence within 5 years (Table 3). Conversely, ~33% patients (32/95) with tumor grade 2 disease and ~51% patients (25/49) with tumor grade 3 disease who had high Ki67 expression (were stratified as high risk based on Ki67 expression) had distant recurrence free survival in excess of 5 years (Table 3). Only 3% (3/95) patients with tumor grade 2 disease and 12% patients (6/49) with tumor grade 3 disease were correctly stratified as high risk by Ki67 and suffered a metastatic relapse within 5 years (Table 3). Taking all these results into account, we found that risk stratification by Ki67 expression in conjunction with tumor grade was not strongly predictive of distant recurrence/clinical outcome within 5 years (Figure 3c and Table 3).

Discussion

Determining which patient receives adjuvant chemotherapy in early stage breast cancer patients is critical, and must be based not just on traditional prognostic factors, but by examination of predictive molecular markers to analyze which patient would benefit the most from chemotherapy [27]. Several gene-expression based tests are now routinely used to stratify patients based on risk of recurrence in hormone receptor positive patients [3,28,29]. Owing to cost, these tests have restricted utility in India and other Low/Medium HDI countries. Because Ki67 is a marker for mitotic cells, high Ki67 expressing tumors are predicted to respond better to chemotherapy [14]. Therefore, Ki67 is routinely used as a surrogate for the more expensive gene expression based assays to assess response to chemotherapy and breast cancer

prognosis in these countries. There are multiple issues in using Ki67 as a prognostic marker, and therefore in this study we assessed the utility of Ki67 as a prognostic marker in an Indian cohort and its correlation to treatment outcome in hormone receptor positive breast cancer.

We found that while baseline prognostic factors including node status, tumor grade and age were significantly associated with DMFS (Figure 2), Ki67 expression was not. High nodal involvement and high grade tumors are prognostic of lower DMFS. Age on the other hand is inversely co-related to DMFS, i.e. younger patients tend to have lower DMFS as compared to older patients. Ki67 expression showed no independent correlation with DMFS. Cox regression analysis further confirmed the significance of Node status and Tumor grade as prognostic factors, but Ki67 expression was not significantly associated with prognosis (Table 2). Analysis of Ki67 expression in patients stratified by Node status showed no association between the two (Figure 3a), indicating that node status is an important prognostic parameter by itself and is independent of the proliferation status of a tumor. This is in keeping with the fundamentals of tumor biology wherein metastatic spread of disease is associated with enhanced capability of tumor cells to invade and migrate through their surrounding tissue [5].

Next, we assessed if there is any correlation between tumor grade and Ki67 expression. Our investigation indicated a linear relationship between tumor grade and Ki67 expression (Figure 3b). This is not surprising since high tumor grade tumors have more number of mitotic figures, and Ki67 expression is associated with mitosis. Interestingly, this association between tumor grade and Ki67 did not translate into worse outcomes in patients with high tumor grade and high Ki67 (Table 3). Thus, in our study we did not find that Ki67 was significantly associated with DMFS, a critical metric of prognosis in breast cancer. Increasing number of studies have recently have shown that intrinsic tumor biology, not just cell proliferation within the tumor is of paramount importance in predicting clinical outcomes [28-30], and our study substantiates this hypothesis. Attempts are ongoing in our laboratory to understand tumor biology in greater detail, so as to stratify cancers based on tumor biology into "good" and "bad" prognosis and provide an accurate predictive platform for personalization of therapy.

Our study was limited by numbers of patients, and the fact that all patients had taken chemotherapy. In fact, the best way to determine the efficacy of a prognostic model is to validate its performance in a chemotherapy naïve cohort. However, it is well known that chemotherapy benefit rates are under 10% [17], and hence our study certainly delivers a substantial point about utility of Ki67 as a prognostic marker even though the entire cohort was treated with chemotherapy. In the future, we plan to extend our study to greater numbers of patients, and to focus on a cohort of patients who received only hormone therapy but not chemotherapy. We also plan to test if different cutoffs for Ki67 perform better in predicting prognosis, since the optimum cutoff is still a source of debate in the field [14,16].

While this study did not find Ki67 to be a significant predictor of prognosis in a small Indian cohort of patients, if the recommendations of the international Ki67 in breast cancer working group are followed rigorously, and standardization of the assay is ensured worldwide, there is potential for defining its prognostic utility with more clarity. Ki67 assessment by IHC is an economical and technically simple method which can be standardized in any medical laboratory, to perform prognostic assessment in breast cancer. It can be performed in parallel with ER/PR/Her2-neu staining on the surgical sample or core biopsy, saving both time and cost. However, more extensive studies on the various molecular subtypes of breast cancer and their association with Ki67 expression in the context of DMFS are necessary before Ki67 can be validated as prognostic marker in breast cancer.

Declarations

Ethics approval and consent to participate

All studies were performed with approval of the Bangalore Ethics Committee (ECR/87/Indt/KA/2013) in accordance with Declaration of Helsinki. The study protocol number is ODPL/BC/001/2014. Informed consent was waived according to Indian Council of Medical Research (ICMR) guidelines since the study was retrospective, observational, non-interventional and anonymized.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Authors' contributions

CR and MB designed the experiments and wrote the manuscript. CR analyzed the data. AK, LM, CP, NN, PR and CB performed the experiments and analyzed the slides.

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