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Original Research Article

Ki-67 Proliferation Index In Carcinoma Breast: Pitfalls In Assessment

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ABSTRACT

Introduction: Ki-67 proliferation index is used as a prognostic-predictive marker in breast carcinoma and determined using visual hotspots or global counts methods. We studied analytical objectivity and reproducibility using the two methods.
Material and Method: 56 cases of carcinoma breast with Ki-67 (MIB-1antibody) staining on same sections were evaluated by two equally experienced pathologists, blinded to each other using both methods. The values obtained were classified into low, intermediate and high categories and statistical analysis was done using SPSS ver21.0 software.
Result: The mean value of hotspot method was higher whereas global method showed better interobserver reliability.
Conclusion: We recommend global method of Ki-67 evaluation as it has better reproducibility and inter-observer agreement as compared to visual hot spot method. The latter which has a higher mean value may result in change in categorization of Ki-67 index especially at higher end of cut-off in intermediate category, thereby affecting treatment protocols.

Keywords: Ki-67 proliferation index, average method, visual hotspot method

Introduction

Ki-67 a molecular marker of cellular proliferation was originally described using mouse monoclonal antibody against a nuclear protein from Hodgkin's lymphoma-descended cell line and is not only used as a prognostic-predictive marker but also as a marker for response to treatment [1, 2]. Ki-67 proliferation index below 15% is associated with decreased incidence of metastasis, recurrence and better survival rates [3, 4]. The molecular classification of breast cancer types Luminal A cancers as those having Ki-67 index of less than 14% and those into Luminal B (Her 2 negative) having Ki 67 index of greater than 20% [5]. As of now Ki67 proliferation index is used for prognosis in breast cancer and for anatomically favorable Estrogen Receptor positive, Progesterone receptor positive / negative and Her 2 negative tumors to decide whether patients shall need adjuvant chemotherapy with majority agreeing

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that in such tumors after taking node status into consideration a Ki67 score of 5 percent or less does not warrant chemotherapy whereas chemotherapy is recommended when a Ki67 score is 30% or more [6, 7]. So accurate and reproducible determination of Ki-67 proliferation index assumes importance in cases of carcinoma breast with respect to decision of treatment and also for response to treatment. Apart from pre-analytical variables involving immunohistochemical staining of Ki-67 on formalin fixed paraffin embedded tissue including choice of antibody, duration of fixation and chemical reagents used, the analytical determination of Ki-67 has many gray areas including areas where to count, visual hotspots, taking average counts, the number of cells to count and visual eyeballing technique versus using manual cell counter for accurate counting in pre-determined fields/areas [8]. The International Ki-67 in Breast Cancer Working Group (IKWG) consensus met in October 2019 and after due assessment gave the recommendation of adopting established standardized visual scoring system known as Global Method [9], where four fields of at least 100 tumor cells representing heterogeneity of total cancer area were selected based upon high, medium and low/negligible Ki-67 staining after comparing the same with hot spot method where the observer counted 500 tumor cells in single field in area with maximal Ki67 staining rate [10]. These are only recommendations for evaluating Ki-67 index, not universally accepted /adopted and can have inter and intra observer variability.

Taking above factors into consideration affecting objective assessment of Ki-67 proliferation index, the current study is proposed to determine analytical/ assessment objectivity with respect to interobserver variation and reproducibility by global method and comparing it to visual hot spot method when other pre -analytical variables are same.

Material and Methods

The present study was carried retrospectively for a period of 18 months from 1 January 2021 to 30 June 2022 after obtaining permission from Institutional Ethics Committee. The study includes 56 cases of breast carcinoma diagnosed on Hematoxylin & Eosin sections and immunohistochemically (IHC) evaluated for Ki-67 positivity using standard immunohistochemistry staining protocol on formalin fixed paraffin embedded sections with Ki-67 (MIB-1 antibody-Mouse monoclonal; Pathnsitu). Section from tonsil had been used as positive control.

We used MIB-1 monoclonal antibody for Ki67 detection which has high sensitivity, specificity, reproducibility and gives comparatively better results across wide range of dilutions and has been considered as gold standard [8].

Inclusion criteria

All diagnosed cases of carcinoma breast where IHC staining using Ki-67 was performed.

Exclusion criteria

All other cases of breast lesions and breast carcinomas where IHC using Ki-67 as marker was not performed or unavailable.

Two histopathologists blinded to each other and with more than five years of experience were assigned to evaluate the Ki-67 positivity using Global method (average of overall positivity of Ki-67 in given section) and standard visual hot spot method [5, 10].

The positively stained tumor cells were identified in a x100 magnification field (as any degree of brown nuclear stain above the background and excluding cytoplasmic brown stained cells and or lymphocytes) and counting done at x400 magnification [5]. In visual hotspot method visual counting in field showing maximal Ki-67 positivity in tumor cells and dividing the total number of positive staining cells with total number of tumor cells in that

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field with effort being made to include a minimal total count of tumor cells of 500. In cases where a minimum count of 500 cells was not possible, fields with maximum number of tumor cells were included. So, the formula for Ki-67 index by visual Hot spot method was

$$\text{Ki-67 proliferation index as \%} = \frac{\text{Total number of positive tumor cells}}{\text{Total number of tumor cells}} \text{ X } 100$$

In case of Global method, similar procedure was followed but four fields of 100 tumor cells each were selected to reflect observed heterogeneity in nuclear staining in given section and average of these was calculated to arrive at final Ki-67 Proliferation index [10] where N is Ki-67 proliferation index in a given field calculated by

$$\text{N (\% age)} = \frac{\text{Total number of positive tumor cells}}{\text{Total number of tumor cells}} \text{ X } 100$$

Average Ki-67 proliferation index (%) (As determined by Global method) = (N1+N2+N3+N4)/4

In this study we chose to classify Ki-67 index into low, intermediate and high categories with up to less than/ equal to 5%, 6-29% and greater than / equal to 30% being three categories for inter observer variation which have a prognostic significance. Subsequently statistical analysis for mean for both methods and each observer along with coefficient of variation (Kappa) for each method was calculated using SPSS Ver.21.0 software.

Observations

A total of 56 cases were included in the study out of which 38 cases were tissue sections from mastectomy specimens and 18 cases were core biopsies. The same tissue sections were used by both observers for evaluation so pre-analytical variables did not affect the observations.
In visual hotspot method, in eight cases a minimum count of 500 cells was not possible fields so fields with maximum number of tumor cells were included and this number of cells varied from 100-400. However, in global method, both the observers were able to find the representative fields in all the cases.
The mean value by visual hotspot method by both observers was 36.68 and 33.57 as compared to global method which was 33.80 and 29.79 respectively.
In visual hot spot method, the difference in low, intermediate and high categories was 1.8, 7.2 and 9.1 percent, respectively but was statistically not significant (p value 0.466). (Table 1, Figure 1)
Similarly in Global method the difference in low, intermediate and high categories was 1.8, 1.8 and nil percent, respectively but was statistically not significant (p value 0.950). (Table 2, Figure 2).
The interobserver reliability coefficient (Kappa- **κ**) for visual hotspot method was 0.791 as compared to that of global method which was 0.902.

Table 1: Categorization into low, intermediate and high categories using Visual hot spot method by both observers

Ki-67 proliferation index	**Visual hotspot method**					
Category	**Number**	**Observer 1**	**Number**	**Observer 2**	**x2 - value**	**P-Value**
Low ≤ 5%	13	23.2	12	21.4	1.525	0.466
Intermediate (6%-29%)	9	16.1	5	8.9		
High ≥ 30%	34	60.7	39	69.6		

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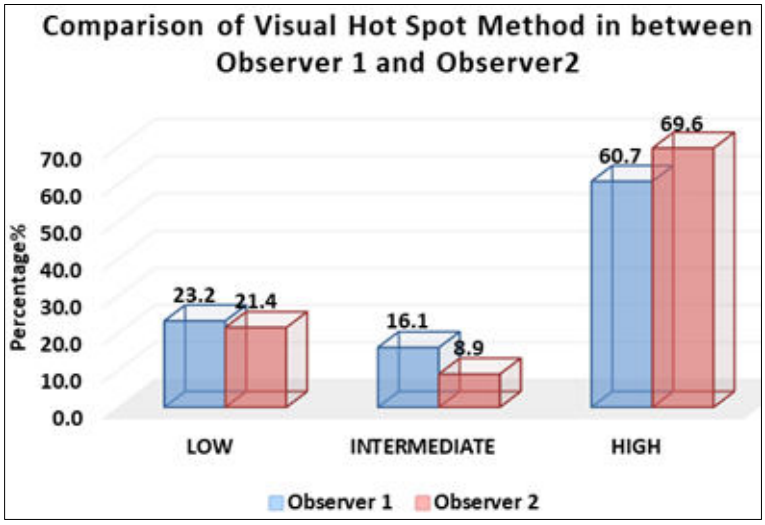

Table 2: Categorization into low, intermediate and high categories using Global method by both observers

Ki-67 proliferation index	Global Method					
Category	Number	Observer 1	Number	Observer 2	x2 - value	P-Value
Low ≤ 5%	14	25.0	15	26.8	0.101	0.950
Intermediate (6%-29%)	8	14.3	7	12.5		
HIGH ≥ 30%	34	60.7	34	60.7		

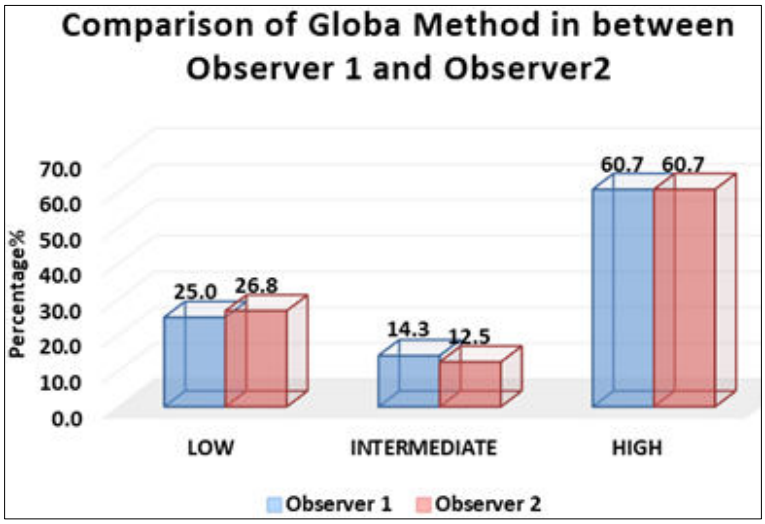

Fig 2: Categorization and comparison using Global method by both observers

Discussion

Ki-67 is a proliferation marker widely used in assessment of malignant neoplasms expressed in all but G0 phase of cell cycle which can be identified by immunohistochemistry with current utility being in separating Luminal A from Luminal B (Her 2 negative) tumors in breast cancer, prognostic evaluation and in deciding use of chemotherapy and subsequent follow up in patients with high Ki-67 index [6, 11]. However, previously in 2011 St Gallen conference the cut off for Luminal A tumors was 14% which was subsequently revised to 20% in 2013 St. Gallens, but with a condition that each laboratory set its own cut off value taking local laboratory value in consideration, due to inter-laboratory variation [8, 12, 13]. Recently the cut off values to classify Ki-67 proliferation index as high or low in breast carcinoma with respect to interobserver variability has been recommended to in very low scores (median-less than/equal to 5%) and in very high scores (median-greater than/equal to

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30%) [6]. So, taking these recommendations into considerations we chose to categorize our observations into three categories as mentioned above and there exists a large gray area in the assessment (6%-29%) which has limited clinical significance.

In our study we found that mean value obtained by visual hot spot method was higher by both observers as compared to global method (36.68 and 33.57 Vs 33.80 and 29.79) which is logically explained as hot spot method counted the field with maximum staining of tumor cells whereas global method took into consideration all the heterogenous areas of tumor section. Honma *et al*. on the basis of prognostic significance in carcinoma breast advocated that Ki-67 evaluation by hotspot method is independently associated with poor outcomes and hence was superior to global method [14], as the values were higher in hotspot method a finding similar to our study.

Subsequently we also assessed the interobserver variability in between two methods across three categories and found similar difference 1.8% for low category which subsequently increased to 7.2 and 9.1% for intermediate and high category in visual hotspot method in comparison with 1.8% and nil for global method although these were statistically not significant (p value greater than 0.05) which may be attributed to low sample size, implying that these differences did not affect categorization ; van den Berg *et al*. when classifying Ki-67 index into high and low for classification into luminal A and Luminal B concluded that at the higher and lower Ki-67 levels, the correlation between the methods of assessment was acceptable, however, close to cut off levels different methods may categorize patient's into different categories which may affect treatment [15]. In our categorization we found that change in categorization was most evident in intermediate category using visual hot spot method between observer 1 and 2 whereas in global method it was found in only one case. So our finding correlates with that of van den Berg *et al*. with respect to intermediate category although they had a very low range of classification (14-20%) when compared with our study (6-29%). These observations point to the fact that in heterogeneous tumors with many hotspots, there may be discordance among observers in selection of hot spots which may cause bias in categorization.

The interobserver reliability coefficient was perfect for Global method (0.902) and substantial for visual hotspot method (0.791) so reproducibility was better with former, a finding shared with that Varga *et al*. who concluded that global method has the best interobserver reliability coefficient when done at low-power magnification evaluating the whole section and draw an average of the stained cells from the tumor periphery respectively from the invasion front [16]. Shui *et al*. found interobserver reliability coefficient as 0.894 and 0.904 for visual hotspot and global method respectively concluding that there is good concordance between both methods. We also found good concordance for both methods with respect to inter-observer reliability but global method achieved the perfect score for reproducibility as observed by Leung *et al*. who found confidence interval of 0.87 for global method as compared to 0.83 for hotspot method.

Conclusions

1. Global and Visual hot spot methods both may be used for categorization of Ki-67 proliferation into high, intermediate and low categories but comparatively variations are more in visual hotspot method especially near high end of assigned cut-off values which may lead to change in categories between two observers.
2. The mean value is higher when visual hotspot method is used because it evaluates only maximally stained tumor areas
3. Reproducibility was better with global method achieving a perfect kappa value of 0.902.

In view of above findings, we recommend use of Global method for estimation of Ki-67 proliferation index as visual hotspot method has potential diagnostic pitfall of higher evaluation of Ki-67 proliferation index because it evaluates only maximally stained tumor

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fields and has less reproducibility as this selection of hotspots may be different for separate observers especially in heterogeneous tumors with multiple hotspots. Subsequently this diagnostic pitfall may cause bias in estimation of Ki-67 proliferation index and subsequent categorization which is one of the factors for instituting chemotherapy and their subsequent follow up for response to therapy, in carcinoma breast.

Limitations

1. In our study sample size was limited so studies with larger sample size are required for further validation.
2. We included all the cases for estimation of Ki-67 index by both methods as per our protocol, irrespective of their treatment status but the average values may be lower when same is estimated in patients undergoing hormonal and or chemotherapy.

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