

# Comparison of Four Different Scoring Methods for Ki-67 Percentage in Breast Carcinoma: A Cross-sectional Study

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## ABSTRACT

**Introduction:** In breast cancer, the prognostic role of Ki-67 has been comprehensively studied, and its usefulness has been proven. Ki-67 expression has a prognostic and predictive value in both adjuvant therapy response and neoadjuvant settings. However, the inter and the intraobserver variability in manual counting limits the accuracy of scoring Ki-67 and consequently its application in treatment.

**Aim:** To examine four different methods of Ki-67 estimation to find the most reliable, reproducible and time-efficient scoring methods for Ki-67.

**Materials and Methods:** The present cross-sectional study was conducted in the Department of Pathology, Sree Mookambika Institute of Medical Sciences, Nagercoil, Tamil Nadu, India, from May 2023 to August 2023. Ki-67 immunostained slides of 30 trucut biopsies of invasive breast carcinoma were retrieved and analysed by two observers in a blinded manner. The four different methods of analysis of Ki-67 expression carried out were the global method, the hotspot method, the Eye-10 method and the stepwise counting strategy. The parameters included in the present study were the mean age of the study population, the pre/postmenopausal status, the histopathological type of invasive breast carcinoma, Estrogen receptor (ER), Progesterone Receptor (PR), and Human Epidermal growth factor Receptor 2 (HER2)/neu status, as well as, the interobserver agreement and the mean time taken by the four methods to analyse the

Ki-67 expression. Data entry was performed using Microsoft Office Excel 2013, and statistical analysis was conducted using Statistical Package for the Social Sciences (SPSS) software version 20.0. Descriptive statistics for qualitative variables and mean and Standard Deviation (SD) for quantitative variables were used for data analysis. Chi-square tests were used for bivariate analysis with a determined statistical significance of 5% ( $p$ -value $<0.05$ ).

**Results:** The mean age of the study population was  $52.9 \pm 9.1$  years. The highest interobserver concordance was observed among the observers using the weighted global scoring method, with an Interclass Correlation Coefficient (ICC) of 0.967. This method was time consuming, with the first observer taking a mean $\pm$ SD time of  $5.5 \pm 0.8$  minutes and the second observer taking  $4.6 \pm 0.9$  minutes. The least time was consumed for performing the Eye-10 scoring, with mean $\pm$ SD time was  $1 \pm 0.87$  minutes. The stepwise counting and hotspot method demonstrated excellent inter-rater agreement, with a kappa of 0.8304 ( $p$ -value $<0.001$ ) between both observers.

**Conclusion:** Although the Eye-10 method and stepwise counting took the least time, they are limited by a gray/intermediate zone for scoring compared to the global scoring method and the hotspot method. As a result, the global and/or hotspot method after proper training is a robust and reliable method of assessing Ki-67, with the hotspot method being the most reliable, as the global method is limited by the use of an online tool.

**Keywords:** Assessment, Breast cancer, Hotspot

## INTRODUCTION

The most common malignancy in women is breast carcinoma, which accounts for up to 11.7% of all malignancies [1]. Ki-67 expression is used to subdivide luminal-like breast cancers into luminal A and luminal B groups [2,3]. Ki-67 staining is thus of critical importance for therapeutic approaches in breast cancer. The St. Gallen International Consensus Guidelines 2021 panel discussed strategies for early breast carcinoma treatment, with importance placed on Ki-67, including refined guidance on local-regional and systemic therapy that builds on its earlier recommendations. However, a standard methodology for the evaluation of Ki-67 has not yet been established [4,5]. The inter and intraobserver variability in the manual counting method of Ki-67 limits the accuracy of its scoring and, therefore, its application in treatment. A computational automated Ki-67 proliferation scoring to improve analysis in digital pathology may not be a feasible option for all laboratories [6]. Ki-67 can be used as an independent predictive marker of pathological response in patients undergoing neoadjuvant chemotherapy in breast carcinoma, underscoring the importance of an efficient scoring method [7]. The aim of the present study was to analyse four different methods of Ki-67 estimation: the global method,

the hotspot method, the stepwise counting strategy, and the Eye-10 method, and thereby find and validate the most reliable, reproducible, and time-efficient scoring system among the four different methods.

## MATERIALS AND METHODS

The present cross-sectional study was carried out in the Department of Pathology, Sree Mookambika Institute of Medical Sciences, Nagercoil, Tamil Nadu, India, from May 2023 to August 2023. In the present study, Ki-67 immunostained slides of 30 trucut biopsies of invasive breast carcinoma were retrieved and analysed. The slides were labelled from 1 to 30 and were interpreted separately for each of the four methods by two pathologists. Institutional Ethical Clearance was obtained before the start of the study (IEC No: SMIMS/IHEC No:1/Protocol No 11/2023).

The four different methods of analysis of Ki-67 expression carried out were the global method, the hotspot method, the Eye-10 method and stepwise counting strategy. The parameters included in the study were the mean age of the study population, the pre/postmenopausal status, the histopathological type of invasive breast carcinoma, ER, PR and HER2/neu status, as well as, the

interobserver agreement and the mean time taken by the four methods to analyse the Ki-67 expression.

**Inclusion criteria:** Trucut breast biopsy samples diagnosed as invasive breast carcinoma for which Ki-67 Immunohistochemistry (IHC) was performed were included in the study.

**Exclusion criteria:** Trucut breast biopsy samples diagnosed as only ductal or lobular carcinoma in situ were excluded from the study.

## Study Procedure

### Staining method and interpretation:

- The antigen retrieval was carried out on paraffin-embedded slides in a microwave oven in citrate buffer for 20 minutes. The Ki-67 antibody (Dako) was diluted 1:500 and incubated for 25 minutes. The slides were then stained with diaminobenzidine and counterstained with hematoxylin.
- Cells with any degree/intensity of brown nuclear staining were considered positive. Cells showing only blue haematoxylin counterstain (absence of brown nuclear staining) were considered negative [4].

**Global method [4,8]:** The International Ki-67 Working Group (IKWG) website was accessed, and the online scoring application (app) was linked. After prespecified training methods, the Ki-67 slides were reviewed using a light microscope with low power magnification using 10x objectives, excluding the areas of carcinoma in situ and non tumour tissue (necrosis and fibrosis). Nuclear staining of any intensity was defined as Ki-67 positive. Estimates for the percent area with negligible, low, medium, or high Ki-67 index were made in relation to overall percentage positivity. Then, 100 nuclei were scored in a typewriter counting pattern from the top of the selected scoring field in one high power field (40x) each as negligible, low, medium, or high in each field type until either 100 invasive tumour nuclei in total had been counted or all invasive tumour nuclei in the entire scoring field had been counted, whichever came first. The weighted global score output as Ki-67 index for the reviewed slide was recorded, and the interobserver variability was assessed. Ki-67 scores of less than 20% were categorised as low Ki-67, and scores of 20% or more were categorised as high Ki-67 [9,10].

**Hotspot method [4,8]:** The online scoring app on the IKWG website was accessed, and the 30 Ki-67 immunostained slides were reviewed by two separate observers. One high-powered field with the highest Ki-67 positivity was selected, and 500 nuclei were counted. The hotspot score report, as calculated by the app, was documented. Interobserver variability of the scored slides was assessed. Ki-67 scores of less than 20% were categorised as low Ki-67 and scores of 20% or more were categorised as high Ki-67.

**Eye-10 method (At a glance) [11]:** Using 10x fields including hotspots, assessments were made in 10% intervals at a glance by two separate observers. Ki-67 scores of less than 20% were categorised as low Ki-67 and scores of 20% or more were categorised as high Ki-67.

**Stepwise counting strategy [12]:** Ki-67 stained slides were initially assessed by counting 50 cells under 40x in a hotspot. 0-2 positive cells were declared as Ki-67 negative, 19 to 50 positive cells as Ki-67 positive. If the number of positive cells was 3-18, another 10 cells were counted. The steps were repeated with 10 tumour cells at a time until the upper or lower regions were assessed. Up to a maximum of 400 cells were evaluated. When the entire field of magnification did not include enough tumour cells, a new field was chosen within the same hotspot and adjacent to the original field. The number of Ki-67 positive cells falling in the green colour coding was taken as low Ki-67 (<20%), and the number of cells falling in the red category was taken as high Ki-67 (≥20%).

## STATISTICAL ANALYSIS

Data entry was performed using Microsoft Office Excel 2013, and statistical analysis was conducted using SPSS software version 20.0.

Descriptive statistics such as frequency for qualitative variables and mean and standard deviation for quantitative variables were used for data analysis. Chi-square tests were used for bivariate analysis with a predetermined statistical significance level of 5% (p-value<0.05).

## RESULTS

The mean age of the study population was 52.9±9.1 years. Approximately 12 (40%) females were premenopausal, while 18 (60%) were postmenopausal. Nine (30%) cases were Modified Bloom-Richardson (MBR) grade 2 and 21 (70%) cases were MBR grade 3. The majority (96.7%) of the cases were invasive carcinoma breast, NST and 1 (3.3%) case was mixed ductal and lobular type carcinoma. Twenty-three (76.7%) cases were ER positive and 7 (23.3%) cases were ER negative. Fifteen (50%) cases were PR and HER2 positive, and 15 (50%) were PR and HER2 negative. Fifteen (50%) cases were luminal A, and 15 (50%) cases were Luminal B as depicted in [Table/Fig-1].

Parameters	Frequency (n)	Percentage (%)
<b>Age group (in years)</b>		
>55	10	33.3
≤55	20	66.7
<b>Menopausal status</b>		
Premenopausal	12	40
Postmenopausal	18	60
<b>Modified Bloom-Richardson grade</b>		
Grade 2	9	30
Grade 3	21	70
<b>Histopathological type of invasive breast cancer</b>		
NST	29	96.7
Mixed ductal and lobular type	1	3.3
<b>ER status</b>		
Positive	23	76.7
Negative	7	23.3
<b>PR status</b>		
Positive	15	50
Negative	15	50
<b>HER2/Neu status</b>		
Positive	15	50
Negative	15	50
<b>Molecular profile</b>		
Luminal A	15	50
Luminal B	15	50

**[Table/Fig-1]:** Clinical profile of the cancer patients.  
NST: No special type

In the global scoring (weighted) method, 19 (63.3%) cases showed high (≥20%) Ki-67 percentage, and 11 (36.7%) cases showed low (<20%) Ki-67 percentage. In the Tumour hotspot method, 21 (70%) cases showed high Ki-67, and 9 (30%) cases showed low Ki-67 expression. The Eye-10 methods showed 21 (70%) cases with high Ki-67 and 9 (30%) cases showed low Ki-67 expression. The stepwise counting method showed 23 (76.7%) of cases with high Ki-67 and 7 (23.3%) cases with low Ki-67 as depicted in [Table/Fig-2].

Method	Cut-off	High, n (%)	Low, n (%)
Global scoring (weighted)	20	19 (63.3)	11 (36.7)
Tumour hotspot	20	21 (70)	9 (30)
Eye-10	20	21 (70)	9 (30)
Stepwise counting	20	23 (76.7)	7 (23.3)

**[Table/Fig-2]:** Distribution of high and low Ki-67 expression, as per the various methods.

The highest interobserver concordance was seen among the observers using the weighted global scoring method with an ICC of 0.967. However, this was also time consuming, with the first observer taking a mean±SD time of 5.5±0.8 minutes and the second observer taking 4.6±0.9 minutes. The tumour hotspot method had concordance with ICCs of 0.951 and 0.936, respectively, as depicted in [Table/Fig-3]. Eye-10 scoring also had good concordance between observers (ICC 0.888). The least time was consumed for doing the Eye-10 scoring with mean±SD time was 1±0.87 minutes. These agreements were also highly statistically significant as described in [Table/Fig-3] below. The stepwise counting method also had excellent inter-rater agreement with a kappa of 0.8304 (p-value<0.001) between both observers. The mean time taken by observer 1 for the stepwise counting method was 1.05±0.37 minutes, while observer 2 took a mean time of 0.98±0.09 minutes.

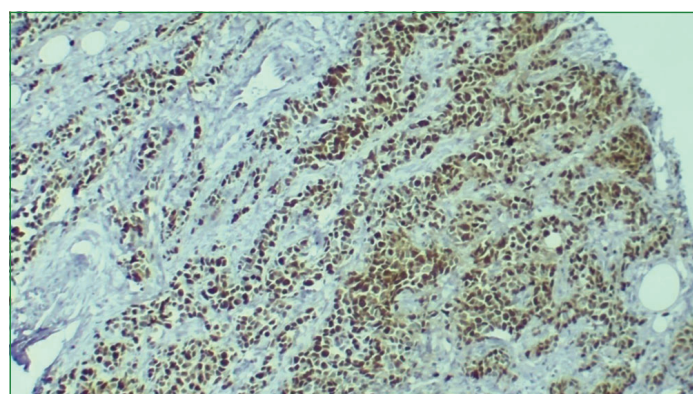
Method	Observer	Time in minutes Mean (SD)	Ki-67 score Mean (SD)	Agreement	Df, p-value
Global weighted score	1	5.5 (0.8)	37.13 (33.3)	0.967	29, <b><0.001</b>
	2	4.6 (0.9)	32.73 (29.7)		
Hotspot score	1	5.37 (0.8)	42.4 (34.3)	0.936	29, <b><0.001</b>
	2	4.2 (1.4)	35.81 (30.5)		
Eye-10 score	1	1 (0)	41.93 (34.1)	0.888	29, <b><0.001</b>
	2	0.87 (0.3)	(27.1)		

**[Table/Fig-3]:** Agreement among the various methods of Ki-67 estimation between observers. The p-value in bold font indicates statistically significant values; Mean times for stepwise counting method are 1.06±0.4 for observer 1±0.93 minute (0.1) for observer 2.

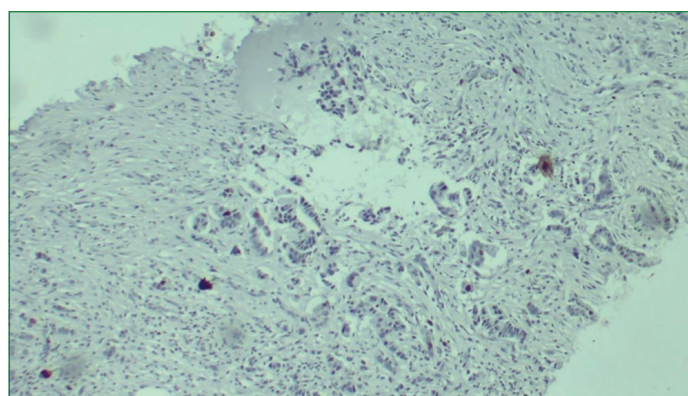
There was substantial agreement between the global weighted scoring method and the stepwise counting method with a kappa of 0.69. The inter-rater agreement between the tumour hotspot method and the stepwise counting method was excellent with a kappa of 0.83. The Eye-10 method also had excellent agreement with the stepwise counting method, with a kappa of 0.83 as depicted in [Table/Fig-4]. The expression of high and low Ki-67 is depicted in [Table/Fig-5,6].

Method	Stepwise counting strategy score of Ki-67		Agreement (κ)
	High	Low	
<b>Global weighted Ki-67 score</b>			
High	19	0	0.6889
Low	4	7	
<b>Tumour hotspot method score</b>			
High	21	0	0.8305
Low	2	7	
<b>Eye-10 method score</b>			
High	21	0	0.8305
Low	2	7	

**[Table/Fig-4]:** Inter-rater agreement between stepwise counting of Ki-67 expression and global scores, hotspot method and Eye-10 scores.



**[Table/Fig-5]:** High Ki-67 index (98%) (H&E, 10x).



**[Table/Fig-6]:** Low Ki-67 index (10%) (H&E, 10x).

## DISCUSSION

Expression of Ki-67 is an important prognostic biomarker for assessing cancer and has predictive significance in its treatment [13,14]. The biological heterogeneity of the tumour and the lack of standardisation in Ki-67 interpretation have resulted in inconsistent interobserver and inter-laboratory reproducibility [4,15]. The type of tissue, warm and cold ischaemic time, fixation medium used and fixation time are some of the preanalytical variables, whereas the choice of antibody used, antibody retrieval, and scoring method are some of the analytical and postanalytical variables in Ki-67 estimation [15-17].

In 2011, the IKWG recommended visual counting of the positive Ki-67 cells among at least 1000 invasive tumour cells; however, it was deliberated that the task may be labour-intensive for pathologists [15,18]. To reduce the variability, the IKWG in 2018 recommended a standardised scoring system with an online application to score Ki-67 with two methods, the global method and the hotspot method [4]. The global method is tedious, time consuming, and requires regular digitalisation of the stained sections, which could be challenging to achieve in routine practice. The intraobserver and interobserver variability due to minor differences in defining and selecting hot spots can make a significant difference in the Ki-67 score by the hotspot method [8]. In the current study, the highest interobserver concordance was seen among the observers using the global weighted scoring method. However, this was time consuming. The tumour hotspot method had high concordance similar to the global method but was less time consuming than the global method.

Hida Al et al., showed that the visual assessment of Ki-67 at a glance with a 10-grade scale (Eye-10) is an easy method and can exclude obviously high and low Ki-67 breast tumours [11]. The Eye-10 scoring in the present study also had good concordance between observers. The least time was consumed for performing the Eye-10 scoring. Romero Q et al., proposed a stepwise counting strategy, a time saving method, which acknowledges small highly proliferative hot spots that could overcome the diluting effect of the Ki-67 labeling index, especially in heterogeneous and highly proliferative cases [12]. The stepwise counting method in the present study had excellent inter-rater agreement and was less time consuming.

The disadvantage of both the Eye-10 method and stepwise counting strategy is the presence of a gray/ intermediate zone (equivocal), which is inevitable and not present in the global method and the hotspot method. In the current study, the Eye-10 method was similar to the study by Romero Q et al., and the stepwise counting strategy had good to excellent inter-rater agreement with high statistical significance and took the least amount of time for scoring compared to the global scoring method and the hotspot method [12]. The global and hotspot method, though having good inter-rater agreement, was labor-intensive and time consuming. Automated digital image analysis of Ki-67 can be done but is not feasible for all institutions [19].

The St. Gallen consensus 2009 proposed three categories of Ki-67: low (15%), intermediate (16-30%) and high (>30%); St. Gallen 2011

held two categories with a cut-off of 14% between luminal A and luminal B [20,21]. The St. Gallen consensus in 2013 changed the cut-off point to 20% with the option to use local laboratory (median) values [22]. In 2015, Ki-67 of  $\geq 20\%$  was chosen to distinguish luminal B-like disease [9]. Polewski MD et al., in their study found that patients with a Ki-67  $\geq 20\%$  had an increased risk of developing invasive disease within two years compared to those with Ki-67  $< 20\%$ , thereby validating the prognostic value of Ki-67 at this specific cut-off point [10]. In the present study, the Ki-67 cut-off point for all the four different methods of estimation is 20%.

### Limitation(s)

The major limitation of the present study was a small sample size, and immunostaining for Ki-67 of corresponding surgical specimens for confirmation was not feasible. Parameters such as distant metastasis and overall survival could not be accessed due to a lack of follow-up.

### CONCLUSION(S)

In conclusion, although the Eye-10 method and stepwise counting took the least time, they are limited by a gray/intermediate zone for scoring compared to the global scoring method and the hotspot method. As a result, the global and/or hotspot method, after proper training, is a robust and reliable method of assessing Ki-67, with the hotspot method being the most reliable, as the global method is limited by the use of an online tool.

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