RESEARCH ARTICLE

Comparison of visual assessment (VA) and automated assessment (Digital Image Analysis, DIA) of Ki-67 in breast carcinoma

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Abstract

Objective: To determine the agreement between visual assessment and automated assessment of Ki-67 labelling index in breast carcinoma.

Method: The observational, prospective study was conducted at the Histopathology Department, Shifa International Hospital, Islamabad, Pakistan, from November 24, 2018, to November 23, 2019, and comprised female patients regardless of age who had been diagnosed with invasive breast carcinoma. Ki-67 immunohistochemical staining was performed on paraffin embedded sections of the breast. Visual assessment of the proliferative index was done using hotspot method, followed by automated assessment of the digital image by ImmunoRatio software. Microscopic images of the maximally positive areas were taken and uploaded onto the software which gave an assessment of the proliferative index. The outcomes of the two assessments were compared. Data was analysed using SPSS 16.

Result: Of the 200 females with mean age 49±5.6 years (range: 29-83 years), 125(62.5%) showed homogeneous 75(37.5%) exhibited heterogeneous staining for Ki-67 on immunohistochemistry. Overall, high Ki-67 index value was noted for 159(79.5%) cases by visual assessment and 168(84%) by digital image analysis. The corresponding value for low Ki-67 index value were 41(20.5%) and 31(16%). Agreement between the two methods among high Ki-67 index value was in 146(73.0%) cases, and the corresponding value for low Ki-67 value was 18(8.5%). Besides, 36(18%) cases showed disagreement in Ki-67 values.

Conclusion: There was a significant agreement between visual assessment and digital image analysis of Ki-67 labelling index in breast carcinoma, suggesting that both the methods can be used in clinical practice.

Key Words: Ki-67 antigen, Immunohistochemistry, Digital image analysis, Proliferative index, Breast cancer.

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Introduction

Breast cancer (BC) is the leading cancer worldwide, including Pakistan.[1] The traditional classification is of limited value as breast malignancies with similar clinicopathological features may behave differently as regards outcome and treatment response.[2] Therefore, to unravel this heterogeneity, a new classification system was developed based on hormone receptor status and gene expression profiling i.e. molecular subtypes of BC. It includes the expression of oestrogen receptors (ERs) and progesterone receptors (PRs), human epidermal growth factor receptor 2 (HER2Neu) and Ki-67 status based on immunohistochemistry (IHC).[2]

Accordingly, BC is divided into four molecular subtypes; Luminal A, Luminal B, HER2Neu, and Triple-negative BC (TNBC), and each shows characteristic clinical behaviour.[3] The Ki-67 proliferative index is important in terms of differentiating HER2-negative Luminal A and Luminal B BC and advise adjuvant chemotherapy for Luminal B cancers, but not for Luminal A4. The cut-off for Ki-67 labelling index (LI) was previously 14% but in recent studies 20% has been proposed[5]. Keeping this in view, a precise and dependable Ki-67 scoring method is needed.[6] To improve the accuracy and reproducibility of Ki-67 assessment, many image-based analysis software programmes have been developed.[6] Most of these are very expensive, and, therefore, are not widely used, especially in developing countries, but some are free and easily available, like ImmunoRatio.[6]

The current study was planned to compare visual assessment (VA) with digital image analysis (DIA) Ki-67LI in BC.

Patients and Methods

The observational, prospective study was conducted at the Histopathology Department, Shifa International Hospital, Islamabad, Pakistan, from November 24, 2018, to November 23, 2019. After approval from the institutional ethics review committee, the sample size was calculated

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using the World Health Organisation (WHO) calculator.[7] The sample was raised using non-probability consecutive sampling technique. The sample comprised female patients regardless of age who had been diagnosed with invasive BC on the basis of tru-cut needle or incisional biopsy, lumpectomy and mastectomy. Patients with in-situ BC only were excluded. Mastectomy specimens of patients who had received neo-adjuvant chemotherapy (NACT) were also excluded, and so were the samples with scanty tumour tissues (<500 tumour cells) and poorly fixed tissues.

All the specimens were formalin-fixed and grossed as per the technique recommended by the College of American Pathologists (CAP) protocols 2018.[8] Blocks best representative of the lesion were selected for IHC. Sections for IHC were cut at 4µ from paraffin embedded blocks, de-paraffinised and rehydrated. IHC for Ki-67 was performed with a multimer-technology-based detection system (ultraView Universal DAB, Ventana, Tucson, United States). The Ki-67 monoclonal antibody (MIB-1) ready to use (RTU) was used in an automated immunostainer (BenchMark XT, Ventana, Tucson, US) with a proprietary buffer (Standard Cell Conditioner 1 [CC1], Ventana, Tucson, US) at 95°C for 64 minutes. Lastly, the sections were exposed to diaminobenzidine (DAB) at 37°C for eight minutes. It was then counter-stained with Mayer's haematoxylin and mounted.

For VA, the entire slide was scanned under low-power microscopy. At least three high-power (x40 objective) fields (HPFs) were selected to represent the spectrum of staining seen on preliminary overview of the whole slide. In slides that were homogenously stained, the area chosen for VA included at least 500 tumour cells, whereas in heterogeneously stained slides, at least three hotspots (maximally stained areas) were selected to assess KI-67LI which was recorded as a percentage.[9,10]

For DIA of Ki-67LI using ImmunoRatio[10], different numbers of photomicrographs of Ki-67 immunostained slides were taken using Nikon microscope-mounted camera (Digital Sight DS-Fi2) Japan, choosing hotspot or average method at high power, depending on the tumour cell distribution in each case, to capture a minimum of 500 cells. The selected images were then uploaded on The ImmunoRatio webpage, which is an ImageJ plugin for image analysis and scoring of immunostained slides.[10] The ImmunoRatio programme was operated in the advanced mode. The brown and blue threshold modifications were set on auto-setting. The most suitable tumour nuclear size was selected. DAB-stained and haematoxylin-stained nuclei were separated by colour threshold, and uploaded. A pseudo-coloured (montage) image corresponding to the area separated by the percentage of Ki-67 proliferative index was generated by the software. After that, the "analyse" button was clicked, and the result was displayed on the screen.[11-13]

The data was recorded on a proforma, including patient's particulars, type of biopsy, VA score and DIA score. The total number of images uploaded per case and Ki-67 staining distribution, like homogeneous and heterogeneous patterns, were also recorded.

Data was analysed using SPSS 16. The Ki-67LI data was divided into two categories; 20% = low and ≥20% = high. Quantitative variables were expressed as mean ± standard deviation, while qualitative variables were expressed as frequencies and percentages. Kappa statistics were used to conclude percent agreement between VA and DIA.

The kappa results were categorised as follows: 0 = no agreement, 0.01-0.20 = none to slight, 0.21-0.40 = fair, 0.41-0.60 = moderate, 0.61-0.80 = substantial, and 0.81-1.00 = nearly perfect agreement.[13] P<0.05 was taken as statistically significant.

Results

Of the 200 females with mean age 49±5.6 years (range: 29-83 years), 125(62.5%) showed homogeneous 75(37.5%) exhibited heterogeneous staining for Ki-67 on IHC. There were 135(67.5%) tru-cut needle biopsies, 37(18.5%) mastectomies, 26(13%) lumpectomy, and 2(1%) incisional biopsies.

Overall, high Ki-67 index value was noted for 159(79.5%) cases by VA and 168(84%) by DIA. The corresponding value for low Ki-67 index value were 41(20.5%) and 31(16%). Agreement between the two methods among high Ki-67 index value was in 146(73.0%) cases, and the corresponding value for low Ki-67 value was 18(8.5%).

Table-1: Cross-tabulation of visual assessment (VA) and digital image analysis (DIA) of Ki-67

		Digital Image Analysis of Ki67 (N=200)		
		Higher Ki67 >20% (%)	**Low Ki67 <20% (%)**	**Total (%)**
Visual assessment of Ki67 (N=200)	Higher KI67 >20%	146 (73.0)	13 (6.5)	159 (79.5)
	Low Ki67 <20%	23 (11.5)	18 (9)	41 (20.5)
	Total (%)	169 (84.5)	31 (15.5)	200 (100)

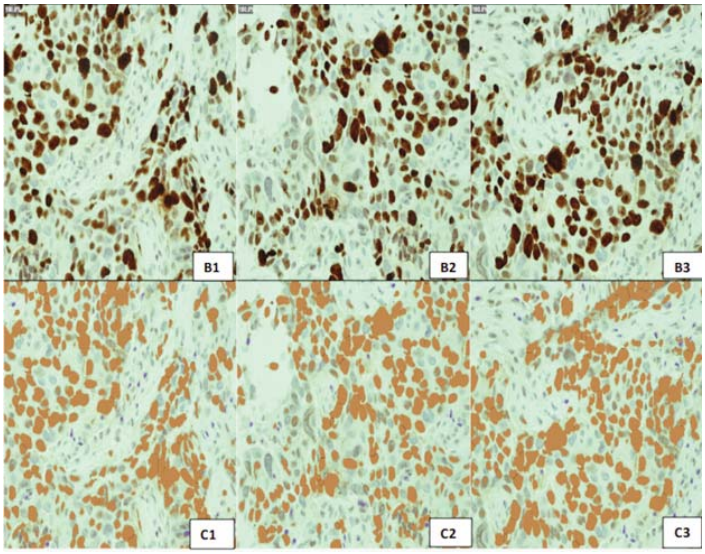

Figure-1: Homogenous Ki-67 staining showing agreement between visual assessment (VA) (100%) and digital image analysis (DIA) (100%) (x40 objective)
A: Estimation of average Ki-67 index for the three sets of uploaded images by DIA.
B1-B3: Original uploaded images with individual Ki-67 index.
C1-C3: Images automatically generated by the software.

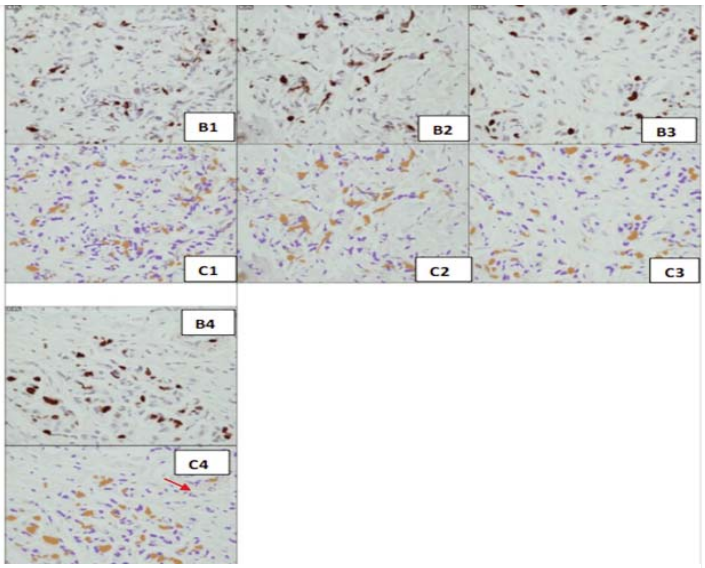

Figure-2: Disagreement between visual assessment (VA) (5%) and digital image analysis (DIA) (counted as 25.4%) due to some stromal cells (red arrow) assessed as Ki-67 positive tumour cells by the software (x40 objective).
A: Estimation of average Ki-67 proliferative index for the four sets of uploaded images.
B1-B4: Original uploaded images with individual Ki-67 index.
C1-C4: Images automatically generated by the software.

(Figure 1). Besides, 36(18%) cases showed disagreement in Ki-67 values. There were 13(6.5%) cases scored as high Ki-67LI by VA and low on DIA (Figure 2). Conversely, 23(12%) cases scored as low Ki-67LI by VA, but high by DIA (p=0.36). Overall kappa value 0.393 showed a fair agreement between DIA and VA (Table 1).

Table-2: Causes of Ki-67 labelling index (LI) discrepancy between visual assessment (VA) and digital image analysis (DIA).

Cause of discrepancy	n=36(%)
Type of biopsy (tru-cut biopsy)	29 (80.5)
Tumour heterogeneity	28 (77.7)
Misidentification of tumour cells/ non tumour cell estimation	16 (44.4)
Visual assessment interpretation error	13 (36.1)
Poor quality immunostaining	05 (13.8)
Technical factors affecting digital image analysis.	02 (5.5)

There were multiple causative factors for disagreement in Ki-67LI scoring in 36(18) cases. The cause of discrepancy was scanty focal tumour in tru-cut biopsies in 29(80.5%), followed by tumour heterogeneity in 28(77.7%) cases, wrong identification of tumour cells / non-tumour cell estimation by DIA in 16(44.4%) cases, VA interpretation error in 13 (36.1%) cases, poor quality immunostaining in 5(13.8%) cases, and technical factors affecting DIA in 2(5.5%) cases. There were multiple causative factors in individual specimens (Table 2).

Discussion

Ki-67LI, expressed as percentage of Ki-67 antigen positive tumour cells, is very important because it is a prognostic marker for response to NACT in BCs.[14] However, there is no standardised methodology to evaluate Ki-67LI. The International Ki-67 Breast Cancer Working Group15 recommended manual scoring, but exact counting of a large number of tumour cells requires a lot of time, and is, therefore, not possible in a busy histopathology setup. To address the issue, many expensive digital image-based analysis programmes have been developed.[14] By using these programmes, lasting digital data is generated, and repeat assessment is available at any time.

ImmunoRatio is an open, computerised, web-based image analysing application for determining Ki-67LI.[16] The current study aimed at evaluating this software and its usage in local hands. The Ki-67LI correlation between ImmunoRatio and VA showed fair agreement (kappa: 0.393), and p=0.36 showed that the variables were not statistically different from each other.

According to the International Ki-67 Breast Cancer Working Group, Ki-67 cut-off should be applicable only after observer validation, and analysis should only be performed in an experienced laboratory.[17] Currently, 20% Ki-67LI is used as a cut-off for molecular classification of invasive BC and as a prognostic factor[17], therefore, the

current study used a cut-off of 20% for Ki-67LI, like in previous studies.[18,19]

There was a reasonable concordance between VA and DIA in previous studies in BC cases as well as in other cancers.[20,21] The main advantages of DIA are the reproducibility, permanence and storage of data. In the current study, majority of cases showed appreciable concordance between both methods, with high Ki-67LI displaying a homogenous staining pattern (79.5% by VA and 84% by DIA) and having the highest concordance. A lower concordance rate was noted among intermediate range group with Ki-67LI values of 10-30%. Similar results were also seen in previous studies.[21] The current study analysed 36 cases with inconsistencies >5-10% between the VA and DIA values, and found multiple factors involved.

The most important factor was the nature of the biopsy. The study showed that tru-cut biopsy specimen analysis yielded more discrepancies between VA and DIA results compared to mastectomy specimens, which was in line with earlier results.[22] This is because tru-cut biopsy specimens have scanty tumour cells interrupted by stroma in most cases, resulting in lesser number of tumour cells available for evaluation.

Heterogeneity of Ki-67LI was the second commonest cause of discrepancy in the current study, as reported by earlier studies.[21,22] To overcome this problem, it is recommended to calculate Ki-67LI in peripheral tumour cells with the highest proliferative index. Therefore, selection of the correct area is the cornerstone of correct assessment. Many researchers have proposed a solution by standardising the selection method, region of interest (ROI) size and the number of cells. Ki-67LI is highly dependent on ROI size, and the number of cells estimated visually. This is also an important parameter for Ki-67 quantification by DIA. Scanning the entire slide is another possible way to resolve this heterogeneity problem. This approach was adopted by Laurinavicius et al.[10]

In the current study, better Ki-67LI agreement was observed in homogeneous (Figure 1) than in heterogeneous slides. The study showed that tumour's heterogeneity affected the consistency between VA and DIA of Ki-67LI. Effort was made to overcome the problem by assessment of Ki-67LI using three hotspot areas with tumour cells >500, as was done by Zhong et al[23] who used an average scoring method, assessing minimum of three selected areas on the entire digital slide compared to the hotspot scoring method. Even then some cases showed disagreement, and Ki-67LI by VA was up to 30% compared to 100% by DIA.

Another cause of discrepancy between VA and DIA was tumour cells identification bias (Figure 2). DIA has benefit of quantifying larger number of cells and stronger objectivity compared to a human eye even with the microscope. However, DIA has its own disadvantages, like recognition of tumour cells. On VA, tumour cells are distinguished from lymphocytes and other stromal cells by histopathologists quite easily. However, occasionally DIA is unable to differentiate tumour cells from lymphocytes and stromal cells. For example, Ki-67 positive lymphocytes may be falsely interpreted as tumour cells, or some negative tumour cells may be miscalculated as lymphocytes, which may lead to inaccurate Ki-67LI. Double-staining can be used as an alternate technique to highlight the tumour cells, and to improve accuracy of DIA in Ki-67LI evaluation.[13]

Another factor leading to inconsistency was VA inaccuracy detected on slide review. Some were interpretational errors, while others were clerical.

Poor quality of Ki-67 immunostaining was another factor due to improper tissue fixation, tissue processing and thick slices because of abundant fat. The spindled morphology of tumour cells attributable to poor handling and fixation led to difficulty in differentiating tumour cells from stromal cells on DIA. The current study omitted most poorly-fixed sections with poor staining, but there was still potential for error due to focal artifacts in some cases.

DIA is an intricate task that involves multiple steps, including pre-processing, accurate outlining objects of interest, and measuring certain shape or textures. It is a tedious process with many confounding factors. Pre-analytical issues for achieving good IHC staining should be addressed even for accurate tumour area assessment which may be done by initial quantification using positive staining for cytokeratins.

VA has many limitations, particularly inter- and intra-observer variability. Ki67LI by DIA probably yields more reproducible and accurate results, and is an adequately effective method in local settings as well. The cost of DIA must be kept in mind, as the better commercially available softwares require a scanner for digitalisation of images. DIA software and a trained pathologist are required to accomplish the analysis. So, before the implementation of DIA for Ki-67LI as a routine, proper training of pathologists and laboratory staff is required. However, with the advent of artificial intelligence, this will soon be a routine methodology in the developed countries.

The current study used the best possible freely available

software at the time. This was a pilot project to see if this methodology was applicable in local settings without the use of a digital slide scanner. As scanners become more widely available, DIA seems to be an achievable objective for routine use in the future.

Conclusion

There was reasonable concordance between VA and DIA of Ki-67LI in BC cases, indicating that both VA and DIA can be used to assess Ki-67 LI in clinical practice. Multiple factors were found to be responsible for disagreement between the two methods. Therefore, proper awareness of confounding factors is required before it can be accurately implemented in routine practice.

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AUTHOR'S CONTRIBUTION:

MZ: Design, data acquisition, analysis, interpretation, drafting, final approval and agreement to be accountable for all aspects of the work.
NM: Concept, design, data interpretation, revision, final approval and agreement to be accountable for all aspects of the work.
IM: Concept, data interpretation, revision, final approval and agreement to be accountable for all aspects of the work.