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Assessment of Ki67 in Breast Cancer: A Comparison Between the Eye-10 Method, Stepwise Counting Strategy, and International System of Ki67 Evaluation

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ABSTRACT

Background & Objective: Ki-67 evaluation is an essential tool to define luminal A and B breast cancers, which is not yet systematized. The International Ki67 in Breast Cancer Working Group suggests the counting of 500 or 1000 cancer cells, which is a time-consuming process. Therefore, novel methods, such as the Eye-10 method and stepwise counting strategy, are proposed to facilitate measurement.

Methods: Immunohistochemical staining of Ki67 was performed on 100 hormone-receptor-positive invasive ductal carcinoma specimens. Ki67LI was evaluated for each case, and then results were dichotomized by a cut-off point of 20%. Next, for each sample, an expert pathologist visually assessed percentages of Ki67-positive cells in 10% intervals at a glance (Eye-10 method). Finally, by using a dynamic process with rejection regions, Ki67 was defined so if the estimate belonged to the upper or lower rejection region, the Ki67 status had been determined and if the rejection region could not be reached after counting the maximum number of 400 tumor cells, the specimen was regarded as equivocal (stepwise counting strategy).

Results: The comparison between Eye-10 and Ki67LI revealed almost perfect agreement (kappa coefficient =0.889), and the concordance between the stepwise counting strategy and Ki67LI was substantial (kappa coefficient =0.639).

Conclusion: Both two methods left some results in the gray/intermediate zone, which is unavoidable. Both methods are much faster and simpler than evaluation of Ki67LI and are also reliable. Regarding the gray zone in both methods, further improvements in the methodology, as well as more analytical studies, are needed.

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Introduction

Breast cancer is the most common malignancy in women all over the world. Nowadays, breast cancer is not treated as a single disease but is divided into different subgroups, which each of them have a different biology, therapeutic plan, and prognosis.

According to the 13th St. Gallen International Consensus Meeting, by using immunohistochemical (IHC) staining of estrogen receptors (ERs), progesterone receptors (PgRs), human epidermal growth factor receptor 2 (HER2), and Ki67, breast cancer is divided into four subtypes, i.e., Luminal A, Luminal B, Erb-B2 overexpression, and Basal-like.

Tumor specimens, with positive IHC staining for ER or PgR, are considered as hormone-receptor-positive (HR+) and classified into luminal subtypes.

Ki-67, a nuclear marker of cell proliferation, defines luminal A and B tumors. Among ER-positive/ HER2 negative tumors, luminal A-like tumors are defined as PgR positive and low Ki67 breast cancers with low recurrence risk based on a multigene expression assay. Meanwhile, luminal B-like tumors are defined as tumors with a negative or low positive reaction for PgR, high Ki67 (≥20%) index, and high recurrence risk (1).

In 2013, St. Gallen recommended the use of adjuvant cytotoxic chemotherapy for luminal B but not for luminal A. After this recommendation, the evaluation of ki67 via the IHC method has become widespread. However, the Ki67 assessment is not yet standardized (2).

Ki-67, a nuclear protein and prognostic factor for luminal-type breast cancers, was first identified by Gerdes *et al.* in the early 1980s, which was by using a mouse monoclonal antibody directed against a nuclear antigen from a Hodgkin's lymphoma-descended cell line. It was shown that the Ki-67 nuclear antigen is expressed in all cell cycles except G0. The most common analysis method of the Ki-67 antigen is the

immunohistochemical evaluation by using the antihuman Ki-67 monoclonal antibody MIB-1 and reporting the percentage of positively stained malignant cells (3).

However, the value of ki67 is limited because of many variations in the preanalytical, analytical, and post-analytical practices. To achieve a harmonized methodology, reduce intra and interobserver variability, and convince application in clinical practice, some recommendations were recently proposed by the International Ki67 in Breast Cancer Working Group for the analysis, reporting, and use of Ki67 in clinical practice. This guideline suggests the counting of 500 or 1000 cancer cells in at least three high-power (×40 objectives) fields, including the invasive edge of the tumor and hot spots (4).

Counting many cells, as a portion of a standard test, is exhaustive and time-consuming. Since this counting method is very labor-intensive, various methods are propounded by scholars all over the world to facilitate Ki67 measurement. In this study, the Ki67 labeling index (Ki67 LI) with manual counting, which has been represented by the International Ki67 in Breast Cancer Working Group, was compared with Eye-10 and stepwise counting strategy methods.

In the Eye-10 method, pathologist visually estimates percentages of Ki67-positive cells in 10% intervals at a glance; and in stepwise counting strategy, Ki67 is obtained by using a dynamic process with upper and lower rejection boundaries, which results in counting fewer cells (more explanation is available in the next section).

Materials and Methods

In this study, at the Pathology Department of Iran University of Medical Sciences, we surveyed 100 mastectomy specimens with the diagnosis of invasive ductal carcinoma with positive estrogen and progesterone receptors. As the names and personal information of the patients were not disclosed, this study obtained ethical approval from the Faculty of Medicine Ethics Committee. The specimens were fixed in buffered formalin 10% and paraffin-embedded. All the patients were female and have not received any neoadjuvant therapies. The paraffin blocks of the specimens were cut at 4 μm, deparaffinized, and rehydrated in graded ethanol. The antigen retrieval was performed in a microwave oven in citrate buffer pH 6 for 20 min. The Ki67 antibody (clone MIB-1, Dako, Glostrup, Denmark) was diluted 1:500 and incubated for 25 min. Then slides were stained with diaminobenzidine (DAB) chromogen and counterstained with hematoxylin.

Ki67 Labeling Index

For the first step, the percent of Ki67 was estimated for all specimens by the standard method of the International Ki67 in Breast Cancer Working Group (Ki67LI). Scoring involved the counting of at least 500 malignant invasive cells. According to the International Ki67 in Breast Cancer Working Group guidelines, only nuclear staining was considered positive, and staining intensity was not relevant (4). The Ki67 index was expressed as the percentage of positive stained cells among the total number of invasive cells in the scored area (Figures 1,2,3,4). Then the results are divided into “high Ki67” and “low Ki67” based on the cut-off point of 20%.

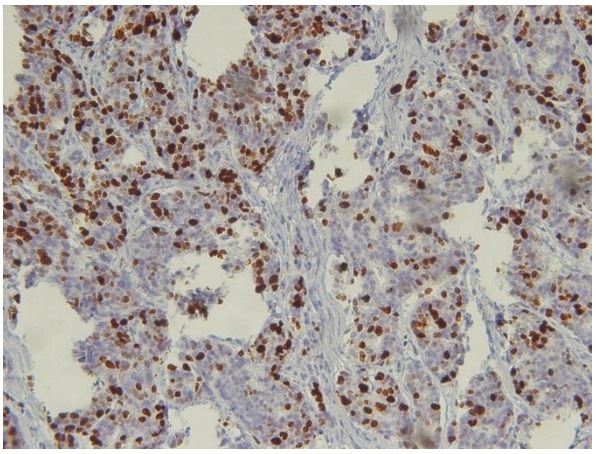

Fig. 1. Invasive edge of the tumor with objective lens *20

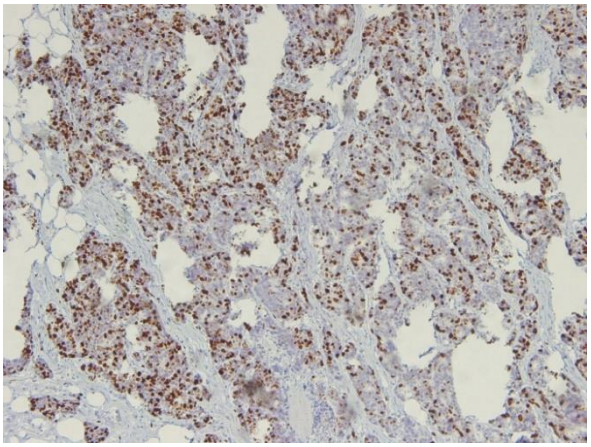

Fig. 2. Invasive edge of the tumor with objective lens *10

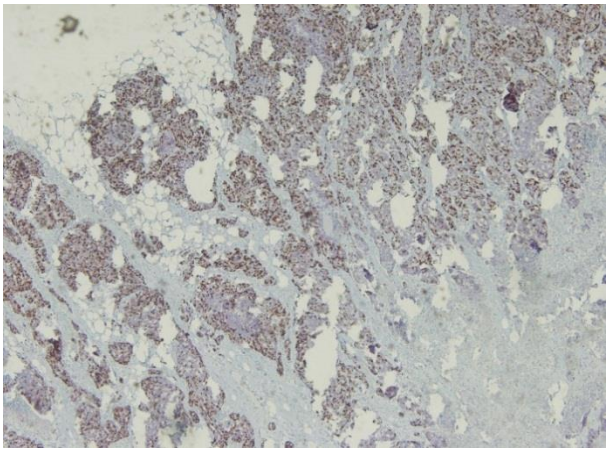

Fig. 3. Invasive edge of the tumor with objective lens *4

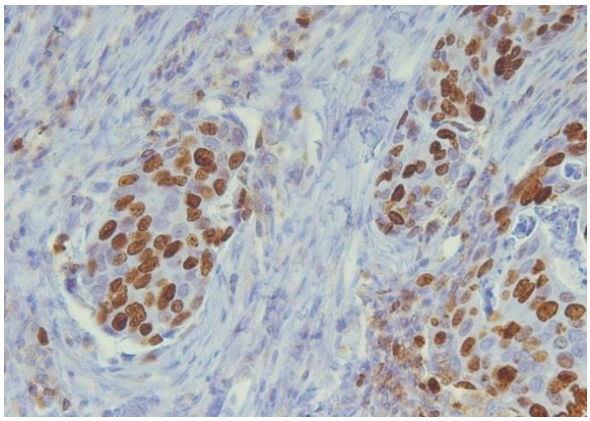

Fig. 4. Invasive edge of the tumor with objective lens *40

Visual Measurement of Ki67 at a Glance (Eye-10)

For the second step, Ki67 was estimated for all specimens by using a procedure similar to the Eye–10 method (5) with the following details:

For each sample, a hot spot was identified by using ×4 objective. Then, at ×10 and ×20 objective fields, an expert pathologist visually assessed percentages of Ki67-positive cells in 10% intervals at a glance (less than 10%, 10%, 20%, 30%, 40% …). Next, these scores were categorized as "low Ki67" (the specimens with Ki67 less or equal to 10%) and "high Ki67" (the specimens with Ki67 equal to 20%, 30%, 40% …).

Stepwise Counting Strategy

In the third step, Ki67 was estimated for all specimens via stepwise counting strategy in the manner described below:

This procedure is based on a dynamic process with rejection regions derived from exact two-sided binomial confidence intervals for proportions. Ki67 was defined by the following parameters: the cut-off (20%), minimum (50) and maximum (400) number of tumor cells to count, and increment (10) and overall significance level of the test procedure (0.05). So that in the hot spot, the minimum number of tumor cells (50 cells) was evaluated, and the fraction of Ki67 positive cells was compared to the rejection boundaries (these boundaries are defined in Table1 and Figure 1 in the original article) (6). If the estimate belonged to the upper or lower rejection region, the Ki67 status had been determined, and the evaluation had ceased. If not, the assessment continued with an additional number of tumor cells (10 cells). Finally, each specimen is categorized in one of these groups, i.e., low proliferative, high proliferative, and equivocal groups (when the rejection region could not be reached after evaluating the maximum number of 400 tumor cells) (6).

Statistical Analysis

The association between each method and Ki67LI was evaluated by SPSS 24 (SPSS Inc., Chicago, Illinois, USA) using Cohen's kappa coefficient of agreement.

Results

Out of the total 100 cases, 11 cases had low Ki67LI (<20%), and 89 cases had high Ki67LI (≥20%).

The lowest value was 1%, and the highest was 99% with the median and mean of 39.9% and 42.10±21%, respectively.

By Eye-10 method assessments, 9 cases had low Ki67 (<20%, that is, cases with Ki67 equal to or less than 10%), and 91 cases had high Ki67 (cases with Ki67 equal to 20%, 30%, 40% …). Actually, the Ki67 results were matched in most cases (98/100, 98%). Only two Eye-10 cases of ≥20% (2/100) showed Ki67/LILI <20% (Figure 5 and Table 1).

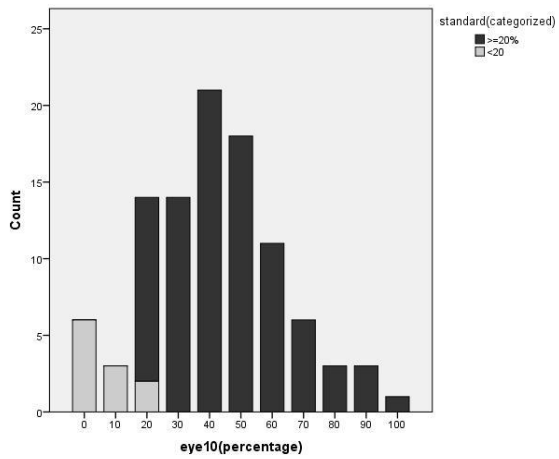

Fig. 5. The comparison between the Eye-10 and standard methods (Ki67 labeling index) shows almost complete agreement (kappa=0.889)

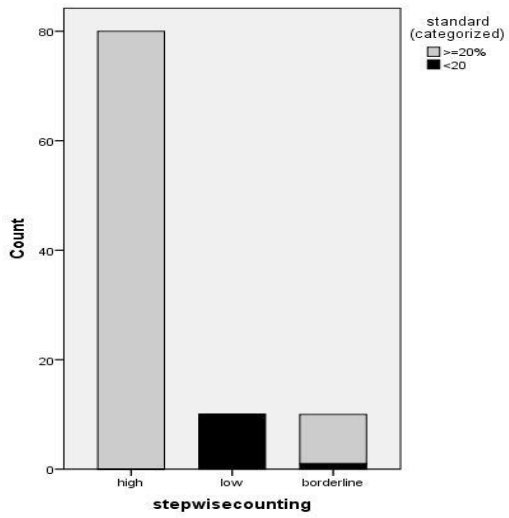

Fig. 6. The comparison between the stepwise counting strategy and standard method (Ki67 labeling index) shows substantial agreement (kappa=0.639).

On stepwise counting strategy, the Ki67 status was determined after the minimum number of 50 tumor cells, which were counted in 63 out of 100 cases. A total of 80 out of 100 samples were classified as high proliferative and 10 cases as low proliferative. A total of 10 out of 100 samples remained unclassified (failed to reach rejection boundaries), even after counting 400 tumoral cells, and were labeled as equivocal (borderline).

For 90 of 100 classifiable samples, the Ki67 status was determined by stepwise counting strategy, which was matched with Ki67LI (80 cases as high and 10 cases as low).

Of the remaining ten unclassified samples, nine samples had high Ki67LI, and one had low Ki67LI (Figure 6 and Table 2).

Table 1. The results of the standard and Eye-10 methods, comparing crosstab

	Eye-10 high	Eye-10 low	Total
Standard high	89	0	89
Standard low	2	9	11
Total	91	9	100

Table 2. The results of the standard method and stepwise counting strategy, comparing crosstab

	Stepwise counting strategy high	Stepwise counting strategy low	Stepwise counting strategy equivocal	Total
Standard high	80	0	9	89
Standard low	0	10	1	11
Total	80	10	10	100

Discussion

As a nuclear protein and essential indicator of uncontrolled cellular proliferation in malignancy, Ki67 has been shown to have prognostic value in breast cancer, and its predictive efficacy, for luminal-type breast cancers, has been proved in several studies (7-9).

According to the 13th St. Gallen International Consensus Meeting, the treatment modality is different for the luminal A and B subtype of breast cancer. The consensus recommended endocrine therapy for luminal A-like tumors with low Ki67 and endocrine plus cytotoxic chemotherapy for luminal B-like (HER2 negative) tumors with high Ki67 (1).

Nowadays, the Ki67 assessment by IHC is an acceptable current choice method to monitor tumor proliferation index in the pathology of breast specimens. However, preanalytical, analytical, and post-analytical issues can affect IHC results. In this regard, preanalytical variables are the tissue type, cold ischemic time shorter than one hour, fixation medium, and time to fixative. In addition, analytical and post-analytical variables are the type of the antibody, antigen retrieval, and scoring method or analysis strategy (4,10,11).

To harmonize the assessment method, minimize the variability, and increase intra and interlaboratory reproducibility, the International Ki67 in Breast Cancer Working Group has proposed guidelines for the evaluation of the ki67 maker in breast cancer (4).

Unfortunately, there is no standard method yet to evaluate ki67 in breast cancer.

Manual counting of at least 500 malignant invasive cells (and preferably at least 1000 cells), proposed by the International Ki67 in Breast Cancer Working Group, is often used to evaluate Ki-67 (12-14).

Counting this number of cells as a portion of a standard test is a massive, labor-intensive, and time-consuming task for pathologists and has a problem of reproducibility (15,16).

To resolve this problem, automated counting by a computer software device might be a candidate. However, differentiating invasive cancer cells from non-invasive carcinoma or non-tumoral cells is not easy work for computers. (2) In addition, the digital image analysis by PC software is not affordable for all countries and institutes.

Against this background, various methods, such as visual assessment (Eye-10) and stepwise counting strategy, are developed by different groups to simplify the counting method.

A study by Akira I. Hida *et al.* showed a significant positive correlation between Eye-10 and Ki67LI with the magnitude of (r=0.94). They showed that the visual assessment of Ki67 at a glance with a 10-grade scale (Eye-10) is an easy method (a rational alternative for Ki67LI) and can exclude obviously high and low Ki67 breast tumors, leaving a gray zone around a cut-off point (5).

The comparison between Eye-10 and Ki67LI in our study reveals almost perfect agreement (k=0.889). This agreement suggests that Eye-10 is a reliable, fast, and

easy method (which could easily classify the proliferation index to low and high), and it can stratify patients into luminal breast cancers.

In the present study, a total of 98 out of 100 cases matched in both Eye-10 class and Ki67LI, and only two cases showed different results. The values of Ki67LI of these two cases were 16.8% and 18%, which fell into a high proliferative category by the Eye-10 method.

In cases with ≤10% or ≥20% ki67 index, the Eye-10 method can discriminate clearly luminal A and luminal B tumors with a 20% cut-off. The Eye-10 assessment, which does not require counting 500-1000 cancer cells, is an appropriate tool for the evaluation of low or high Ki67 breast cancers.

The most challenging point is between 10-20%. Tumors in this range cannot be divided easily with the Eye-10 method, which is based on 10-percent intervals.

This group of luminal-type breast cancers might be regarded as a ‘‘gray zone” (5).

A study by Quinci Romero *et al.* revealed that stepwise counting strategy is a time-saving method, which could overcome the diluting effect of the ki67 labeling index, especially in heterogeneous and highly proliferative cases (6).

In our study, the agreement between the stepwise counting strategy and the Ki67LI was substantial (k=0.639). The reason for the lower Kappa coefficient in this method (in comparison to the Eye-10 method) is the fact that some of the cases remained equivocal. The main advantage of this method is that many cases are categorized by counting the fewer number of cells than the standard method (for 63 out of 100 cases, the Ki67 status was determined in the first round by counting 50 cells). Therefore, it is a time-saving and faster method, which simplifies the work of pathologists. The debating issue is around the cut-off point (20%), as all ten unclassified samples have Ki67LI around 20%, and the cases with Ki67 value far from 20% are grouped in the same way as the standard method (Ki67LI).

Therefore, cases with ki67 value, which are too close to the cut-off point, might fail to reach the rejection boundaries and will be considered as equivocal (gray zone).

The disadvantage of both the Eye-10 method and stepwise counting strategy is the presence of a gray/intermediate zone, which is unavoidable. Manual counting of 500-1000 cancer cells could be used here, but it is a huge task and may have a low reproducibility (5).

In these cases, considering other factors, such as the histological grade, nodal status, tumor size, lymphovascular invasion, and patient preferences, should be taken into account for a better decision about adjuvant therapy.

Conclusion

In this study, both the Eye-10 method and stepwise counting strategy are useful in stratifying luminal-type breast cancers. Both methods are much faster (taking less than one minute for each slide), simpler, and reliable than the evaluation of Ki67LI.

Considering the gray zone in both methods, where the precise evaluation is complicated, further improvements in the methodology and more analytical studies are needed.

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At the end, we are pleased to show our regards to all contributors during this study.

Conflict of Interest

The authors declared that there is no conflict of interest regarding the publication of this article.

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