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Semi-Automation Of The Ki-67 Proliferative Index Quantification Method In Breast Cancer

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Abstract— **In this retrospective study, the primary aim was to assess Ki-67 expression levels in breast cancer slide samples and analyze agreement among values obtained by different counting methods, including semi-automated approaches. The Ki-67 proliferative index, a crucial marker for assessing cell proliferation and predicting prognosis in breast cancer, was evaluated through three distinct methods: visual counting, manual counting by two calibrated observers, and a semi-automated approach. All mean values of the Intra-Class Correlation Coefficient obtained were statistically significant ($p < 0.01$). The lowest coefficient occurred in the comparison between the semi-automated program count and visual counting (0.791), while the highest correlation was between the program count and manual counting (0.996). This study underscores the importance of standardization and reliable methods in Ki-67 evaluation for precise interpretations and consistency in breast cancer cases, where the Ki-67 index plays a critical role in predicting disease progression and guiding treatment decisions.**

Keywords— **Breast cancer, Digital pathology, Ki-67, Proliferative index, Prognosis**

I. Introduction

Breast cancer is the most frequently diagnosed malignancy among young women and one of the leading causes of death globally [1]. The commonly used evaluation method to determine the specific cell subtype and its molecular classification is immunohistochemistry (IHC), which consists in amplifying the visualization of specific binding between antibodies and antigens located in cells and tissues. In breast cancer, the main biomarkers used for predictive and prognostic value are progesterone receptors (PR), estrogen receptors (ER), epidermal growth factor receptors (EGFR), epidermal growth factor receptors 2 (HER2), specific cytokeratins, and nuclear expression of Ki-67. Ki-67 is a non-histone protein encoded by the MKI67 gene, located on the long arm of chromosome 10 [2]. It is highly expressed in the nuclear region of proliferative cells and only in those with compromised DNA repair processes [3]. The Ki-67 index is also related to the molecular classification of breast cancer, with luminal-A characterized by a low proliferative index and luminal-B (HER2 negative) characterized by a high proliferative index. Therefore, it has a direct correlation with treatment options and clinical outcomes, with luminal-A being responsive to isolated endocrine therapy and luminal-B dependent on adjuvant chemotherapy, in most cases [4].

The counting of tumor cells marked with the Ki-67 antigen is generally done in an analog and subjective manner. Pathologists perform the count in the microscopic field during analysis, providing opinions that can vary from 5% to 30% intra- and inter-observer, according to the International Ki-67 in Breast Cancer Working Group (IKBCWG) [5]. Given this, the 14th edition of the St. Gallen International Breast Cancer Conference suggested that each laboratory establish its own threshold values for classifying the Ki-67 index as high, moderate, or low based on the median value obtained through their analyses [6].

For this purpose, there are computational tools in the market used to perform a similar activity; however, they are either labor-intensive or expensive, tied to closed computational systems and dependent on exclusive hardware and programming languages. Therefore, the development of semi-automatic and automatic, accessible systems on open platforms for reading and calculating the proliferative index has a significant social appeal and could be highly beneficial for the treatment of individuals with breast cancer.

II. Methods

This work is a retrospective study conducted at a single institution. Slides stained with the Ki-67 immunohistochemistry technique and hematoxylin and eosin staining were selected from the year of 2020 to 2023, totaling 54 cases. They correspond to core biopsies or anatomopathological specimens from surgical excisions.

The slides were digitized at a magnification of 20x using the Zeiss® AxioScan Z1 microscope model and identified as DIS.

A. *Visual Counting*

Each slide was evaluated by three pathologist experts who provided an analog opinion on the proliferative index, defined by the average obtained from the three independent evaluations.

B. *Manual Counting*

Additionally, DISs underwent manual counting using the open-source software Image-J Fiji to define the proliferative index, comparing them with values obtained by experts and the semi-automated program. For this, four sections of areas of interest (1000px x 1000px) were made for each slide, which were analyzed by two calibrated researchers in accordance with the IKBCWG's visual counting protocol. Image 1 shows sections from slides with different proliferative indices. The first is considered "low" (<5%), the second "intermediate" (5%-30%), and the third "high" (>30%) [7]. The total proliferative index value for each DIS was calculated by the simple average of the values assigned to each of the four sections.

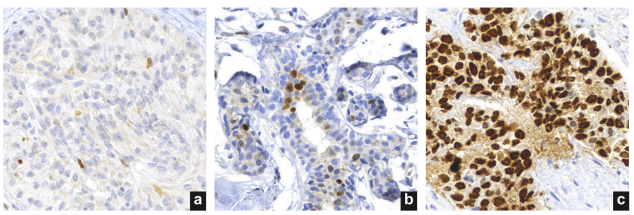

Fig 1. Demonstration of immunohistochemistry sections evaluated by manual counting. (a) Ki-67 index assessed at 3.69%; (b) Ki-67 index assessed at 24.9%; (c) Ki-67 index assessed at 98.76%.

C. *Semi-automated Counting*

The same image cutouts were also analyzed by the program developed in collaboration with the Department of Informatics. The Ki-67 values returned by the program were subjected to comparison. A set of 33 slides underwent analysis using color parameters individually adjusted for each cutout (modified parameters), and another set of 15 slides was analyzed using identical parameters for all cutouts (standardized parameters).

Figure 2 depicts the color subtraction performed by the program's algorithm, which depends on 2 parameters assigned to the brown color ("Brown 1" and "Brown 2") and one to the blue color ("Blue"), all related to the brightness and color saturation of the image. Ki-67-marked cells (brown) and cells negative for immunohistochemistry (blue) are identified.

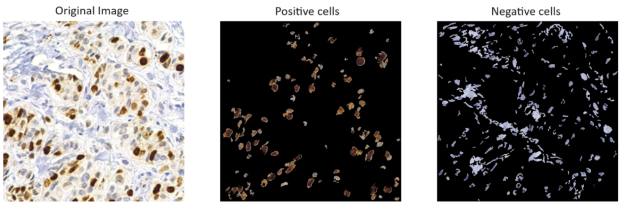

Fig. 2. Demonstration of the color separation performed by the program's algorithm.

D. *Statistics*

The data were collected and tabulated in Microsoft Excel® spreadsheets and analyzed using the Statistical Package for the Social Sciences - SPSS® (IBM® SPSS® Statistics v. 25.0, SPSS Inc, Chicago, USA) computational program. Quantitative variables were expressed as means, medians, and standard deviations. For inferential analyses, the Intraclass Correlation Coefficient (ICC) was utilized. This coefficient is employed to analyze the agreement of continuous variables among different observers. ICC values less than 0.4 are considered "poor," values between 0.4 and 0.6 are deemed "fair," values from 0.6 to 0.75 are considered "good," and values from 0.75 to 1.0 are considered "excellent" [8]. The higher the ICC value, the closer the viewpoints of different observers on the same sample. Values of $p < 0.05$ were considered significant.

III. RESULTS

Table 1 presents the mean, median, and standard deviation values for the set of slides analyzed by manual counting compared to those analyzed by the pathologists and the program. The highest mean and median were obtained through manual cell counting (44.90% and 41.51%, respectively), while the lowest values were visually assigned by the pathologists (36.94% and 30.00%, respectively).

Table 1 Mean Ki-67 Values Found

	Manual Ki-67 (%)	Pathologist Ki-67 (%)	Semi-automated (MP) (%)	Semi-automated (SP) (%)
Mean	44.90	36.94	43.66	39.50
Median	41.51	30.00	38.75	33.50

Standard deviation	24.61	24.92	24.00	25.86

MP: Modified parameters, SP: Standardized parameters

Table 2 represents the mean ICC values among the slides analyzed by the pathologists versus manual counting, and the program. All values were statistically significant, with a p-value < 0.01. The lowest ICC resulted from the comparison of the program (standardized parameters) with the visual analysis by the pathologists, being 0.791 (95% CI 0.371-0.930). It was followed by the comparison of the pathologists' analyses with manual counting, 0.805 (95% CI 0.637-0.891). The highest correlation was found between the program's counting (modified parameters) and manual counting, with an ICC of 0.996 (95% CI 0.992-0.998). However, even the lowest values obtained remained above 0.75, a standard considered excellent.

Table 2 Comparison Between Obtained Ki-67 Values

	ICC (95% IC)
Pathologist x Manual	0.805 (0.637-0.891)
Program (MP) x Pathologist	0.809 (0.607-0.907)
Program (MP) x Manual	0.996 (0.992-0.998)
Program (SP) x Pathologist	0.791 (0.371-0.930)
Program (SP) x Manual	0.880 (0.482-0.964)

MP: Modified parameters, SP: Standardized parameters

IV. Discussion

According to the International Agency for Research on Cancer of the World Health Organization (WHO), in 2020, the incidence of breast cancer was over 2 million cases, representing 11.7% of all neoplasms [9]. Staging, prognosis assessment and indication of adjuvant therapies remain as the main topic of numerous research efforts and new discoveries. In this regard, immunohistochemical markers have been in increasing demand, such as Ki-67.

In our set of slide samples, we obtained Ki-67 values determined by manual counting, visual counting and semi-automated counting, with modified and standardized parameters. The average Ki-67 values found were 44.90%, 36.94%, 43.66%, and 39.50%, respectively. The median values were 41.51%, 30.00%, 38.75%, and 33.5%, following the same order.

Obtained data were comparable to the study conducted by Meermira et al., which analyzed the Ki-67 index of 200 cases, ranging between 0% to 89%. The average Ki-67 value for manual counting was 29.81%, and for computer-assisted counting (CAT) was 38.27%. The median values obtained were 28.35% and 35.45%, respectively. In contrast to our study, the mean proliferative index values attributed by the program were higher than those obtained by manual counting. The image analysis was conducted automatically using the ImmunoRatio software [3].

In Skjervold et al.'s study, the median for visual counting was also lower, determined to be 22.3%, while for computer-assisted counting, it was 30%. The analysis employed the QuPath software, involving manual extraction of regions of interest from the slides guided by heatmaps [10].

Corroborating with the proportion obtained by us, another study conducted in South Korea with 997 breast cancer slides revealed a median of 22.86% for visual counting and 23.43% for computer-assisted counting, using the Ventana Virtuoso software [11].

Regarding the agreement between methods, our ICC obtained between visual counting and manual method was 0.805. Similarly, between visual counting and semi-automated counting using standardized parameters, the ICC was 0.809. The highest correlation was observed between manual counting and semi-automated counting using modified parameters, with a value of 0.996.

Kwon et al. reported an ICC of 0.982 for visual counting compared to CAT. This same study elucidated the main causes of disagreement between methods, namely: tumor heterogeneity, errors in visual interpretation, misidentification of tumor cells, low quality of immunohistochemistry, and counting of non-tumor cells [11].

V. Conclusions

Automated methods for scoring the Ki-67 index involve, in addition to the scanning of the slides *per se*, the use of programs with the ability to distinguish between malignant and benign cells, as well as positive and negative cells for a specific marker. Furthermore, artificial intelligence and deep learning methods are capable of assessing the most suitable cell counting area for Ki-67 analysis, known as "hot spots" [12].

However, there are still challenges to be overcome, such as the high costs of digitization equipment, the large storage space required by these slides, and the adaptation process for physicians to deal with human-machine interface [13]. In our study, it was possible to observe differences between the mean and median values of Ki-67 attributed by different methodologies. Thus, it emphasizes

the necessity of establishing individual thresholds for indices categorized as “low”, “intermediate”, or “high” for each laboratory and, potentially, for each counting method employed [6].

Nonetheless, it was possible to perceive an expressive accordance between Ki-67 values attributed through visual, manual and semi-automated counting techniques. We obtained ICCs ranked as “excellent” for all the comparisons made, with even higher values than those documented in previous studies.

Considering this result, the three methods were regarded as equivalent for identifying the Ki-67 proliferative index in breast cancer slides. In parallel, the visual counting performed analogically by the pathologist is validated; it suggests that it can be equated to the semi-automated counting achieved by the program.

Nevertheless, variations in specimen processing, reagents, protocols, and procedures inherent to each laboratory contribute to the inter- and intra-observer variability perceived in this and other studies regarding Ki-67 counting [10]. Some limitations of the present study include human imprecision during the manual counting and the lack of slides with a low Ki-67 index analyzed by the program. In order to overcome these discrepancies and enable more efficient technology implementation, one could recommend encouraging national research initiatives and promotion of multidisciplinary multicenter collaborations.

Conflict of Interest

The authors declare that they have no conflict of interest.

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