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Original Article

Calculation of Ki-67 Proliferation Index in Lymph Node Excision Biopsy of Non-Hodgkin's Lymphoma: Comparative Analysis of Four Methodologies

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Abstract

Background: Ki67 antigen, identified by Scholzer and Gerdes in 1983. In Non Hodgkin lymphoma, found to be in high concentrations in rapidly multiplying cells. Due to this property the Ki-67 proliferation index is used in numerous malignancies for grading and staging. Its prognostic value is well studied and documented. Many methods can be used to count the Ki67 proliferation index - Eyeballing, Manual counting with microscope, Manual counting in printed image of slide & Automated counting to name a few. In this study we will evaluate all the above mentioned methods of counting Ki67 proliferation index and correlate one another.

Design: 50 ki-67 proliferation index done on lymph node excision biopsy of Non Hodgkin's lymphoma collected & calculated by Eyeballing, Manual counting with microscope, Manual counting in printed image of slide & Automated counting (Qupath - 0.2.3) in the same slide. Eyeballing and Manual counting with microscope is done using Olympus BX43 microscope. Whole slide image (WSI) is captured using Morphle Optimus 6T, printed image is taken and used for manual counting and the WSI of Ki67 is analyzed using QuPath - 0.2.3

Results: Ki67 proliferation index calculated using the above 4 methodologies and analysed using Epi Info™ - 7.2. Pearson correlation coefficient was obtained for each methodologies and correlated with each other.

Conclusion: Our study demonstrated that all the 4 methodologies were correlating with each other statistically. Hence any of the above methods can be used to calculate the Ki67 proliferation index. While in comparison with Manual counting with microscope, Manual counting in printed image of slide correlates more than other methods used in this study..

Key words: Digital pathology, Ki-67, MIB1, Morphle, Non-Hodgkin's lymphoma, Qupath

INTRODUCTION

Ki-67 antigen was discovered by Scholzen and Gerdes in 1983.[1] This antigen expression is a strong indicator of mitotic activity, hence cellular proliferation, during the event of cellular mitosis, in the interface these isoforms are detected in high concentration on the nucleus.[2] Thus used in numerous malignancies for staging and grading including breast, prostate, neuroendocrine, and non-hodgkin's lymphoma (NHL).[3-5] Higher Ki-67 labeling index correlates with bad prognosis in some variants of lymphoma, while other variants particularly diffuse large B-cell lymphoma show no association or the reverse results.[6-10] Many counting methods are used to count the Ki-67 including, eyeballing, manual counting with microscope, manual counting with printed image, and Automated (Qupath).[11] There are discrepancies in the Ki-67 proliferation index counting among the observers and also among the method used to count them. Therefore with this background, we

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compared the four different methodologies for counting Ki-67 in various types of NHL. Thus in this study, we studied the Ki-67 proliferation index using four independent methods and correlated them with each other.

MATERIALS AND METHODS

Ki-67 (MiB-1) immunohistochemistry stained slides (5 micrometer sections of Formalin-Fixed Paraffin-Embedded blocks - MiB-1 clone, Prediluted; Biogenex (Fremont, California, USA). Antigen retrieval was conducted in Tris buffer at pH 8 by heat retrieval method. Diaminobenzidine as the chromogen and Hematoxylin as the counterstain) of 50 cases of NHL were taken from departmental archives. Nuclear brown labeling is taken as positive staining. The Ki-67 index was counted using four methodologies [Figure 1].

"Eye-balling" The most widely used method is estimating the Ki-67 proliferation index. An estimate of the Ki-67 proliferation index is made by scanning the slide in the hotspot without counting the individual positive and negative cells. The scanning of the entire slide is made in Low power (100× magnification). European Neuroendocrine Tumor Society and the North American Neuroendocrine Tumor Society recommend this method in routine reporting.[12,13] "Automated counting" The Ki-67 labeling index of all the slides was counted using Qupath - 0.2.3 (Open source software for digital pathology and whole slide image analysis) [Figure 2]. The slides were scanned using Morphle Optimus 6T (Whole slide scanner) Hotspots were selected and Ki-67 quantification was calculated by the Qupath - 0.2.3.[11] Positive cell detection and percentage calculation are done after stain vector calibration. "Manual counting with microscope" Hotspot area is identified in 100× magnification (Low power) and individual cells are counted for positive and negative at 400x magnification (High power). Hand counter is used for counting the individual cells (Differential counter used to count in peripheral smear). This method was used as the standard method to correlate and evaluate the other methodologies. "Manual counting with printed image" Hot spot was selected in 100× magnification. The static color image of the hot spot was captured via microscope mounted camera (Q imaging, British Columbia) at 400× magnification then it is color printed on photographic paper. Ki-67 - Negative and Positive stained cells were then counted and immediately marked off once counted with circling of the Ki67 - positive and - negative tumor cells. Pale-stained and equivocal nuclei were ignored during counting.

Analysis of the above-mentioned methods will be assessed based on these parameters:

1. The practicality, as well as cost of performing each method, were recorded
2. Pearson's correlation (R) for comparative analysis of the results from individual methods.

RESULTS

The Ki-67 index calculated by different methods were correlated by Pearson's correlation, which revealed that all the methods are statistically correlating with each other and hence can be used for reporting but the manual counting with printed image of the slide was found to have the highest Pearson's correlation index, hence has the highest agreeability factor when compared with manual counting of the slide [Figures 3 and 4].

"Eye-balling" method appears to be the most practical, the speed of this method comes with the price of lack

S. No.	Specimen ID	Eyeballing	Qupath	Microscope	Print
41	H-92/2020	10	12.32	13.67	13.87
42	H-89/2017	40	37.27	42.13	41.29
43	H-86/2020	95	92.12	96.72	95.02
44	H-80/2020	40	35.23	43.3	40.87
45	H-733/2019	70	90.24	86.87	84.24
46	H-700/2019	80	76.25	78.98	75.03
47	H-67/2020	70	79.67	75.9	77.12
48	H-662/2019	50	55.78	53.87	52.34
49	H-640/2019	60	64.68	70.21	67.12
50	H-63/2020	95	98.63	97.19	96.23

Figure 1: Table containing Ki-67 value from 4 different methodologies

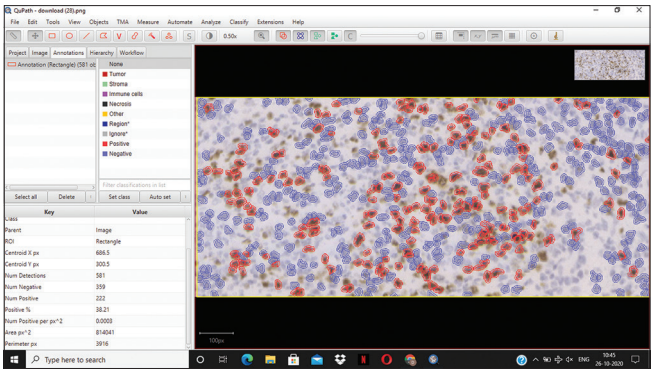

Figure 2: Screenshot of Qupath while performing Ki-67 automated counting

		eyeballing	qupath	microscope	print
eyeballing	Pearson Correlation	1	.950**	.984**	.985**
	Sig. (2-tailed)		.000	.000	.000
	N	50	50	50	50
qupath	Pearson Correlation	.950**	1	.982**	.983**
	Sig. (2-tailed)	.000		.000	.000
	N	50	50	50	50
microscope	Pearson Correlation	.984**	.982**	1	.996**
	Sig. (2-tailed)	.000	.000		.000
	N	50	50	50	50
print	Pearson Correlation	.985**	.983**	.996**	1
	Sig. (2-tailed)	.000	.000	.000	
	N	50	50	50	50

**. Correlation is significant at the 0.01 level (2-tailed).

Figure 3: Table showing Pearson correlation between each different method

of accuracy. Manual counting of printed images is time-consuming and exhausting. It has the upper hand by keeping a permanent record of the labeling index. The manual counting with the light microscope by an expert pathologist is considered as standard but it also is exhausting and time-consuming. Automated method has the undue advantage of repeatability; it is moderate in terms of time consumption factor as the time taken to slide is 40 min. It is non-exhaustive and does not require a pathologist at all times. Although the automated method cannot differentiate the tumor cells from tumor-infiltrating lymphocytes and endothelial cells. Considering the balance of practicality, speed, and accuracy, this study concludes that all four methods are equally effective and correlates with each other. Manual counting of printed images stands above the rest in all the terms plus it has an undue advantage of permanent storage for documentation of counted printed images for future references. In monetary terms, "Eye-balling" and "manual counting" methodologies do not have any additional cost as they were performed by routine microscopic methods. Automated slide scanner costs about US$20,000. However, the digital slide analyzing software was free (Open source). The Printed image method, which was found to be the most desirable, commercially affordable, estimated to be US$6000 for the camera mounted on Olympus microscope. With the arrival of cell phone microscope adapters, this cost will be significantly lower and be restricted only to the purchase of an adapter (which on average costs US$50). Color printers printing images in photographic papers were used (average cost US$10) as an operating cost.

DISCUSSION

The prognostic capabilities of the Ki-67 index in NHL is well documented and studied.[6-10,14] In this study, we focused on the assessment of four of the most widely used Ki-67 index counting methodologies and assessed their practicality and applicability, and the following conclusions were reached. Eye-Balling: College of American Pathologists stated that Ki-67 index estimation is acceptable.[15] In a recent study, showed similar pitfalls in the "eye-balling" method as evidenced by poor interobserver agreement when grading well-differentiated neuroendocrine tumors.[16] The eyeballing method has poor repeatability and interpersonal acceptance. This method depends on stain factors, section thickness, and tumor cell overlapping for better results. The fact that this is the least time-consuming and method with no additional equipment or cost cannot be neglected, but this comes with a hefty price. Manual Counting: The most tiresome and exhaustive method when compared with the rest, not to mention difficult to employ.[17] Even with handheld differential counters it is highly cumbersome and time-consuming to use in routine practice based on workload and other operational factors. We have found that after 2-3 slides of continuous counting it becomes dizzy and puts a lot of strain on the already strained eyes of a pathologist. Manual counting of printed image: This method has an additional cost of US$6000 as an initial investment of microscope mounted camera and color printer. The cost is expected to come down considering the arrival of mobile camera adapters and high-quality mobile cameras. This method also has an undue advantage of keeping a permanent record of the counted photographic sheet for future reference.[18] This method has the highest correlation factor when compared with the so-called standard method of manual counting by an expert pathologist. Automated Counting: This method proved to be an effective alternative to the other routine methods, but it comes with its unique sets of disadvantages, cost factor, time taken to train the laboratory personnels and its false-positive counting (lymphocytes and endothelial cells).[11] This method still requires a pathologist to identify the hotspot for the software to calculate the index. Although the calculation software is an open-source, the need for a whole slide scanner, its operation factors have to be weighed in before opting for this method for routine reporting. The findings of our study were found to be in correlation with various similar studies correlating Ki-67 counting with different methodologies, including manual and automated techniques, where values of manual counting and automated counting methodologies are in agreeable correlation [Figure 5].

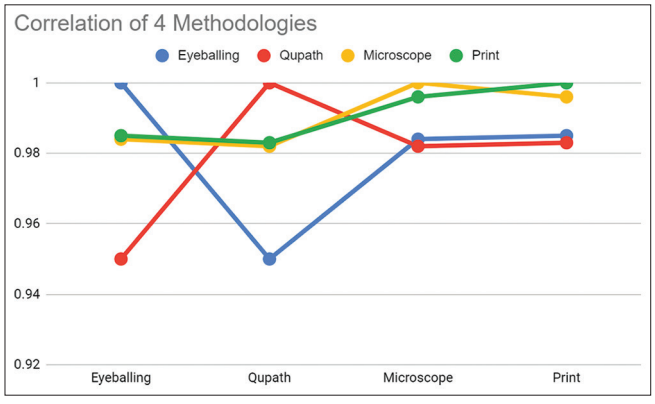

Figure 4: Correlation of Pearson correlation coefficient between four methods

Study	Manual Versus Machine correlation
Reid *et al.*[19]	Pearson's correlation (R = 81.7%)
González-González *et al.*[20]	*P*<0.05
Kinra and Malik[21]	Accuracy and reproducibility (99.9%)
Koopman *et al.*[22]	*P*<0.001

Figure 5: Reference table

CONCLUSION

After analyzing the four different methodologies to calculate the Ki-67 labeling index on the same slide the most practical, accurate, and documentable method is the manual counting of printed images of the slide. Though the manual counting by an expert pathologist is considered as standard it is not employable in routine practice where the caseload and burden placed on pathologists are high. Manual counting of printed images of the slide is not the one without disadvantages, but considering the overall factor, this method is recommended for routine practice with the fact that with the arrival of handheld camera adapters, the time and cost factor will be considerably reduced in the near foreseeable future.

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