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Research Article

Factors affecting the assessment of mitotic count in histopathological sections of tumors: a study of interobserver and intraobserver variability

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ABSTRACT

Background: Mitotic count is the most commonly used method of assessing the proliferative activity of a tumor. It is usually done in routine Hematoxylin & Eosin stained sections and is used for classification, grading, prognostication of tumors and sometimes as a decision factor for treatment. There are numerous variables that can influence the mitotic count like delay in fixation, thickness of the section, size of the high power field of the microscopes and so on. This study is designed to assess the relative importance of Intra-observer variability and Inter-observer variability on mitotic counting by keeping all different procedural factors constant.
Methods: Sections from forty cases of breast cancer and twenty cases of high grade lymphoma formed the study material. Mitotic counts done by the principal investigator under standard conditions were compared with counts done by another experienced observer under standard conditions to evaluate the inter-observer variability. The principal investigator will make all counts twice at intervals of one month to assess intra-observer variability. Paired t test and linear regression were the statistical tests used in analysis. Ki-67 immunohistochemistry was also done in sections fixed at different intervals.
Results: Our study showed that inter-observer ($p=0.261$) and intra-observer variation ($p=0.261$) is not statistically significant. In case of inter-observer variability the correlation is weak and not significant.
Conclusions: Reproducibility in mitotic counting can be achieved by following a strict morphological criteria as well as a strict counting protocol.

Keywords: Observer, Mitosis, Proliferation

INTRODUCTION

The range of therapeutic options for the treatment of patients with primary breast cancer has widened considerably in recent years. The mammographic screening has led to the identification of large numbers of small, stage 1 cancers. It is therefore important that clinicians are given the most accurate prognostic information on which lies the selection of the optimum therapy for each woman. Histological grade provides prognostic information in many tumours, including breast cancers. In routine practice in Nottingham histological grade remains the most important prognostic factor in patients with primary operable breast carcinomas. Proliferative activity assessed by mitotic counts lies at the centre to this grading system

There is an increasing awareness that proliferation is the one of the most characteristic features of malignant tumors. Counting of mitoses is the classic method used to

determine proliferative activity in normal as well as neoplastic tissues. Even though simple, the method of counting mitotic figures in H&E sections have many shortcomings. This method registers only the M phase of the cell cycle. The number of identifiable mitoses also depends upon the period of time between surgical removal and fixation of the specimen and again strict morphological criteria for mitoses have to be used to avoid confusion with nuclear pyknosis and karyorrhexis.[1-6] Another problem lies in the heterogeneous distribution of mitoses.[4,7] All these factors have its effect on various levels and it will lead to false counts and poor reproducibility of mitotic counts.

Fixation is the first and most essential step in tissue preparation for microscopic analysis. A well fixed tissue is the key for a good slide and so for a good diagnostic interpretation. Our previous study showed that lack of prompt fixation ($p<0.001$) led to significant change in mitotic counts. Both mitotic counts and ki-67 index were significantly higher in immediately fixed specimens compared to those fixed after 1 hour. There was only weak correlation between Ki-67 index and mitotic count. Better precision can be obtained in mitotic counts by prompt fixation of the surgical specimens.[8]

Commonly mitotic count is reported as number / HPF. But the size of HPF shows a variation between microscopes up to 200% and the mitotic counts may vary up to 250% because of variation in the area of the high-power fields of different microscopes.[9] Even after following a strict counting protocol mitotic count cut-offs are subject to important sampling variation.[10,11] Mitotic counts can varies according to section thickness also.

Malignant tumors are heterogenous. Influence of tumor heterogeneity on mitotic count is significant and only way to reduce this is to take multiple blocks per tumor and carefully scan all sections for the highest proliferative area and again when counting the mitotic figures, apoptotic cells and neutrophilic granulocytes can mimic mitotic figures.[7] So it is also necessary to adhere to a strict morphological criterion. More accurate counts can be obtained by accessing 20-30 high power fields instead of just 10.[12]

In addition to all the above mentioned procedural factors, error can also occur at the individual level who interprets the counts. The term observer variation can be defined as failure by the observer to identify or measure a value accurately, which will result in an error. It may be due to false interpretation of the data, or due to faulty technique leading to incorrect measurement. Two types of observer variation are inter-observer variation and intra-observer variation

Inter observer variation means the amount by which one observer varies from another when interpreting the same data. Intra-observer variation means the difference in the observations when the same person interprets the original data more than once. In our present study we tried to find out relative contribution of intra-observer and inter-observer variability in mitosis counting by keeping other procedural factors like fixation time and section thickness constant. Investigators counted mitotic figures on same sections using same type of microscope.

METHODS

Study conducted in the Department of Pathology, Sree narayanana institute of medical sciences during a period of 2014. Our study sample included 50 cases composed of 40 cases of Infiltrative duct carcinoma, Breast & 20 cases of high grade Non-Hodgkin's lymphoma.

All specimens were collected from the operation theatre immediately after removal. They were cut and examined and specimen immersed in fixative after 1 hour and sent for routine grossing. Sections at 4-5 micrometer were taken in all cases for comparison. Mitotic counts were made in Labomed microscope with high power field having 0.1325 mm^2 area (standard count). Counting is done in a systematic fashion. We counted 20 consequent high power fields; counts starting from a field in which the first mitosis is seen on eyeballing. A typical basophilic metaphase in a clear slightly basophilic / eosinophilic background can be regarded as a mitotic figure. The counts made by the principal investigator on 4-5 micrometer thick sections on a Labomed microscope using systematic counting method in sections fixed 1 hour after removal will be considered as the standard.

All counts are also be made by the co-investigator to evaluate the inter-observer variability. The principal investigators made all counts twice at intervals of one month to assess intra-observer variability. The proliferating cells are labelled by MIB-1 antibody and a Polymer-HRP IHC Detection System (Biogenex). The results are expressed as percentage of positive tumor cells. Data storage and Analysis is done with EPIINFO software.

RESULTS

The mean mitotic counts obtained by observer 1 were 8.95, while the second observer got a mean value of 8.3. When the counts were repeated by first observer after one month a mean count was only slightly changed 8.55. On correlating the mitotic counts, correlation co-efficient was 0.651 for the counts obtained by first observer after 1 month and it was 0.245 for the counts obtained by second observer.

DISCUSSION

Counting mitosis and expressing it as a quantitative figure per a set number of high per fields is a time honoured method used by histopathologists in the assessment of cell proliferation. Though extremely common in practice, this method has been criticized for

its imprecision or lack of reproducibility. Many of the factors associated with method, instrument and observer have in turn been blamed for this variation. In this study we have tried to delineate the effect of interobserver and intra observer difference on mitotic counting by keeping other factors like fixation time, section thickness and microscopes constant.

Table 1: Comparison between mitotic counts obtained in sets of data in paired parameters.

Parameter 1	Mean (95% CI)	Parameter 2	Mean (95% CI)	Paired t test	
				t	p
Observer 1	8.95(8.1 -9.8)	Observer 1after 1 month	8.55 (7.9-9.2)	1.1	0.261
Observer 1	8.95(8.1 -9.8)	Observer 2	8.3 (7.3 - 9.3)	1.1	0.261

Table 2: Correlation of standard mitotic counts (Done by observer 1, fixation after 1 hr, 5 micrometer sections, standard microscope, systematic counting) with counts under different circumstances.

Parameter	Correlation coefficient (R)	F	p
Observer 1 after 1 month	0.615	23.1	<0.0001
Observer 2	0.245	2.2	0.144

Sections from forty cases of breast cancer and twenty cases of high grade lymphoma formed the study material. A mixture of two types of lesions was selected to reduce chances of systematic error and biological variation. Most of the patients included in this study belonged to 50-60 age groups. In carcinoma breast 70% of the cases belonged to grade 2, 10% to grade 1 and 20% grade 3 categories. All Non-Hodgkin lymphoma studied were high grade.

Among the 40 cases of breast cancer, there was one metaplastic carcinoma and one apocrine carcinoma. All the rest were infiltrating duct carcinomas - not otherwise specified. Among the lymphomas there were 13 diffuse large cell lymphomas (DLBCL) and 7 anaplastic large cell lymphomas (ALCL).

Mitotic counts done by the principal investigator under standard condition were compared with counts done by another experienced observer under standard conditions. To assess the effect of intraobserver variation, principal investigator repeated the counts after an interval of 1month on these sections. Paired t test and linear regression were the statistical tests used in analysis

The specimen was received immediately after removal in the operation theatre and cut and the main tumor fixed after 1 hour. The period of 1 hour was considered the standard, since it usually took this much time to fix the specimen in the theatre before being sent at leisure to the pathology department.

Sections were cut at 4-5 micrometer thickness. Mitotic counts were made in Labomed microscope with high power field having 0.1325 mm^2 area. It is well known that tumors can be heterogeneous in their proliferative activity. Some regions of a section may contain many mitosis and others only few. One way of minimizing errors due to this is by increasing the number of fields counted. It is the convention that areas with higher number of mitosis are chosen because logically it is the maximum proliferative potential of a tumor that is important. In our study the standard method was to start counting from the field in which the first mitosis is identified.

The mean mitotic counts obtained by observer 1 were 8.95 (8.1-9.8), while the second observer got a mean value of 8.3 (7.3-9.3). When the counts were repeated by first observer after one month a mean count was only slightly changed. 8.55 (7.9-9.2) It has shown that inter-observer (p=0.261) and intra-observer variation (p=0.261) was not statistically significant in our study (Table 1). This is in line with recent studies which show that the application of strict morphological criteria, as well as a strict counting protocol, will result in reproducible counting.[13] By taking mitotic counts/mm2 of viable tissue rather than mitotic counts/HPF may also reduce interobserver variation.[14]

Apart from looking for significant differences in mitotic counts obtained by standard and alternative conditions in counting by the paired t test, we also obtained correlations between these variables. In case of inter-observer variability (between investigator and guide) the correlation is weak and not significant despite the fact the difference between means was not significant (Table 2).

We saw that with thickness of histologic sections, staining, and the area measured kept constant, the degree

of reproducibility was found to be dependent largely on the experience of investigator. For investigators at similar levels of experience, the mitosis rates obtained were of the same order of magnitude.

CONCLUSION

Mitotic count indicating the proliferative activity of a tumor is subjected to errors related to the procedural and observer factors. By keeping all procedural factors like section thickness, staining and area measured same we have seen that interobserver variation is insignificant. Experienced investigators obtained similar mitotic counts and intraobserver variation is also insignificant among experienced investigators. Along with good experience application of a strict morphological criteria as well as a strict counting protocol will also help us to reach reproducible counts.

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