Article Type: Original Article.

Computerized image analysis of the Ki-67 proliferation index in mantle cell lymphoma

Yngvild Nuvin Blaker\textsuperscript{1,2}, Marianne Brodkorb\textsuperscript{1,2,3}, John Maddison\textsuperscript{4}, Tarjei Sveinsgjerd Hveem\textsuperscript{1,4,5}, John Arne Nesheim\textsuperscript{1,4}, Hans Martin Mohn\textsuperscript{4}, Arne Kolstad\textsuperscript{3}, Christian Hartmann Geisler\textsuperscript{6}, Knut Liestøl\textsuperscript{5}, Erlend Bremertun Smeland\textsuperscript{1,2}, Harald Holte\textsuperscript{1,3}, Jan Delabie\textsuperscript{7,8}, Håvard Danielsen\textsuperscript{1,4,5,9}

\textsuperscript{1}Centre for Cancer Biomedicine, Faculty of Medicine, University of Oslo, Oslo, \textsuperscript{2}Department of Immunology, Institute for Cancer Research, Oslo University Hospital, Oslo, \textsuperscript{3}Department of Oncology, Division of Cancer Medicine, Surgery and Transplantation, Oslo University Hospital, Oslo, \textsuperscript{4}Institute for Cancer Genetics and Informatics, Oslo University Hospital, Oslo, \textsuperscript{5}Department of Informatics, University of Oslo, Oslo, \textsuperscript{6}Department of Hematology, Rigshospitalet, Copenhagen, \textsuperscript{7}Department of Pathology, Division of Cancer Medicine, Surgery and Transplantation, Oslo University Hospital, Oslo, \textsuperscript{8}Department of Pathology, University of Toronto, Toronto, \textsuperscript{9}Nuffield Division of Clinical and Laboratory Sciences, University of Oxford

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1111/his.12624

This article is protected by copyright. All rights reserved.
Corresponding author:

Yngvild Nuvin Blaker

Department of Immunology, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo University Hospital, Po box 4953, Nydalen, 0424 Oslo, Norway.

Tel: +47 22 78 14 19/ +47 481 09 481

E-mail: ynblak@rr-research.no

Conflict of Interest

The authors declare no conflict of interest.

Abstract

AIMS: Manual counting of the fraction of Ki-67 positive cells (the Ki-67 index) in 1000 tumor cells is considered the “gold standard” to predict prognosis in mantle cell lymphoma (MCL). This time-consuming method is replaced by the faster, but less accurate semiquantitative estimation in routine practice. The aim of this study was to investigate the use of computerized image analysis software for scoring of Ki-67 in MCL.

METHODS AND RESULTS: We developed an automated method for determining the Ki-67 index by computerized image analysis and tested it using a cohort of 62 MCL patients. The data were compared to Ki-67 scores obtained by semiquantitative estimation and image based manual counting. When using the Ki-67 index as a continuous parameter, both image based manual
counting and computerized image analysis were inversely related to survival ($p = 0.020$ and $p = 0.025$, respectively). Ki-67 index obtained by semiquantitative estimation was not significantly associated with survival ($p = 0.093$). The results were validated in a second patient cohort with similar results.

CONCLUSION: Computerized image analysis of the Ki-67 index in MCL is an attractive alternative to semiquantitative estimation and can easily be introduced in a routine diagnostic setting for risk stratification in MCL.

**Keywords:** Ki-67, mantle cell lymphoma, computerized image analysis, malignant lymphoma

**Introduction**

Mantle cell lymphoma (MCL) is an aggressive non-Hodgkin’s lymphoma (NHL) and accounts for about 6% of NHLs in Western countries\(^1\)-\(^2\). MCL is molecularly characterized by the translocation t(11;14)(q13;q32) which leads to overexpression of the cell cycle regulator cyclin D1 (CCND1). In addition, MCL tumor cells may carry a large number of secondary chromosomal and molecular alterations, targeting proteins that regulate cell cycle progression and interfere with the cellular response to DNA damage\(^3\). MCL is considered incurable with conventional chemotherapy and has a median survival of 3-5 years\(^4\)-\(^7\). The clinical course of the disease varies considerably, and survival up to 10 years has been reported\(^8\)-\(^10\). This biological variability can reliably be predicted by the proliferation index of the lymphoma. Gene expression
analyses have identified a proliferation signature that predicts survival in MCL\textsuperscript{11}. In addition, the fraction of Ki-67 positive tumor cells (Ki-67 index) by immunohistochemistry has also been shown to predict prognosis in MCL both before and after the introduction of immunotherapy and high-dose chemotherapy with autologous stem cell transplantation\textsuperscript{9,12-18}. Ki-67 is a nuclear antigen expressed in all active phases of the cell cycle and is hence associated with proliferating cells\textsuperscript{19-21}. Manual counting of the Ki-67 index in 2 x 500 tumor cells in immunostained sections is considered the “gold standard” for prediction of prognosis\textsuperscript{16,22}. This time-consuming method is replaced by semiquantitative estimation (“eyeballing”) of Ki-67 index in routine diagnostics. Semiquantitative estimation is less precise due to larger inter- and intraobserver variability\textsuperscript{22}. Efficient and practical tools for scoring the Ki-67 index with less inter- and intraobserver variability would therefore be of great interest for risk stratification in MCL.

In this study we applied computerized image analysis software to determine the proliferation rate in two cohorts of MCL patients and compared the results to data obtained by image based manual counting and semiquantitative estimation from routine diagnostics.

**Materials and Methods**

**Patient selection and lymphoma samples**

Sixty-two patients from two prospective multicenter phase-II clinical trials conducted by the Nordic Lymphoma Group- MCL\textsuperscript{2} and MCL\textsuperscript{3} were included for analysis of the Ki-67 index in each case. All patients were newly diagnosed with stage II to stage IV disease, and fulfilled
the diagnostic WHO criteria of MCL\textsuperscript{1}. The great majority of patients who responded to induction chemotherapy consisting of dose-escalated CHOP in combination with rituximab alternating with high dose cytarabine – rituximab, altogether six courses, received consolidating high dose chemotherapy with autologous stem cell transplantation. The high dose regimen consisted of BCNU, etoposide, cytarabine and melphalan (BEAM). Additionally, MCL3 patients in unconfirmed complete response or partial response after induction received Zevalin\textsuperscript{®} one week before the BEAM regimen\textsuperscript{23}.

A validation of the computerized image analysis protocol was performed on Ki-67 immunostained sections from 29 Norwegian MCL patients included in a retrospective study performed by the Leukemia and Lymphoma Molecular Profiling Project (LLMPP) consortium\textsuperscript{11}. These patients were treated with a less intensive regimen consisting of either chlorambucile – prednisolone continuous per oral monotherapy or with the CVP regimen (cyclophosphamide, vincristin, prednisolone) at our institution between 1981 and 1996. The histological diagnoses were reviewed as described\textsuperscript{11}.

All the patients included had formalin-fixed paraffin-embedded tumor tissue available for immunohistochemical analysis (Table 1). Details of the immunostaining for Ki-67 are provided in the online Supporting Information. The study was approved by the relevant national ethics committees (REC Reference number S-05145, 2001/10, S-05115, Norway, and KF02-008/01, H-KF-02295714, Denmark).

This article is protected by copyright. All rights reserved.
Software

Two software applications were developed for this project; “ImmunoPath” and “Manual Counter”. Details of the software development are provided in the online Supporting Information. The image analysis software “ImmunoPath” enables direct selection and annotation of representative tumor areas within scanned histopathologic slides of MCL, and provides a tool for computerized image analysis of Ki-67 positive cells within this representative area. The annotated areas are broken down to smaller square sub-areas/ images of interest for efficient processing. Granted that the pathologist has access to digitalized histopathologic slides, the actual computerized image analysis of Ki-67 positive cells only takes seconds, depending on the size of the annotated area (approximately 2000 cells per second). Still, the time needed to make an annotation on a digitalized histopathologic slide may vary, depending on the experience of the pathologist and the histologic complexity of the case, as for analysis by traditional microscopy.

The square sub-areas extracted using “ImmunoPath” were exported and loaded into a separate software system, “Manual Counter”, to allow for image based manual counting by the human eye and marking of positive and negative nuclei with a plus or a minus, respectively (Figure 1A). This image based manual recording of the number and location of nuclei provides the opportunity for direct comparison of identical images from independent observers, and furthermore direct comparison to computerized image analysis of identical images.

The software “ImmunoPath” is made available for free use as a cloud application at www.immunopath.net.
Computerized image analysis and image based manual counting

Representative areas from each tumor biopsy were annotated digitally by an experienced pathologist together with a PhD student. Areas containing a high density of lymphoma cells were selected, avoiding hotspot regions and residual germinal centers as previously recommended\textsuperscript{22}. Smaller images were extracted from the representative tumor area as described in the previous section, and identical images were randomly selected for image based manual counting and computerized image analysis. The whole annotated representative areas were further analyzed by computerized image analysis.

Image based manual counting was performed on digitalized images in all cases. Between two and five randomly selected images (median 3, mean 2.9) were exported from each representative area and counted manually to obtain a minimum of 1000 cells counted in each case. Each image was counted as a whole to avoid selection bias. Image based manual counting was performed by one observer in the first patient cohort and by two observers in the validation cohort.

In some of the cases, a heterogeneous distribution of Ki-67 positive tumor cells was seen. This is illustrated in Figure S1, where the Ki-67 index differed with as much as 47\% in the image with the highest Ki-67 index compared to the image with the lowest Ki-67 index. In such cases with a consistent heterogeneous pattern of Ki-67 expression, all cells were counted within a representative area, but hotspot regions and residual germinal centers were avoided.
Semiquantitative estimation of Ki-67 positive cells

Semiquantitatively estimated Ki-67 indices were available from the two studies in the first patient cohort (MCL2 and MCL3). These scores were obtained in a central pathology review by one hematopathologist in the representative countries\(^{14,23}\). The representative areas were chosen by the pathologists.

Eight of the tumor biopsies had a Ki-67 index score by semiquantitative estimation of 20%, which was the median value. These patients were excluded from the survival analysis to avoid a selection bias.

Statistical considerations

All information on the statistical analyses used in this study is provided in the online Supporting Information.

Results

Computerized image analysis, image based manual counting, and semiquantitative estimation of Ki-67 index: correlation to survival.

Figure 1 illustrates image based manual counting (Figure 1A) and computerized image analysis (Figure 1B) on identical images randomly selected from a representative area in one case.
The median Ki-67 index assessed by image based manual counting of minimum 1000 cells was 14.8% and the mean was 20.8% (range 0.1-84.2%). Patients with a Ki-67 index below median showed a significantly longer OS compared to the patients with a Ki-67 index above median ($p = 0.002$) (Figure 2A). When using Ki-67 index as a continuous parameter in a Cox regression analysis, the Ki-67 index showed a statistically significant inverse relevance for OS ($p = 0.020$).

The median Ki-67 index assessed by computerized image analysis of identical images as assessed by image based manual counting was 17.2%, while the mean was 26.5% (range 0.1-97.4%). Also here, the patients with a Ki-67 index below median showed a significantly longer OS compared to the patients with a Ki-67 index above median ($p = 0.013$) (Figure 2B), and the Ki-67 index was inversely relevant for OS in a Cox regression analysis ($p = 0.025$).

The Ki-67 index obtained by semiquantitative estimation showed a median of 20% and the mean was 25.7% (range 1-80%). Patients with a Ki-67 index below the median showed a borderline significantly longer OS compared to the patients with a Ki-67 index above the median ($p = 0.063$) (Figure 2C). The Ki-67 index was not significant for survival when analyzed as a continuous parameter in Cox regression analysis ($p = 0.093$).

Computerized image analysis was also performed on the whole annotated representative area in each case (Figure S1). The median and mean numbers of digitally counted cells in each case was 42 262, and 48 005, respectively (range 1659-131 021). The median Ki-67 index was 21.2% and...
the mean was 28.8% (range 0.8-98.9%). The Ki-67 index was inversely correlated with OS in the Cox regression analysis ($p = 0.036$), while the Log Rank test showed borderline significant improved OS for patients with a Ki-67 index below median ($p = 0.062$) (Figure S2).

**Correlation between image based manual counting, computerized image analysis and semiquantitative estimation**

There was a strong correlation between image based manual counting and computerized image analysis of the same images (Spearman’s rho = 0.959, $p < 0.001$, Figure 3A).

There was also a significant correlation between image based manual counting of the Ki-67 index and semiquantitative estimation, albeit weaker than observed between image based manual counting and computerized image analysis (Spearman’s rho = 0.807, $p < 0.001$, Figure 3B). Furthermore, we found a significant correlation between computerized image analysis of the whole representative area on the slide and semiquantitative estimation (Spearman’s rho = 0.845, $p < 0.001$, data not shown).

Bland-Altman plots comparing image based manual counting with computerized image analysis of identical images show a trend towards higher estimates of the Ki-67 index at high index values by the computerized image analysis (Figure S3A). The Bland-Altman plots that compared image based manual counting of the Ki-67 index with the semiquantitative scores showed no trend toward over- or underestimation (Figure S3B).
Validation of computerized image analysis

To validate the computerized image analysis protocol, image based manual counting and computerized image analysis was performed on a separate independent series of 29 Norwegian patients with MCL.

We tested the computerized image analysis on the whole representative area in each case from the validation cohort. The median number of counted cells in each case was 14 159, while the mean was 20 385 cells (range 2798-110 996). The Ki-67 index had a median value of 7.7%, while the mean value was 15.5% (range 0.0-90.1%). The patients with a Ki-67 index below median showed a significantly longer OS compared with the patients with a Ki-67-index above median ($p = 0.043$) (Figure 4A). In addition, a higher Ki-67 index was negatively associated with OS in Cox regression analysis ($p = 0.017$).

Image based manual counting of at least 1000 tumor cells from each case in the validation cohort revealed a median Ki-67 index value of 15.1% and a mean value was 19.1% (range 0.1-84.5%). The patients with a Ki-67 index below median did not show a superior OS compared with the patients with a Ki-67 index above median in the Log Rank test ($p = 0.093$) (Figure 4B), but the Ki-67 index was inversely correlated with OS in the Cox regression analysis ($p = 0.037$).
Of interest, the introduction of a software application for image based manual counting demonstrates a very low interobserver variability (Spearman’s rho = 0.978, \( p < 0.001 \)) (Figure 5). The Bland-Altman plots comparing the image based manual counting of the Ki-67 index performed by two observers showed a very high degree of agreement with a trend towards a slightly higher Ki-67 index counted by Investigator 1 (Figure S4).

**Discussion**

The prognostic value of the proliferation index in MCL as determined by manual counting or semiquantitative estimation of Ki-67 expressing cells has been shown in several studies\(^9;12-18\). In this study, we demonstrate the feasibility of computerized image analysis software and image based manual counting to determine the Ki-67 index in two well-defined cohorts of MCL patients.

We showed that Ki-67 scoring by computerized image analysis and image based manual counting of digital images was inversely related to survival in both our patient cohorts as assessed by Cox regression analysis. Of importance, the computerized image analysis was more accurate for prognosis prediction, and showed a better correlation with image based manual counting, than the routinely used semiquantitative estimation of the proliferation index.

Computerized image analysis of Ki-67 index enables counting of larger tumor areas compared to manual counting and hence a more representative estimation of the percentage of Ki-67 positive cells within a tumor. Furthermore, computerized image analysis is much faster than traditional manual counting and creates reproducible results for later review.
The computerized image analysis showed a trend towards higher estimates of the Ki-67 index at high index values compared to image based manual counting in the Bland-Altman plots (Figure S3A). Computerized scoring uses an absolute threshold for scoring a cell as positive whereas the human eye uses a relative threshold, which makes the computerized scoring susceptible to poor staining quality. In fact, the two cases from the first cohort with the greatest discrepancy of the Ki-67 index between the computerized image analysis and the image based manual counting both showed low staining quality with one case with strong background staining (with poor signal/noise ratio) and one case with weak staining of the presumed Ki-67 negative tumor cells. These two cases are clearly seen as outliers in Figure S3A. In these cases, the computerized image analysis software overestimated the number of Ki-67 positive cells and underestimated the number of Ki-67 negative cells, respectively. The human eye appears to correct for differences in staining quality. Computerized image analysis would therefore probably benefit from a rigorous quality control of the immunohistochemical staining method.

One limitation of computerized image analysis systems is their inability to differentiate between specific cell types such as tumor cells and normal cells. Therefore, representative areas of the lymphoma must be carefully selected by the pathologist before the procedure. However, as long as the pathologist selects a representative part of the lymphoma, this should not be a problem for scoring the proliferation index in MCL, as the number of reactive cells in MCL is low\textsuperscript{25}.
The challenges of selecting a representative area have been discussed in previous articles\textsuperscript{22}. Some of the cases showed a heterogeneous distribution of Ki-67 positive cells, providing an obvious explanation for the differences between image based manual counting of a limited area and computerized image analysis of large areas in our material (Figure S1). A consensus meeting has previously advised to exclude residual germinal centers and proliferation hot spots from the cell counting to establish the proliferation index\textsuperscript{22}.

Scanning and digital viewing of histopathologic slides is increasingly being incorporated into diagnostic routine in the pathology laboratory\textsuperscript{26;27}. It is likely that the use of standardized tissue staining protocols together with computerized image analysis software is to become part of the diagnostic tool kit of the pathologist. We have demonstrated in this study that computerized image analysis of the Ki-67 proliferation index shows statistically significant survival difference in MCL. Furthermore, this analysis is more accurate than the routinely used semiquantitative estimation. The technique seems therefore to be well suited for use in clinical trials including risk stratification based on the proliferation index, and can easily be incorporated into routine practice in centers that have access to digitalized images.

**Acknowledgements**


This article is protected by copyright. All rights reserved.
This work was supported by grants from the South-Eastern Norway Regional Health Authority (No. 39444), and The Norwegian Cancer Society (No. 33260). The work was partly supported by the Research Council of Norway through its Centers of Excellence funding scheme, project number 179571.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Digital annotation of a representative tumor area and heterogeneity.

Figure S2. Overall survival related to Ki-67 index by computerized image analysis of the whole representative area in each case.

Figure S3. Bland-Altman plots comparing Ki-67 index by image based manual counting with (A) computerized image analysis and (B) with semiquantitative scores.

Figure S4. Bland-Altman plot comparing image based manual counting of Ki-67 index by two independent observers in the validation cohort.

Data S1. Immunostaining of Ki-67.

Data S2. Software development.

Data S3. Statistical considerations

Data S4. Figure legends to Supplementary Figures.

This article is protected by copyright. All rights reserved.
Reference List


This article is protected by copyright. All rights reserved.


This article is protected by copyright. All rights reserved.


<table>
<thead>
<tr>
<th>Tissue type</th>
<th>First patient cohort N=62 (%)</th>
<th>Validation cohort N=29 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph nodes</td>
<td>38 (61)</td>
<td>26 (90)</td>
</tr>
<tr>
<td>Tonsils</td>
<td>8 (13)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>11 (18)</td>
<td></td>
</tr>
<tr>
<td>Extranodal tissue</td>
<td>5 (8)</td>
<td>2 (7)</td>
</tr>
</tbody>
</table>

Table 1. Tumor tissue available for immunohistochemical analysis
**Titles and legends to figures**

**Figure 1.** (A) Image based manual counting and (B) computerized image analysis of Ki-67 positive tumor cells in identical images randomly selected from a representative area in one case. Image based manual counting was performed by applying the software tool “Manual Counter”, where positively stained nuclei (brown) and negative nuclei (blue) were marked with plus and minus, respectively. Computerized image analysis was performed by applying the software tool “Immunopath”, where positively stained nuclei (brown) and negative nuclei (blue) were marked with red and green dots, respectively. The images were captured with a 40x objective lens and extracted with dimensions of 160 μm x 160 μm.

**Figure 2.** Overall survival related to Ki-67 index below (solid line) and above (dashed line) median by (A) image based manual counting and (B) computerized image analysis in identical images, and by (C) semiquantitative estimation of a representative area chosen by the pathologist.

**Figure 3.** Correlation between (A) image based manual counting and computerized image analysis of the Ki-67 index in identical images (Spearman’s rho = 0.959) and (B) image based manual counting and semiquantitative estimation of the Ki-67 index (Spearman’s rho = 0.807).

**Figure 4.** Overall survival (OS) related to Ki-67 index below (solid line) and above (dashed line) median in the validation cohort. (A) OS related to Ki-67 index below and above median by computerized image analysis of the whole representative area in each case. (B) OS related to Ki-67 index below and above median by image based manual counting.

**Figure 5.** Correlation between image based manual counting of Ki-67 index by two independent observers in the validation cohort (Spearman’s rho = 0.978).
A)  B)  C)