

Automated Immunohistochemistry as an Alternative to Conventional Immunohistochemistry for Analyzing the Expression of BCL2 and Ki-67 in Diffuse Large B-Cell Lymphoma Patients

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ABSTRACT

OBJECTIVE Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin lymphoma. Immunohistochemistry (IHC) is widely used to assess prognostic markers such as BCL2 and Ki-67. Conventional IHC relies on manual staining and visual estimation by pathologists, while automated IHC incorporates computer-based image analysis. This study aimed to evaluate the level of agreement between these two IHC workflows in assessing BCL2 and Ki-67 expression in DLBCL patients.

METHODS Formalin-fixed, paraffin-embedded tissue samples from nodal and extranodal sites of 42 and 60 DLBCL cases were analyzed for BCL2 and Ki-67, respectively. Conventional IHC involved manual staining and evaluation by a pathologist in 10.00% increments. Automated IHC used the Ventana BenchMark ULTRA system with digital image analysis. Expression levels of the two systems were compared using paired t-tests, intraclass correlation coefficients (ICC), Pearson's correlation, and Bland-Altman plots.

RESULTS For BCL2, no significant difference was found between the two methods (mean difference = 5.79%, $p = 0.103$), with moderate to good agreement (ICC = 0.739) and strong correlation ($r = 0.772$). For Ki-67, pathologist-based assessment yielded significantly higher values (mean difference = 13.91%, $p < 0.001$), with moderate agreement (ICC = 0.569) and strong correlation ($r = 0.688$). Bland-Altman analysis confirmed acceptable limits of agreement, although a slight underestimation in the automated Ki-67 results was observed.

CONCLUSIONS Automated IHC showed moderate to good agreement with conventional IHC, particularly for BCL2. Despite some differences in Ki-67 quantification, both methods demonstrated consistent trends. Automated IHC offers reproducibility and scalability, making it a promising complementary tool in pathology.

KEYWORDS conventional immunohistochemistry, automated immunohistochemistry, BCL2 expression, Ki-67 expression, diffuse large B-cell lymphoma

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INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin lymphoma (NHL), accounting for approximately 30.00–40.00% of all NHL cases worldwide (1, 2). DLBCL is an aggressive form of B-cell malignancy that often requires immediate treatment due to its rapid progression (3). Understanding the molecular landscape of DLBCL is critical for the selection of appropriate treatment strategies, particularly as it pertains to the expression of key biomarkers such as B-cell lymphoma 2 (BCL2) and the proliferation marker Ki-67.

BCL2 is an anti-apoptotic protein that prevents programmed cell death (4), making it a crucial marker for the evaluation of chemoresistance and prognosis in DLBCL patients. Increased expression of BCL2 is frequently associated with adverse clinical outcomes (5, 6), as it can promote tumor survival despite aggressive therapies. In parallel, Ki-67 is a nuclear protein expressed during cell division (7, 8) and serves as a proliferation indicator in various malignancies, including DLBCL. A higher Ki-67 index correlates with increased tumor cell proliferation and, in some cases, with poor overall survival, making it an important diagnostic and prognostic marker (9, 10). Concurrent overexpression of BCL2 and Ki-67 suggests that lymphoma cells exhibit both enhanced survival and increased proliferative activity. This biological profile reflects a more aggressive disease behavior and is commonly associated with poor clinical outcomes and resistance to standard therapies (11). A recent study among upper northern Thai DLBCL patients found that a $\geq 50.00\%$ cut-off was the most effective threshold for distinguishing the expression of Ki-67 and the prediction of three-year overall survival (12).

Immunohistochemistry (IHC) is a widely used technique in pathology for the detection of specific protein expressions, such as BCL2 and Ki-67, within tissue samples. Conventional IHC involves manual processes for tissue section staining, antibody incubation, and subsequent evaluation by a pathologist. While conventional IHC remains the gold standard, its reliance on manual steps can lead to variability in staining intensity, antibody performance, and human interpretation, potentially impacting diagnostic accuracy. In recent years, automated IHC has emerged as a viable

alternative to conventional IHC, offering improved standardization and reproducibility through the automation of staining procedures. Automated IHC employs programmable systems that minimize human error, allowing for consistent antibody staining, reduced variability between tests, and more objective results due to the use of integrated digital analysis software. Automation in IHC holds great potential for improving diagnostic workflows, especially in high-throughput laboratories where time and efficiency are critical. This study aimed to evaluate the agreement between conventional pathologist-based IHC and automated computer-based IHC methods for assessing the expression levels of BCL2 and Ki-67.

METHODS

Sample collection

A retrospective study was performed on formalin-fixed paraffin-embedded tumor tissue specimens obtained from both nodal and extranodal sites of patients with de novo DLBCL who were diagnosed and treated at Maharaj Nakorn Chiang Mai Hospital in Chiang Mai, Thailand, between January 2018 and December 2019. Forty-two samples were included for BCL2 analysis and sixty for Ki-67 analysis. As the study was retrospective, it was conducted with a waiver of written consent following ethical approval from the Ethics and Research Committee of the Faculty of Medicine, Chiang Mai University (Certificate No. 248/2021, Study Code ANA-2563-07765).

Conventional immunohistochemistry study

All the tissue samples were analyzed and evaluated by experienced pathologists. Thin sections, each 3- μm thick, were cut from the paraffin block for conventional IHC assessment. The sections were placed on silane-coated slides and dried in a hot air oven at 60°C for 40 minutes. The sections were then dewaxed, rehydrated, and subjected to antigen retrieval by boiling in citrate buffer pH 6.0 for 20 minutes in a microwave oven at high power, maintaining a temperature of approximately 95–100°C. They were then washed with phosphate-buffered saline (PBS) for three 5-minute cycles. Endogenous peroxidase was blocked by immersing the slides in hydrogen peroxide on a shaker for 30 minutes at room temperature. To block non-specific binding sites, the slides were

treated with 5% normal goat serum (NGS) and 1% bovine serum albumin (BSA) in 0.1 M PBS for 1.5 hours. Next, the slides were incubated with primary antibodies (mouse anti-human BCL2, clone 124, and mouse anti-human Ki-67, clone MIB-1, both from Dako, Carpinteria, CA, USA, RTU) on a shaker for 1 hour, followed by overnight incubation at 4°C. The following day, the slides were shaken for 1 hour at room temperature, then washed with PBS for three 10-minute cycles. They were then incubated with a biotinylated secondary antibody and Streptavidin-HRP (horseradish peroxidase) for 1.5 hours at room temperature, followed by three 10-minute PBS washes. The signal was detected using 3,3'-diaminobenzidine (DAB), and the reaction was stopped with tap and distilled water. Mayer's hematoxylin was used for counterstaining, followed by alcohol dehydration and clearing with xylene. Coverslips were mounted on the slides, and a pathologist evaluated the slides by grading the percentage of positive tumor cells in 10.00% increments from 0.00-100.00% at 40X magnification.

Automated immunohistochemistry study

For the automated IHC assessment, sections of 3- μ m thick, formalin-fixed, paraffin-embedded tissue were cut and placed on Superfrost Plus slides. These slides were heated for one hour at 60°C in a dry oven to enhance tissue adhesion and soften the paraffin. IHC was performed using a Ventana BenchMark ULTRA autostainer (Roche Diagnostics, Tucson, AZ, USA), following standard protocol. The tissue sections underwent deparaffinization, rehydration, and antigen retrieval with Ventana's CC1 solution (pH 8.0). Primary antibodies were applied, including mouse anti-human BCL2 (clone 124, Dako, Carpinteria, CA, USA, RTU) and mouse anti-human Ki-67 (clone MIB-1, Dako, Carpinteria, CA, USA, 1:200 dilution). BCL2 and Ki-67 were incubated for 32 minutes. Detection was carried out using the Ultraview universal DAB kit, followed by hematoxylin counterstaining and bluing. The slides were cleaned using tap water, dehydrated in ethanol and xylene, and mounted for microscopy. BCL2 and Ki-67 slides were scanned at 40X magnification using the Aperio Digital Pathology Slide Scanner (Leica Biosystem, Buffalo Grove, IL, USA). The digital slides were reviewed with Aperio ImageScope Ver.12.4.6.5003 (Leica

Biosystem, Buffalo Grove, IL, USA). Regions of interest were selected manually within the full section of each case at 10X magnification, and ten equal-sized fields were selected for analysis at 40X magnification. Image analysis was carried out using Membrane Algorithm v.9 for BCL2 expression and Nuclear Algorithm v.9 for Ki-67 expression.

Statistical analysis

Mean expression percentages of BCL2 and Ki-67 were compared between conventional and automated IHC using paired t-tests. Pearson correlation coefficients and intraclass correlation coefficients (ICC, two-way mixed-effects model, absolute agreement) were used to assess the strength and consistency of agreement. Bland-Altman plots were constructed to visualize agreement and identify any systematic bias between methods. Statistical significance was defined as a $p < 0.05$. All statistical analyses were conducted using IBM SPSS Statistics version 23 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 9 (GraphPad Software, LLC, San Diego, CA, USA).

RESULTS

The BCL2 expression showed membranous staining, while Ki-67 expression demonstrated nuclear staining. These staining patterns were clearly visualized in both the conventional and automated IHC techniques, as illustrated in Figure 1.

The pathologist-scored BCL2 percentages ranged from 0.00 to 90.00%, with a mean ($\pm$ standard deviation, SD) of $42.38\% \pm 35.39\%$ and a median interquartile range (IQR) of 40.00% (0.00-80.00%). Ki-67 percentages ranged from 10.00 to 90.00%, with a mean $\pm$ SD of $55.67\% \pm 20.45\%$ and a median IQR of 60.00% (40.00-70.00%). The computer analysis showed BCL2 percentages ranging from 0.02% to 79.85%, with a mean $\pm$ SD of $36.59\% \pm 27.32\%$ and a median IQR of 41.36% (6.04-61.66%). Ki-67 percentages ranged from 2.79% to 88.70%, with a mean $\pm$ SD of $41.76\% \pm 22.68\%$ and a median IQR of 41.45% (24.27-60.99%). When comparing the mean percentages between the two methods (Figure 2), the results indicated no significant difference in the percentage of BCL2 protein expression between the pathologist and computer-based analyses (mean difference = 5.79; 95%CI: -1.22 to 12.79; $p =$

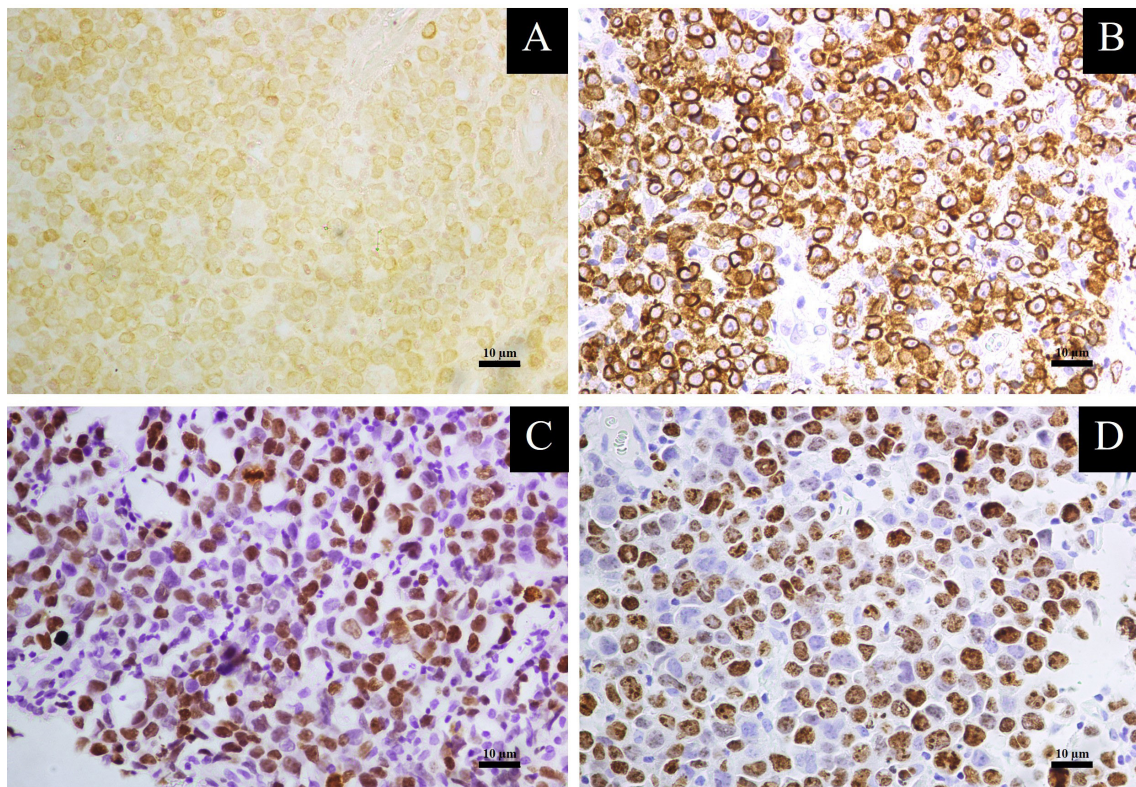


Figure 1. Representative immunohistochemical analysis of BCL2 and Ki-67 expression in DLBCL, captured at 40X magnification. (A) BCL2 membranous staining using conventional IHC technique, (B) BCL2 membranous staining using Automate IHC technique, (C) Ki-67 nuclear staining using conventional IHC technique, (D) Ki-67 nuclear staining using Automate IHC technique

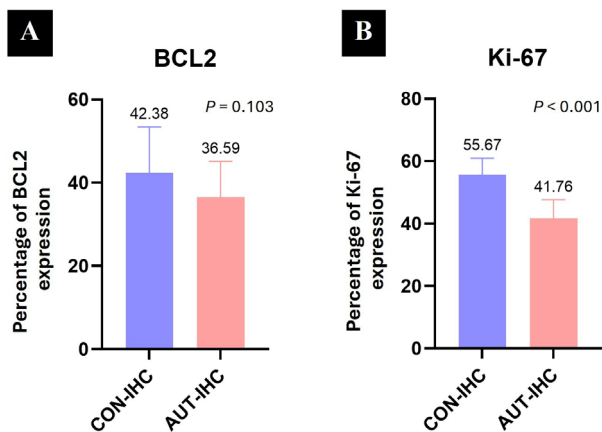


Figure 2. Comparison of the percentage expression of BCL2 (A) and the percentage expression of Ki-67 (B) analyzed using conventional IHC and automated IHC techniques. CON-IHC, conventional immunohistochemistry technique; AUT-IHC, automated immunohistochemistry technique

0.103). However, the analysis by the pathologist revealed a significantly higher percentage of the expression of the Ki-67 protein compared

to computer-based analysis (mean difference = 13.91; 95%CI: 9.48 to 18.34; $p < 0.001$).

The agreement between the two methods was further evaluated using the ICC and Bland-Altman analysis. For BCL2, the ICC was 0.739 (95%CI: 0.564–0.851, $p < 0.001$), indicating moderate to good agreement between the conventional and automated assessments. For Ki-67, the ICC was 0.569 (95%CI: 0.137–0.779, $p < 0.001$), indicating moderate agreement.

The Bland-Altman plots (Figure 3) demonstrated a mean difference (bias) of 5.79% for BCL2, with limits of agreement (LoA) ranging from –38.29% to 49.87%. For Ki-67, the mean difference was 13.91%, with LoA ranging from –19.72% to 47.54%.

Additionally, a strong Pearson correlation was observed between the percentages of protein expression analyzed by the pathologist using conventional IHC and those from the computer-based automated IHC method for both BCL2 ($r = 0.772$, $p < 0.001$) and Ki-67 ($r = 0.688$, $p < 0.001$). The correlation data are illustrated in Figure 4.

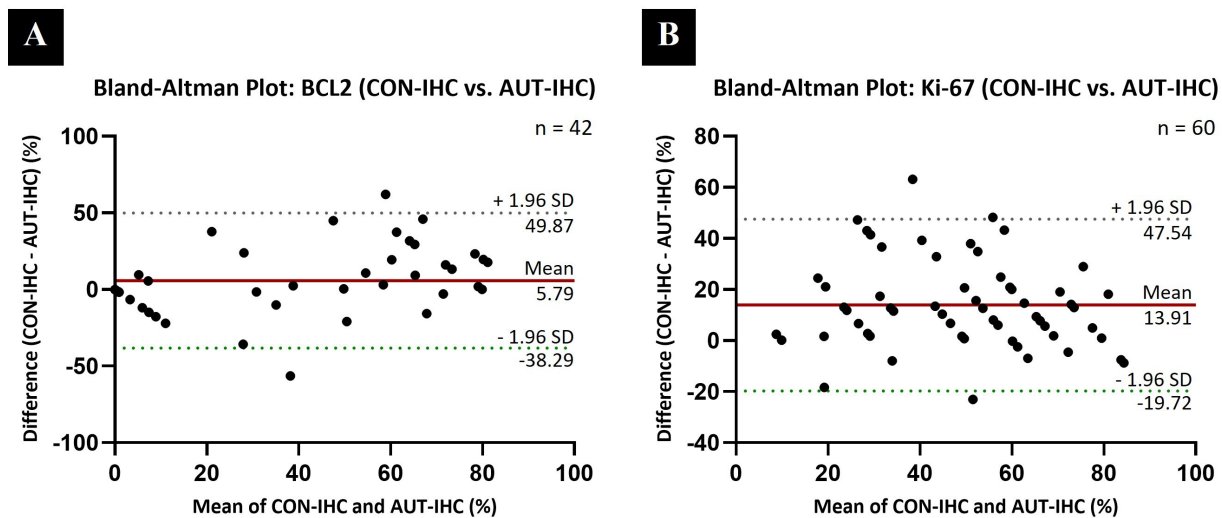


Figure 3. Bland-Altman plots showing the agreement between conventional IHC (CON-IHC) and automated IHC (AUT-IHC) for BCL2 (A) and Ki-67 (B). The solid line represents the mean difference (bias), while the dashed lines indicate the upper and lower limits of agreement (mean $\pm$ 1.96 SD).

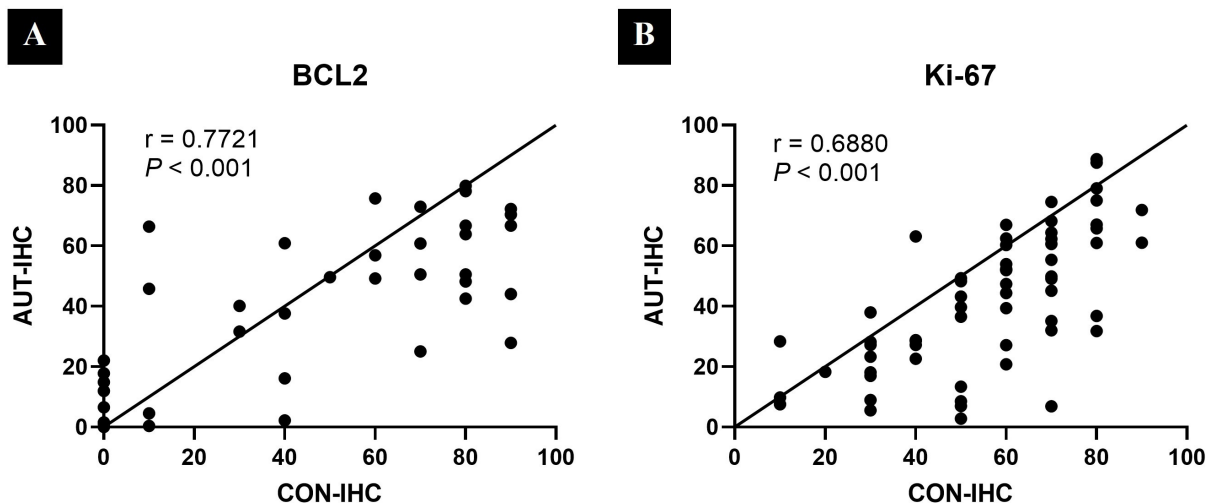


Figure 4. Scatter plots showing the correlation between conventional IHC (CON-IHC) and automated IHC (AUT-IHC) for the expression of BCL2 (A) and of Ki-67 (B). Each dot represents an individual sample. A strong positive correlation was observed for both markers, indicating consistency between manual and automated evaluation methods.

DISCUSSION

Immunohistochemical staining methods have become essential tools in the daily practice of anatomic pathology worldwide. These techniques allow for the identification and localization of specific proteins within tissue sections, enabling and facilitating disease diagnosis, prognosis, and therapeutic decision-making. Traditionally, pathologists have manually evaluated IHC slides, but advancements in digital pathology have led to the development of automated analysis systems. These systems offer the potential for increased efficiency, consistency, and objectivity. However, questions remain about the accuracy and reliability of computer-based methods in comparison to

human evaluation. In this study, we evaluated the agreement between conventional pathologist-based IHC assessment and automated computer-based IHC analysis of BCL2 and Ki-67 expression in patients with DLBCL.

The comparable results observed with the two methods for BCL2 suggest that both manual and automated analyses provide similarly reliable quantification of this marker. However, the results of the analysis revealed a notable difference in the measurement of expression of Ki-67, where pathologist-based assessment yielded a significantly higher percentage expression compared to the computer-based approach.

These results can be attributed to several key factors related to interpretative differences between human and automated evaluation. First, pathologists often focus on areas with the highest proliferative activity and estimate Ki-67 positivity in increments of 10.00%, relying on approximate visual categorization. In contrast, computer-based analysis typically assesses a broader region of the tissue, including both high and low proliferative zones, produces more continuous and specific percentages (e.g., reporting 12.00% rather than rounding to the nearest 10.00%), providing finer numerical detail that does not rely on set increments (13). Second, this discrepancy may also be influenced by biological variability in Ki-67 staining intensity, which fluctuates depending on the cell cycle phase (7, 14–16). Pathologists may recognize and incorporate such variability during evaluation and are generally more flexible in interpreting faint or borderline staining. In contrast, automated systems may treat such variations as below-threshold signals and exclude them from the final count. Additionally, pathologists typically exclude non-viable tissue and non-tumor elements such as endothelial cell nuclei or inflammatory cells from the analysis. In contrast, automated systems may not always effectively differentiate or exclude these areas, which can dilute the calculated Ki-67 index.

To assess agreement, we employed both ICC and Bland–Altman analysis. The ICC indicated moderate to good agreement for BCL2 and moderate agreement for Ki-67, suggesting generally consistent measurements across both methods. Bland–Altman plots confirmed that most observations fell within acceptable limits of agreement, although a mild negative bias for Ki-67 was observed. Strong Pearson correlations for both markers further supported the consistency in expression trends between the two methods.

Overall, the findings highlight the potential of computer-based methods as a reliable complement to conventional pathologist evaluations, particularly in the case of BCL2. However, they also emphasize the need to improve automated methods for markers like Ki-67 to achieve the level of detail provided by human experts. A prior study suggested that Ki-67 percentages assessed by pathologists may correlate more closely with survival outcomes than those from image analysis.

This may be due to the pathologist's ability to focus on large tumor cells and exclude background lymphocytes, whereas current algorithms may lack the precision to distinguish such subtle histological features (13).

It should be noted that this study was designed to compare two integrated IHC workflows: conventional (manual staining and manual evaluation) and automated (automated staining and computer-based evaluation). Because both the staining and evaluation steps differ simultaneously, the observed differences may reflect the combined effect of these components. While this workflow-based design provides a comprehensive comparison, it limits the ability to isolate the specific impact of either staining or evaluation alone. Future studies should aim to assess these steps independently using standardized conditions to better determine their individual contributions.

Despite this limitation, automated IHC offers improved reproducibility and suitability for high-throughput applications, though it tends to be less flexible and more costly. In contrast, conventional IHC is more cost-effective and adaptable but introduces greater variability due to subjective interpretation. Notably, this study involved a single pathologist, and intra- or inter-observer variability was not assessed. Future work should incorporate multiple evaluators to determine the reproducibility of manual scoring.

From a clinical perspective, BCL2 and Ki-67 are not definitive diagnostic markers for DLBCL, but are widely used as prognostic indicators. BCL2 is associated with the regulation of apoptosis, while Ki-67 reflects cellular proliferation. Because both markers are also expressed in normal tissues, careful interpretation is essential. Accurate quantification of these proteins has important implications for risk stratification and treatment planning in patients with DLBCL.

CONCLUSIONS

In conclusion, our findings demonstrate that automated and conventional IHC workflows show good overall agreement in the assessment of BCL2 and Ki-67 expression. While minor differences exist, particularly in Ki-67 quantification, both approaches yielded broadly comparable results. Rather than positioning one method as superior, it is important to note that both methods

yielded results trending in the same direction, with each method having distinct advantages and limitations. Automated systems enhance reproducibility and standardization, while conventional methods benefit from the expert judgment of trained pathologists. These complementary strengths suggest that both methods can play important roles in modern pathology, and that their integration should be guided by clinical context, workflow capacity, and biomarker-specific considerations.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

AUTHOR CONTRIBUTIONS

P.Y.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, software, validation, visualization, writing – original draft, writing – review & editing; K.B.: conceptualization, data curation, formal analysis, funding acquisition, investigation, project administration, supervision, validation, writing – review & editing; A.T.: conceptualization, data curation, formal analysis, investigation, resources, validation, writing – review & editing; T.R.: conceptualization, data curation, formal analysis, investigation, resources, validation, writing – review & editing; S.A.: conceptualization, data curation, writing – review & editing; T.D. conceptualization, data curation, formal analysis, investigation, methodology, project administration, resources, software, supervision, validation, writing – review & editing.

DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

INSTITUTIONAL REVIEW BOARD STATEMENT

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics and Research Committee of the Faculty of Medicine, Chiang Mai University (Certificate No. 248/2021, Study Code ANA-2563-07765).

INFORMED CONSENT STATEMENT

Patient consent was waived due to the retrospective nature of the study, as approved by the Ethics and Research Committee of the Faculty of Medicine, Chiang Mai University.

REFERENCES

1. de Leval L, Hasserjian RP. Diffuse large B-cell lymphomas and burkitt lymphoma. *Hematol Oncology Clin of North Am.* 2009;23:791-827.
2. Leich E, Hartmann EM, Burek C, Ott G, Rosenwald A. Diagnostic and prognostic significance of gene expression profiling in lymphomas. *APMIS.* 2007;115: 1135-46.
3. Kim K, Jorge VM, Tiu A, Mousaa P, Gupta S, Djibo D, et al. Early Mortality after Diagnosis of Diffuse Large B-Cell Lymphoma : A Retrospective, Single Urban-Community Hospital Study. *Blood.* 2019;134(Supplement_1):5335.
4. Klanova M, Kazantsev D, Pokorna E, Zikmund T, Karolova J, Behounek M, et al. Anti-apoptotic MCL1 protein represents critical survival molecule for most burkitt lymphomas and BCL2-negative diffuse large B-cell lymphomas. *Mol Cancer Ther.* 2022;21:89-99.
5. Roh J, Cho H, Pak H-K, Lee YS, Lee S-w, Ryu J-S, et al. BCL2 super-expressor diffuse large B-cell lymphoma: a distinct subgroup associated with poor prognosis. *Mod Pathol.* 2022;35:480-8.
6. Punnoose E, Peale FV, Szafer-Glusman E, Lei G, Bourgon R, Do AD, et al. BCL2 Expression in first-line diffuse large B-cell lymphoma identifies a patient population with poor prognosis. *Clin Lymphoma Myeloma Leuk.* 2021;21:267-78:e10.
7. Harlendea NJ, Harlendo K. Ki-67 as a Marker to Differentiate Burkitt Lymphoma and Diffuse Large B-cell Lymphoma: A Literature Review. *Cureus.* 2022;16: e72190. PubMed PMID: 39583511
8. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. *J Cell Physiol.* 2000;182:311-22.
9. Sadeghipour A, Taha SR, Shariat Zadeh M, Kosari F, Babaheidarian P, Fattahi F, et al. Expression and Clin-

- ical Significance of Ki-67, CD10, BCL6, MUM1, c-MYC, and EBV in Diffuse Large B Cell Lymphoma Patients. *Appl Immunohistochem Mol Morphol*. 2024;32:309-21.
10. Broyde A, Boycov O, Strenov Y, Okon E, Shpilberg O, Bairey O. Role and prognostic significance of the Ki-67 index in non-Hodgkin's lymphoma. *Am J Hematol*. 2009;84:338-43.
11. Tang YL, Zhou Y, Cheng LL, Su YZ, Wang CB. BCL2/Ki-67 index predict survival in germinal center B-cell-like diffuse large B-cell lymphoma. *Oncol Lett*. 2017;14:3767-73.
12. Yimpak P, Bumroongkit K, Tantiworawit A, Rattana-athammethree T, Aungsuchawan S, Daroontum T. Immunohistochemistry-based investigation of MYC, BCL2, and Ki-67 protein expression and their clinical impact in diffuse large B-cell lymphoma in upper Northern Thailand. *PLoS One*. 2024;19:e0307253. PubMed PMID: 39038016.
13. Chabot-Richards DS, Martin DR, Myers OB, Czuchlewski DR, Hunt KE. Quantitative image analysis in the assessment of diffuse large B-cell lymphoma. *Mod Pathol*. 2011;24:1598-605.
14. Li LT, Jiang G, Chen Q, Zheng JN. Ki67 is a promising molecular target in the diagnosis of cancer (review). *Mol Med Rep*. 2015;11:1566-72.
15. González-González R, Molina-Frechero N, Carreón-Burciaga RG, López-Verdín S, Robles-Bonilla C, Pereira-Prado V, et al. Comparison between Manual and Automated Methods for Ki-67 Immunoexpression Quantification in Ameloblastomas. *Anal Cell Pathol (Amst)*. 2016;2016:7486989. PubMed PMID: 27843757
16. Remes SM, Tuominen VJ, Helin H, Isola J, Arola J. Grading of neuroendocrine tumors with Ki-67 requires high-quality assessment practices. *Am J Surg Pathol*. 2012;36:1359-63.