Ki-67 expression in mature B-cell neoplasms: a flow cytometry study

Natália Marcondes[1,2]
Flavo Fernandes [2]
Gustavo Faulhaber [1,2,3]

1. Programa de Pós Graduação em Medicina: Ciências Médicas, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil
2. Laboratório Zanol, Porto Alegre, RS, Brasil
3. Departamento de Medicina Interna, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil

SUMMARY

OBJECTIVE: *Ki-67 is a nuclear protein associated with cellular proliferation in normal or leukemic conditions that can help identify more aggressive diseases and is usually evaluated with immunohistochemistry. The aim of this was to assess Ki-67 expression on mature B-cell neoplasms samples with flow cytometry immunophenotyping.*

METHOD: *After surface staining with CD19 and CD45, intracellular staining for Ki-67 was performed in leukemic mature B-cells. Ki-67 expression was evaluated with flow cytometry.*

RESULTS: *Ki-67 expression was higher in mantle cell lymphoma, Burkitt lymphoma, and diffuse large B-cell lymphoma cases. It was also associated with CD38 mean fluorescence intensity.*

CONCLUSIONS: *Ki-67 expression evaluated by flow cytometry can be a useful tool in the diagnosis of mature B-cell neoplasms. More studies are needed to validate Ki-67 assessment with flow cytometry immunophenotyping.*

KEYWORDS: *Flow cytometry. Immunophenotyping. Ki-67 Antigen.*

INTRODUCTION

Mature B-cell neoplasms (MBCN) are a heterogeneous group of diseases have rearranged immunoglobulin gene, characterized by a monoclonal B-cell lymphoid population that usually has kappa or lambda restriction[1]. In the course of illness, there is a progressive accumulation of clonal cells, causing lymphocytosis, infiltration of bone marrow and other tissues[2]. The current classification of MBCN is based on the assessment of clinical, morphological, immunophenotypic, and genetic data [3].

Uncontrolled proliferation is a key feature of tumor cells and, in most cases, the percentage of proliferating cells provides the biological behavior and clinical course of the disease[4]. Ki-67 is a nuclear protein associated with cell proliferation that is expressed in all active stages of cell division, both in normal and leukemic cells, and contributes to the cell cycle regulation[5-7]. Therefore, Ki-67 is an excellent marker to establish the growth fraction of a cell population at a certain time, and the percentage of tumor cells expressing Ki-67 is used as a proliferation index for evaluating several types of cancer[8,9]. Most studies assessing Ki-67 expression in MBCN were performed with immunohistochemistry, which can be influenced by the presence of non-malignant reactive cells between tumor cells, different methodologies used in sample processing, and interoperator variations[10,11].

This study was conducted to establish the expression of Ki-67 on B-cell lymphocytes of MBCN with a flow cytometry methodology.

METHOD

Flow cytometry staining for intracellular Ki-67 was performed in samples from 147 patients diagnosed with MBCN, according to the World Health Organization criteria[3]. The exclusion criteria were known previous treatment for B-cell neoplasms and presence of other hematological malignancies. Data were obtained in reports provided by the medical assistants.

The flow cytometric analysis was processed as reported elsewhere[12]. Briefly, samples were stained with fluorochrome-conjugated monoclonal antibodies (MoAbs) against CD45 FITC and CD19 APC. Red blood cells were lysed with Human BD Phosflow Lyse/Fix Buffer. Cells were washed and incubated with Fix/Perm Buffer (Transcription Factor Buffer Set). Cells were washed and incubated with Perm/Wash Buffer (Transcription Factor Buffer Set) and 5µL of Ki-67 PerCP-Cy 5.5 MoAb (clone B56). Cells were washed and resuspended in phosphate buffered saline (PBS). All samples were processed within 48-hours of collection[13]. All reagents were purchased from BD Biosciences (San Diego, CA, USA). Cells were acquired on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences, San Diego, CA, USA). Analyses were carried out with CytoPaint Classic 1.1 (Leukobyte, Pleasanton, CA, USA). Expression intensities of CD19 and CD45 were used to gate B-cell population; the percentage and relative mean fluorescence intensity (MFI) of Ki-67 expression in CD19^{+}/CD45^{bright} B-cell lymphocytes were recorded. MFI of CD5, CD10, CD19, CD20, CD23, CD38 and CD45 of MBCN cells were also registered.

Statistical analysis was performed with ANOVA and comparisons were adjusted by the Bonferroni test. The association of variables with Ki-67 MFI was evaluated by the correlation test of Spearman. Backward multiple linear regression, including variables associated with MFI of Ki-67 with $P<0.2$, was performed to identify factors independently associated with it. When variables had co-linearity, the more representative one was included in the analysis. Data were analyzed with SPSS v.18.0 (Chicago, IL, USA), and differences were considered significant when $P<0.05$.

This study was conducted from October 2014 to October 2015 in accordance with the Declaration of Helsinki and current laws in Brazil. This study was performed after approval of the Research Ethics Committee of the Hospital de Clínicas de Porto Alegre (14-0104). Written informed consent was deemed unnecessary.

RESULTS

This study included 94 chronic lymphocytic leukemias (CLL) (63.9%); 5 atypical chronic lymphocytic leukemias (aCLL) (3.4%); 19 marginal zone lymphomas (12.9%); 9 lymphoplasmacytic lymphomas

TABLE 1. CHARACTERISTICS OF THE SAMPLES INCLUDED IN THE STUDY, SEPARATED BY DISEASE.

	CLL (n=94)	aCLL (n=5)	LPL (n=9)	HCL (n=2)	MCL (n=9)	FL (n=5)	MZL (n=19)	BL (n=2)	DLBCL (n=2)
Sample									
Bone marrow	38 (40.4%)	1 (20.0%)	8 (88.9%)	2 (100%)	6 (66.7%)	4 (80.0%)	12 (63.2%)	1 (50.0%)	1 (50.0%)
Peripheral blood	56 (59.6%)	4 (80.0%)	1 (11.1%)	0	3 (33.3%)	0	7 (36.8%)	1 (50.0%)	1 (50.0%)
Lymph node	0	0	0	0	0	1 (20.0%)	0	0	0
Sample counts									
Lymphocytes (%)	69.53 (±18.23)	76.87 (±14.30)	39.10 (±24.82)	60.75 (±0.57)	58.73 (±26.19)	64.28 (±26.75)	45.68 (±21.40)	49.21 (±19.34)	62.55 (±24.02)
CD19+ (% of lymphocytes)	62.72 (±20.34)	66.78 (±19.51)	25.11 (±22.85)	20.46 (±17.09)	50.76 (±26.81)	51.60 (±23.80)	33.15 (±20.80)	38.30 (±29.95)	48.23 (±34.90)
MFI of CD20 on neoplastic cells	111.22 (±119.28)	89.16 (±31.73)	489.01 (±271.68)	787.59 (±354.94)	512.60 (±250.64)	397.76 (±211.61)	753.42 (± 484.89)	247.85 (±162.25)	203.13 (±105.90)
MFI of CD38 on neoplastic cells	7.75 (±8.00)	11.78 (±7.50)	22.42 (±20.46)	7.50 (±3.54)	30.48 (±26.71)	34.80 (±44.69)	9.86 (±8.05)	103.19 (±19.06)	40.46 (±49.17)

Data are shown as mean ±SD or number (n). Abbreviations: CLL: chronic lymphocytic leukemia; aCLL: atypical chronic lymphocytic leukemia; MZL: marginal zone lymphoma; LPL: lymphoplasmacytic lymphoma; MCL: mantle cell lymphomas; FL: follicular lymphoma; HCL: hairy cell leukemia; BL: Burkitt lymphoma; DLBCL: diffuse large B-cell lymphoma.

(6.1%); 9 mantle cell lymphomas (MCL) (6.1%); 5 follicular lymphomas (3.4%); 2 hairy cell leukemias (1.4%); 2 Burkitt lymphomas (1.4%) and 2 diffuse large B-cell lymphomas (DLBCL) (1.4%). Sample characteristics separated per disease category are shown in table 1. The expression of Ki-67 of MBCN samples included in the study is shown in table 2.

MCL cases had higher Ki-67 expression compared to CLL, aCLL, lymphoplasmacytic lymphoma, follicular lymphoma and marginal zone lymphoma cases (P<0.05). The MFI and percentage of Ki-67 expression were significantly higher in Burkitt lymphoma and DLBCL samples compared to the remaining cases, and Burkitt lymphoma cases had the highest Ki-67 MFI (P<0.001) (figure 1). Statistical analysis demonstrated the association of Ki-67 MFI in CD19+ events with MFI of CD38 and CD20, and the percentage of CD19 and lymphocytes on the samples. After multivariable analysis, the MFI of CD38 expression remained significantly associated with the MFI of Ki-67 (P<0.001).

DISCUSSION

In our study, the MFI of Ki-67 among different MBCN was associated with the MFI of CD38 in leukemic cells. Also, the proliferation index – measured by the Ki-67+ percentage and the Ki-67 MFI in CD19+ events – was higher in MBCN that have more aggressive clinical courses compared to indolent diseases.

Few studies have evaluated Ki-67 expression with flow cytometry. Landberg and Roos[14] evaluated Ki-67 expression in non-Hodgkin lymphoma (NHL) cells and the percentage of expression allowed for the discrimination between high and low-grade lymphomas. In another study, Ki-67 staining was able to help differentiate between low, intermediate and high-grade NHL[15]. Our results are similar, with more aggressive diseases presenting higher proliferative index.

The Ki-67 expression in samples from CLL were evaluated with flow cytometry. Most studies ev-

idenced low proliferative index, and higher Ki-67 expression was identified in samples from patients with advanced clinical stage or after cytokine stimulation[16-22]. Similar results were obtained with an in vitro proliferation study of MCL cells[23]. Those are in accordance with the low Ki-67 expression of CLL samples from our study, since we only included patients recently diagnosed. Given that our MCL group included some blastic variant samples, it had a higher and more variable Ki-67.

In a previous study, CLL subclones were separated according to their CD38 expression, Ki-67 expression was found with more frequency in the CD38+ population and, cells with higher CD38 MFI presented higher Ki-67 percentage[24]. Lin et al.[25] sorted CLL cells from bone marrow or peripheral blood according to their Ki-67 expression; Ki-67+ B-cells compartment contained a significantly higher number of CD38+ leukemic cells compared to B-cells not expressing Ki-67. Interestingly, they did not find differences in Ki-67 or CD38 expression in samples collected on the same day from different sites of the same patient. CD38 is expressed by activated B-cells, and it was observed that CD38 expression was significantly higher in the proliferative fraction of CLL cells, this subset also had a higher number of cells with Ki-67 expression[26]. We found an association between the MFI of Ki-67 and CD38, considering all neoplasms and different sample types included in our study.

Herishanu et al.[27] used flow cytometry to evaluate the Ki-67 expression in CLL cells from different sites and identified higher expression on lymph nodes, compared to peripheral blood and bone marrow samples. We only had one lymph node sample from a follicular lymphoma case and its Ki-67 expression was low (5.5%, MFI of 10.2).

Immunophenotyping by flow cytometry is a method of cytological analysis that allows for the identification and characterization of cells in suspension[28,29]. A major advantage of flow cytometry

TABLE 2. KI-67 EXPRESSION ON MATURE B-CELL NEOPLASMS, SEPARATED BY DISEASE.

	CLL (n=94)	aCLL (n=5)	LPL (n=9)	HCL (n=2)	MCL (n=9)	FL (n=5)	MZL (n=19)	BL (n=2)	DLBCL (n=2)
Ki-67+ / CD19+ (% of total events)	1.63 (±1.32)	1.67 (±2.07)	0.81 (±0.70)	0.99 (±0.95)	11.99 (±16.72)	3.28 (±2.84)	1.56 (±1.25)	35.79 (±31.57)	38.10 (±30.17)
MFI of Ki-67 in CD19+ events	7.3 (±12.0)	4.6 (±1.7)	7.8 (±3.8)	8.8 (±1.3)	63.3 (±114.1)	21.3 (±32.9)	9.0 (±5.0)	501.6 (±490.8)	210.6 (±55.9)

Data are shown as mean ±SD. Abbreviations: CLL: chronic lymphocytic leukemia; aCLL: atypical chronic lymphocytic leukemia; MZL: marginal zone lymphoma; LPL: lymphoplasmacytic lymphoma; MCL: mantle cell lymphomas; FL: follicular lymphoma; HCL: hairy cell leukemia; BL: Burkitt lymphoma; DLBCL: diffuse large B-cell lymphoma.
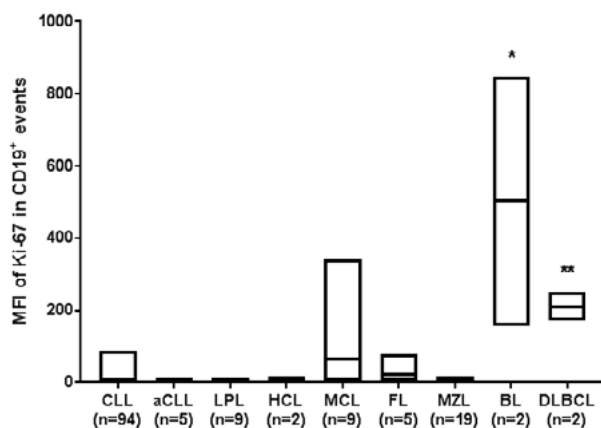
FIGURE 1 Box-plot diagram of Ki-67 mean fluorescence expression in mature B-cell neoplasms (the line shows the mean). Abbreviations: CLL: chronic lymphocytic leukemia; aCLL: atypical chronic lymphocytic leukemia; MZL: marginal zone lymphoma; LPL: lymphoplasmacytic lymphoma; MCL: mantle cell lymphomas; FL: follicular lymphoma; HCL: hairy cell leukemia; BL: Burkitt lymphoma; DLBCL: diffuse large B-cell lymphoma. * BL MFI was higher than all other groups, $P<0.001$. ** DLBCL MFI was higher than other groups, except BL cases, $P<0.001$.

is the possibility of methodology standardization for assessing multiple parameters on a single cell, as well as staining of more than one antigen[30]. The International Lunenburg Lymphoma Biomarker Consortium investigated the impact of immunohistochemical staining procedures and interoperator variation for the quantification of several markers in DLBCL and observed that Ki-67 expression had low reproducibility among the participant laboratories[11]. In our study, we used a flow cytometry approach to assess Ki-67 expression in different MBCN, flow cytometry has a methodological advantage compared to the current standard methodology for evaluation of Ki-67 expression, considering that immunophenotyping procedures can be standardized and validated for use in laboratories worldwide, the cell of interest can be gated, and test results are ready within hours.

Our study has some limitations. The major one is the inclusion of normal residual B-cells within the heterogeneous population of clonal lymphocytes on samples. Besides, MCL cases were considered together as one group, regardless of the variant type, and we had few cases of some types of MBCN. Also, we only had access to immunophenotyping and biopsy results as complementary tests for the disease entity definition, the latter provided by different laboratories.

CONCLUSION

In summary, in our study, Ki-67 expression was higher in MCL, Burkitt lymphoma and DLBCL cases and was associated with the MFI of CD38. The assessment of Ki-67 expression with flow cytometry has the potential to be used in the differential diagnosis of MBCN and other neoplasms. More studies are needed to compare Ki-67 expression with flow cytometry and immunohistochemistry in order to validate the flow cytometry methodology for diagnostic and research purposes.

ACKNOWLEDGMENTS

RESUMO

OBJETIVO: *Ki-67 é uma proteína nuclear associada à proliferação celular em condições normais ou leucêmicas que pode ajudar a identificar doenças mais agressivas. Este marcador é geralmente avaliado com imuno-histoquímica. O objetivo deste estudo foi avaliar a expressão de Ki-67 em amostras de neoplasias de células B maduras com imunofenotipagem por citometria de fluxo.*

MÉTODO: *Após marcação de superfície com CD19 e CD45, foi realizada marcação intracelular para Ki-67 em células B maduras leucêmicas. A expressão de Ki-67 foi avaliada por citometria de fluxo.*

RESULTADOS: *A expressão de Ki-67 foi maior em células de linfomas de manto, linfoma de Burkitt e linfoma difuso de grandes células B. Também houve associação de Ki-67 à intensidade de fluorescência média de CD38.*

CONCLUSÃO: *A expressão de Ki-67 avaliada por citometria de fluxo pode ser útil no diagnóstico de neoplasias de células B maduras. São necessários mais estudos para validar a avaliação de Ki-67 com imunofenotipagem por citometria de fluxo.*

PALAVRAS-CHAVE: *Citometria de fluxo. Imunofenotipagem. Antígeno Ki-67.*

REFERENCES

1. Kaushansky K, Williams WJ. Williams hematology. 8th ed. New York: Mc-Graw-Hill Medical; 2010.

2. van Dongen JJ, Lhermitte L, Böttcher S, Almeida J, van der Velden VH, Flores-Montero J, et al; EuroFlow Consortium (EU-FP6, LSHB-CT-2006-018708). EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes. Leukemia. 2012;26(9):1908-75.

3. Swerdlow SH, International Agency for Research on Cancer, World Health Organization. WHO classification of tumours of haematopoietic and lymphoid tissues. 4th ed. Lyon: International Agency for Research on Cancer; 2008.

4. Sheval EV, Churakova JV, Dudnik OA, Vorobjev IA. Examination of the proliferative activity of tumor cells in human lymphoid neoplasms using a morphometric approach. Cancer. 2004;102(3):174-85.

5. Chen WJ, He DS, Tang RX, Ren FH, Chen G. Ki-67 is a valuable prognostic factor in gliomas: evidence from a systematic review and meta-analysis. Asian Pac J Cancer Prev. 2015;16(2):411-20.

6. Schmidt MH, Broll R, Bruch HP, Duchrow M. Proliferation marker pKi-67 affects the cell cycle in a self-regulated manner. J Cell Biochem. 2002;87(3):334-41.

7. Van Bockstaele DR, Lan J, Snoeck HW, Korthout ML, De Bock RF, Peetermans ME. Aberrant Ki-67 expression in normal bone marrow revealed by multiparameter flow cytometric analysis. Cytometry. 1991;12(1):50-63.

8. Klapper W, Hoster E, Determann O, Oschlies I, van der Laak J, Berger F, et al; European MCL Network. Ki-67 as a prognostic marker in mantle cell lymphoma-consensus guidelines of the pathology panel of the European MCL Network. J Hematop. 2009;2(2):103-11.

9. Landberg G, Tan EM, Roos G. Flow cytometric multiparameter analysis of proliferating cell nuclear antigen/cyclin and Ki-67 antigen: a new view of the cell cycle. Exp Cell Res. 1990;187(1):111-8.

10. Martinez AE, Lin L, Dunphy CH. Grading of follicular lymphoma: comparison of routine histology with immunohistochemistry. Arch Pathol Lab Med. 2007;131(7):1084-8.

11. Jong D, Rosenwald A, Chhanabhai M, Gaulard P, Klapper W, Lee A, et al; Lunenburg Lymphoma Biomarker Consortium. Immunohistochemical prognostic markers in diffuse large B-cell lymphoma: validation of tissue microarray as a prerequisite for broad clinical applications - study from the Lunenburg Lymphoma Biomarker Consortium. J Clin Oncol. 2007;25(7):805-12.

12. Marcondes NA, Fernandes FB, Alegretti AP, Faulhaber GAM. Expression of Bruton's tyrosine kinase in B-cell neoplasms evaluated by flow cytometry. Clin Exp Med. 2017;17(4):499-504.

13. Davis BH, Dasgupta A, Kussick S, Han JY, Estrellado A; ICSH/ICCS Working Group. Validation of cell-based fluorescence assays: practice guidelines from the ICSH and ICCS - part II - preanalytical issues. Cytometry B Clin Cytom. 2013;84(5):286-90.

14. Landberg G, Roos G. Expression of proliferating cell nuclear antigen (PCNA) and Ki-67 antigen in human malignant hematopoietic cells. Acta Oncol. 1991;30(8):917-21.

15. Brons PP, Raemaekers JM, Bogman MJ, van Erp PE, Boezeman JB, Pennings AH, et al. Cell cycle kinetics in malignant lymphoma studied with in vivo iododeoxyuridine administration, nuclear Ki-67 staining, and flow cytometry. Blood. 1992;80(9):2336-43.

16. Aydin A, Elnaggar A, Robertson L, Keating M, Huh Y. IL-2 receptor expression and ki-67 flow cytometric analysis in B-chronic lymphocytic-leukemia. Int J Oncol. 1995;7(6):1433-6.

17. Koníková E, Kusenda J. Altered expression of p53 and MDM2 proteins in hematological malignances. Neoplasma. 2003;50(1):31-40.

18. McManus AP, Desai ZR, Lavabre-Bertrand T. B-cell chronic lymphocytic leukaemia populations respond stochastically to combinations of growth signals in vitro. Leuk Res. 1993;17(6):477-81.

19. Bennett F, Rawstron A, Plummer M, Tute R, Moreton P, Jack A, et al. B-cell chronic lymphocytic leukaemia cells show specific changes in membrane protein expression during different stages of cell cycle. Br J Haematol. 2007;139(4):600-4.

20. Morabito F, Cutrona G, Gentile M, Loiacono F, Matis S, Recchia AG, et al. More on the determination of Ki-67 as a novel potential prognostic marker in B-cell chronic lymphocytic leukemia. Leuk Res. 2010;34(12):e326-8.

21. Cheng S, Ma J, Guo A, Lu P, Leonard JP, Coleman M, et al. BTK inhibition targets in vivo CLL proliferation through its effects on B-cell receptor signaling activity. Leukemia. 2014;28(3):649-57.

22. Mittal AK, Chaturvedi NK, Rohlfsen RA, Gupta P, Joshi AD, Hegde GV, et al. Role of CTLA4 in the proliferation and survival of chronic lymphocytic leukemia. PLoS One. 2013;8(8):e70352.

23. Castillo R, Mascarenhas J, Telford W, Chadburn A, Friedman SM, Schattner EJ. Proliferative response of mantle cell lymphoma cells stimulated by CD40 ligation and IL-4. Leukemia. 2000;14(2):292-8.

24. Damle RN, Temburni S, Calissano C, Yancopoulos S, Banapour T, Sison C, et al. CD38 expression labels an activated subset within chronic lymphocytic leukemia clones enriched in proliferating B cells. Blood. 2007;110(9):3352-9.

25. Lin TT, Hewamana S, Ward R, Taylor H, Payne T, Pratt G, et al. Highly purified CD38 sub-populations show no evidence of preferential clonal evolution despite having increased proliferative activity when compared with CD38 sub-populations derived from the same chronic lymphocytic leukaemia patient. Br J Haematol. 2008;142(4):595-605.

26. Calissano C, Damle RN, Marsilio S, Yan XJ, Yancopoulos S, Hayes G, et al. Intraclonal complexity in chronic lymphocytic leukemia: fractions enriched in recently born/divided and older/quiescent cells. Mol Med. 2011;17(11-12):1374-82.

27. Herishanu Y, Pérez-Galán P, Liu D, Biancotto A, Pittaluga S, Vire B, et al. The lymph node microenvironment promotes B-cell receptor signaling, NF-kappaB activation, and tumor proliferation in chronic lymphocytic leukemia. Blood. 2011;117(2):563-74.

28. Duarte AJS. Citometria de fluxo: aplicações no laboratório clínico e de pesquisa. São Paulo: Atheneu; 2013.

29. Tute RM. Flow cytometry and its use in the diagnosis and management of mature lymphoid malignancies. Histopathology. 2011;58(1):90-105.

30. Soares A, Govender L, Hughes J, Mavakla W, Kock M, Barnard C, et al. Novel application of Ki67 to quantify antigen-specific in vitro lymphoproliferation. J Immunol Methods. 2010;362(1-2):43-50.