Original Article

BrdU Incorporation in Multiparameter Flow Cytometry: A New Cell Cycle Assessment Approach in Multiple Myeloma

Guilhem Requirand,[1,2] Nicolas Robert,[1,2] Stéphanie Boireau,[1,2] Laure Vincent,[3] Anja Seckinger,[4,5] Salaheddine Bouhya,[3] Patrice Ceballos,[3] Guillaume Cartron,[3,6] Dirk Hose,[4,5] Bernard Klein,[1,2,6] and Jérôme Moreaux[1,2,6,7]*

[1]CHU Montpellier, Department of Biological Hematology, Montpellier, France
[2]IGH, CNRS, University of Montpellier, Montpellier, France
[3]CHU Montpellier, Department of Clinical Hematology, Montpellier, France
[4]Medizinische Klinik und Poliklinik V, Universitätsklinikum Heidelberg, Heidelberg, Germany
[5]Nationales Centrum für Tumorerkrankungen, Heidelberg, Germany
[6]University of Montpellier, UFR de Médecine, Montpellier, France
[7]UMR CNRS 5235, University of Montpellier, Montpellier, France

Background: Mutiple myeloma (MM) is a neoplasia characterized by the accumulation of malignant plasma cells (PC) in the bone marrow. Although proliferation markers have been studied in MM, none of the current staging systems include them. Moreover, approaches used to analyze proliferation do not separate MM cells (MMCs) from normal PC.

Methods: In this study, we combined multiparameter flow cytometry and BrdU incorporation or Ki67 staining to analyze MM cell proliferation in 44 monoclonal gammopathy of undetermined significance (MGUS), 153 newly diagnosed MM patients and 69 MM patients at relapse. The prognostic value of proliferation assessment was analyzed in 60 newly diagnosed patients treated with high-dose chemotherapy supported by autologous hematopoietic stem cell transplantation.

Results: The median number of proliferating malignant PC significantly increases during MM disease progression. MM patients with a percentage of proliferating MMCs greater than 1.42% using BrdU/DAPI or greater than 1.1% using ki67/DAPI, are associated with a *significantly* shorter event free survival compared with patients with a lower percentage of proliferating MMCs.

Conclusions: Combination of flow cytometry with BrdU or ki67/DAPI staining could become a standard for the determination of MM cell proliferation. Furthermore, in the context of new effective myeloma treatment options, assessment of MM cell proliferation may be valuable, in clinical trials, to identify novel agents that could significantly affect the small proliferative compartment of MM cells. © 2018 International Clinical Cytometry Society

Key terms: proliferation; prognosis; multiparameter analysis; cytometry; monoclonal

How to cite this article: Requirand G, Robert N, Boireau S, Vincent L, Seckinger A, Bouhya S, Ceballos P, Cartron G, Hose D, Klein B, and Moreaux J. BrdU Incorporation in Multiparameter Flow Cytometry: A New Cell Cycle Assessment Approach in Multiple Myeloma. Cytometry Part B 2018; 9999: 1–6.

Additional supporting information may be found online in the Supporting Information section at the end of the article.

*Correspondence to: Jerome MOREAUX, Laboratory for Monitoring Innovative Therapies Department of Biological Hematology Hôpital Saint-Eloi - CHRU de Montpellier 80, av. Augustin Fliche 34295 Montpellier Cedex 5 IGH - Institute of Human Genetics CNRS-UM UMR 9002 Email: jerome.moreaux@igh.cnrs.fr

Grant sponsor: Institut National du Cancer, Grant numbers: R14026FF, PLBIO15-256
Received 3 April 2018; Revised 19 July 2018; Accepted 27 July 2018
Published online 00 Month in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/cyto.b.21730

INTRODUCTION

Multiple myeloma (MM) is characterized by an accumulation of malignant plasma cells (PC) in the bone marrow (BM) leading to bone destruction, renal failure, and organ dysfunction. Within the last decade, new treatment strategies have significantly improved the outcome of MM patients (1). However, despite these new treatments using drugs such as proteasome inhibitors or immunomodulatory agents (IMiDs), most patients will eventually relapse and die (2). A key biological question is the ability to perform an accurate appreciation of the tumor burden, in terms of infiltrating plasma cells, for the initial staging of patients and for the monitoring of the disease. Flow cytometry is widely used for the diagnosis and monitoring of hematological disorders including MM (3–5). Proliferating PC, that is, the growing fraction of MM cells, have been evaluated by flow cytometry detection of newly synthesized DNA using techniques based on the uptake of tritiated thymidine or bromodeoxyuridine (BrdU) (6–8). The so-called plasma cell labeling index (PCLI) based on propidium iodide incorporation (9) has been shown to be a powerful and independent predictor of survival in MM (10,11) as the assessment of Ki-67 expressing cells in the malignant fraction (12). Although proliferation markers have been studied in MM, none of the current prognostic scores or staging systems, available to physicians nowadays, include these markers. Moreover, the approaches used to analyze proliferation in MM do not separate MMCs from their *normal cell counterparts*. Therefore, easy-to-perform markers of proliferation remain to be developed for routine practice. Novel multiparameter flow cytometry (MFC) enables increased sensibility and depth of malignant PC detection (13). Furthermore, assessment of proliferating myeloma cells is of special interest, as proliferating MMCs can be targeted by available treatments and upcoming therapeutic treatment options (14). The study aimed to combine MFC and BrdU incorporation or Ki67 staining to analyze MM cell proliferation in samples from MGUS and MM patients. We also investigated the prognostic value of proliferation assessment in a cohort of newly diagnosed MM patients.

MATERIALS AND METHODS

Primary Multiple Myeloma Cells

Bone marrow samples were collected after patients' written informed consent in accordance with the Declaration of Helsinki and institutional research board approval from Montpellier University hospital (DC-2008-417). In particular, bone marrow samples were collected from 44 MGUS patients, 153 MM patients at diagnosis, and 69 at relapse. For 60 newly diagnosed patients treated with high dose Melphalan (HDM) and autologous stem cell transplantation (ASCT), MMCs were purified using anti-CD138 MACS microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany), and their gene expression profile (GEP) obtained using Affymetrix U133 plus 2.0 microarrays.

Antibodies

Anti CD45 R Phycoerythrin-TexasRed (ECD) (clone-J33), anti CD138 allophycocyanin (APC) (clone-BB4), anti CD38 Pe-Cyanin 5.5 (PeCy5.5) (clone-LS198–4-3), CD20 allophycocyanin-AlexaFluor 750 (APC-AF 750) (clone-B9E9), CD19 R Phycoerythrin-Cyanine 7 (PC7) (J3–119), CD56 R Phycoerythrin-Cyanine 7 (PC7) (clone-N901), CD117 R Phycoerythrin-Cyanine 7 (PC7) (clone-104D2D1), CD27 R Phycoerythrin-Cyanine 7 (PC7) (clone 1A4CD27) monoclonal antibodies (mAbs), were purchased from Beckman Coulter (Miami, FL, USA), CD200 Phycoerythrin-Cyanine 7 (clone-MRCOX-104), Kappa immunoglobin light chain phycoerythrin (PE) (clone-G20-193), Lambda immunoglobin light chain fluoroscein isothiocyanate (FITC) (clone-JDC-12), KI67 allophycocyanin (APC) (clone-B56), and anti BrdU-APC (BrdU Flow Kit from BD Biosciences) mAbs were purchased from Becton Dickinson Biosciences (San Jose, CA, USA).

Cell Staining for Immunophenotyping of MM

BM aspirates were harvested in heparin-containing syringes. Samples were filtered through a 100 μm filter to remove bone debris. Whole bone marrow was incubated with or without BrdU (10 μM), (Flow Kit APC, Biosciences, San Jose, CA, USA) at 37°C during 2 h. To lyse red blood cells, samples were incubated with a four-fold volume excess of ammonium chloride for 20 min. Then, leukocytes were washed and incubated in phosphate buffered saline (PBS) (Sigma-Aldrich, Dorset, United Kingdom) containing 10% goat serum albumin (PAA Laboratories, Paris, France) for 10 min as blocking step. Erythrocytes-lysed bone marrow samples after saturation step using goat serum (PPA laboratories, Austria), were labeled with optimal concentrations of antibodies (Tables 1 and 2). Cells were stained with anti-CD19, anti-CD20, anti-CD27, anti-CD38, anti-CD56, anti-CD200, and anti-CD45 mAbs, during 20 min at 4°C, washed twice in PBS/10% goat serum, fixed and permeabilized (BrdU Flow Kit from BD Biosciences, BD Biosciences), and washed using PermWash buffer (BD Biosciences). Cells were incubated during 1 h at 37°C in PBS containing DNAse I (300 μg/mL). Cells were then stained with anti-Kappa light chain, anti-Lambda light chain, and anti-BrdU-APC mAb (BrdU Flow Kit from BD Biosciences) during 20 min at 4°C (Tables 1 and 2). For Ki67 staining, cells were labeled as described in Tables 1 and 2. After fixation and permeabilization, cells were stained with an APC conjugated anti-KI67 antibody or isotypic APC (BD Biosciences) and anti-Kappa light chain, anti-Lambda light chain mAb. Nuclei were stained in PermWash containing DAPI (2 μg/mL) (4′,6 Diamido-2-phenylindole, dilactate from Invitrogen, Life Technologies) during 20 min at 4°C. Cells were analyzed using Cyan cytometer (Beckman

Table 1
BrdU Panel Composition

Tubes	DAPI	FITC	PE	ECD	PeCy5.5	PeCy7	APC	APC-AF750
1 – Control	DAPI	Lambda	Kappa	CD45	CD38	CD19	Anti BrdU	CD20
2 – Negative Pool	DAPI	Lambda	Kappa	CD45	CD38	Negative Pool CD19/CD27	Anti BrdU	CD20
2 – Positive Pool	DAPI	Lambda	Kappa	CD45	CD38	Positive Pool CD56/ CD117/CD200	Anti BrdU	CD20

The panel comprises three tubes that contain DAPI, anti-CD45, anti-CD20, anti-CD38, anti-BrdU, anti-kappa, anti-lambda antibodies, a positive antibody pool against markers that are overexpressed in malignant PC (CD56, CD117 and CD200) and a negative antibody pool against markers that are downregulated in malignant PC (CD19 and CD27) compared with normal PC.

Coulter, FL, USA). Contaminating events were removed on both FCS and SSC plots. Singlets were plotted on FSC-A versus FSC-H and SSC-A versus SSC-H plots to remove debris and to select the total leukocyte population. PC and B cells were selected on CD45/CD38 and CD20/CD19 plots, respectively. Abnormal PC were selected based on the CD19, CD27, CD56, CD117, and CD200 signals (13). 5,000,000 events were acquired per tube and the minimum number of abnormal plasma cells needed was 20. The maximum sensitivity of the method is 0.0004% (13). The analyses have been done using FlowJo software (FlowJo, LLC).

Gene Expression Profiling and Statistical Analyses

Gene expression data were normalized with the MAS5 algorithm and analyses processed with GenomicScape (http://www.genomicscape.com) (15) the R.2.10.1 and bioconductor version 2.5 programs (16). Gene Set Expression Analysis (GSEA) was used to identify genes and pathways differentially expressed between populations (17). Univariate and multivariate analysis of genes prognostic for patients' survival was performed using the Cox proportional hazard model. Difference in event free survival between groups of patients was assayed using Maxstat algorithm (18) and survival curves plotted using the Kaplan–Meier method.

RESULTS

MFC was used to delineate malignant and normal plasma cells (13) (Supporting Information Fig. S1A). PCs were selected on CD45/CD38 plot. Specific malignant markers including CD117, CD56, and CD200 combined with CD19, CD27 normal PC markers and kappa or lambda light chains were used to identify malignant from normal plasma cells (Supporting Information Fig. S1A) (Tables 1 and 2). In order to assess proliferation of myeloma cells specifically, we combined MFC with BrdU incorporation or Ki67 staining (Tables 1 and 2) (Supporting Information Fig. S1B).

MFC and BrdU incorporation was investigated in 44 MGUS, 153 newly diagnosed MM patients and 69 MM patients at relapse (Fig. 1A). The median percentage of malignant PC in the S phase of the cell cycle in MGUS, newly diagnosed MM patients and relapsing patients was 0.2% (range: 0%–1.23%), 0.4% (range: 0%–17.3%), and 0.83% (range: 0%–33.8%), respectively. The median number of proliferating malignant PC was 2-fold higher in newly diagnosed patients compared with MGUS (P = 3E-5) and two-fold higher in MM at relapse compared with patients at diagnosis (P = 0.03) (Fig. 1A). We also investigated the interest of Ki67 staining to assess proliferation of malignant plasma cells. MFC incorporating Ki67/DAPI staining was also investigated in 11 MGUS, 49 newly diagnosed MM patients, and 18 MM patients at relapse (Fig. 2A). The median percentage of $Ki67^+/DAPI^+$ (cells in S-G2-M phases) malignant PC in MGUS, newly diagnosed MM patients and relapsing patients was 0.38% (range: 0.07%–1.75%), 0.59% (range: 0%–18.8%), and 0.91% (range: 0%–17.6%), respectively. The median number of $Ki67^+/DAPI^+$ malignant PC was significantly higher in newly diagnosed patients compared with MGUS and in MM at relapse compared with MGUS (P = 0.04). MMC proliferation assessment using BrdU incorporation or Ki67+/DAPI+ staining was highly correlated (r = 0.95, $P < 0.001$) (Supporting Information Fig. S2).

Table 2
Ki67 Panel Composition

Tubes	DAPI	FITC	PE	ECD	PeCy5.5	PeCy7	APC	APC-AF750
1 – Control	DAPI	Lambda	Kappa	CD45	CD38	CD19	Ki67	CD20
2 – Negative Pool	DAPI	Lambda	Kappa	CD45	CD38	Negative Pool CD19/CD27	Ki67	CD20
2 – Positive Pool	DAPI	Lambda	Kappa	CD45	CD38	Positive Pool CD56/ CD117/CD200	Ki67	CD20

The panel comprises three tubes that contain DAPI, anti-CD45, anti-CD20, anti-CD38, anti-Ki67, anti-kappa, anti-lambda antibodies, a positive antibody pool against markers that are overexpressed in malignant PC (CD56, CD117, and CD200) and a negative antibody pool against markers that are downregulated in malignant PC (CD19 and CD27) compared with normal PC.

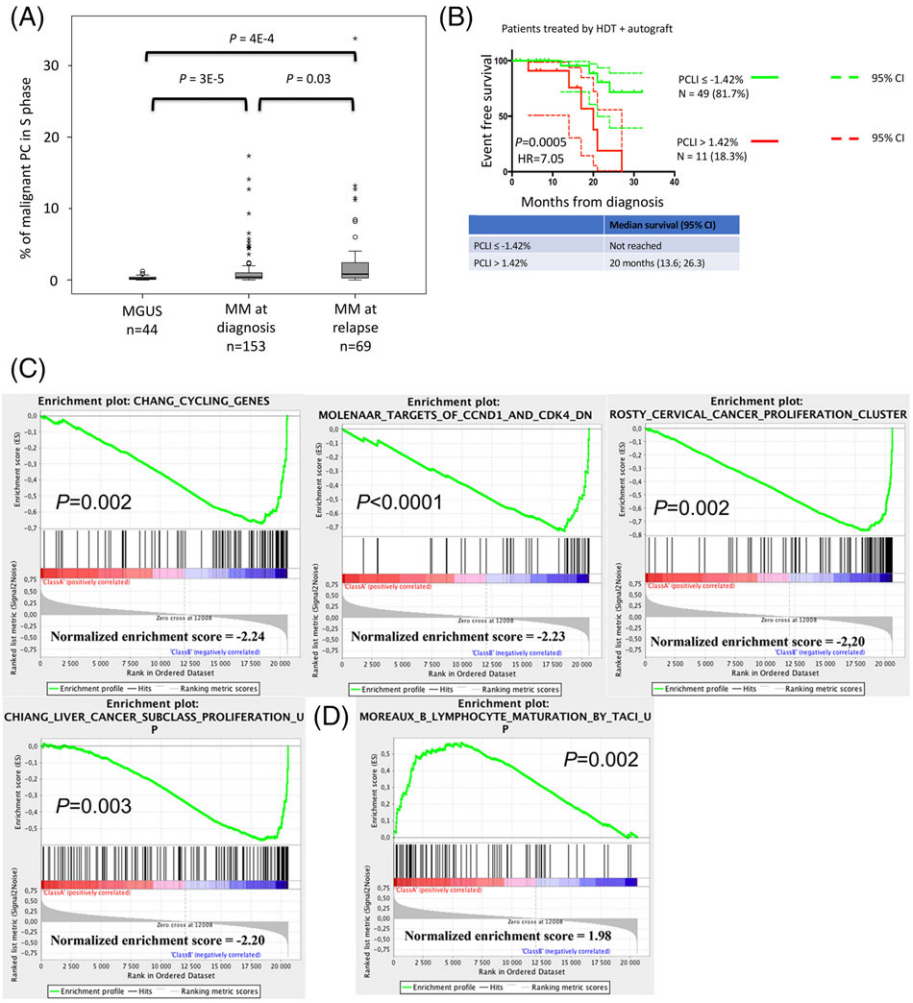

FIG. 1. Assessment of malignant plasma cell proliferation using BrdU incorporation. (A) The percentage of malignant proliferative MM cells was analyzed using BrdU incorporation and multiparameter flow cytometry in bone marrow samples from 44 patients with MGUS, 153 newly diagnosed patients and 69 patients at relapse. (B) A percentage of proliferating MM cells >1.42% could predict for shorter event free survival. Patients of a cohort of 60 newly diagnosed patients were ranked according to increasing proliferation defined by BrdU incorporation/DAPI and a maximum difference in event free survival (EFS) was obtained using the Maxstat R function. (C) Top gene sets significantly associated with high risk proliferation assessment in MM. GSEA enrichment plots with the absolute enrichment *P* value and the normalized enrichment score of the gene set. (D) Top gene sets significantly associated with low risk proliferation assessment in MM. GSEA enrichment plots with the absolute enrichment *P* value and the normalized enrichment score of the gene set.

The prognostic value of proliferation assessment using BrdU incorporation was analyzed in 60 newly diagnosed patients treated with high dose chemotherapy supported by autologous hematopoietic stem cell transplantation (HSCT). Using R Maxstat algorithm (18), allowing to determine the optimal cutpoint for continuous variables (19–21), we identified that MM patients with a percentage of proliferating MMCs >1.42 are associated with a significant shorter event free survival (20 months median EFS) (P = 0.0005) compared with patients with a percentage of proliferating MMCs ≤ 1.42 (not reached median EFS) (Fig. 1B). Using the same methodology, MM patients with a percentage of ki67 +/DAPI+ MM cells greater than 1.1% were significantly associated with an adverse EFS (19 months median EFS) compared with patients with a percentage of ki67 +/DAPI+ MM cells ≤1.1% (not reached median EFS) (P = 3E-5) (Fig. 2B). Therefore, proliferation staining using BrdU incorporation or ki67/DAPI was significantly higher in newly diagnosed patients classified in the proliferation subgroup of the myeloma molecular classification (22) (Supporting Information Fig. S3). MM cell proliferation using BrdU incorporation or KI67/DAPI was also significantly higher in patients with high growth proliferation gene index (GPI) (23), UAMS-high risk score (HRS) (24), high RS GEP-based score (25), and high IFM score (26) associated with an adverse outcome in MM (Supporting Information Figs. S4 and S5).

Gene Set Expression Analysis (GSEA) was performed comparing gene expression profiles of patients with high risk proliferation assessment (S phase >1.42%) to patients with low risk proliferation (S phase <1.42%).

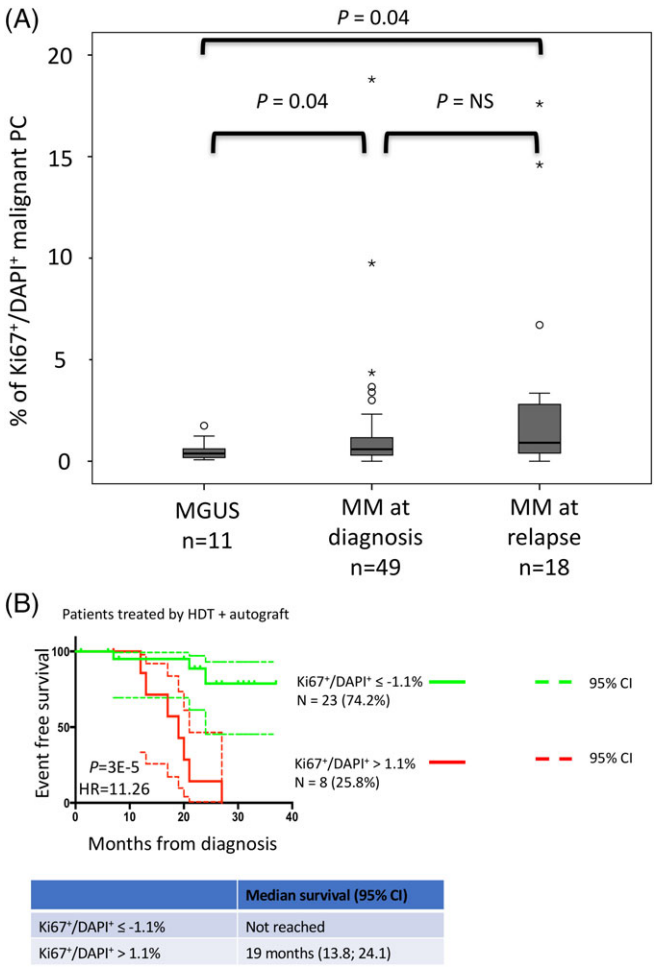

FIG. 2. Assessment of malignant plasma cell proliferation using ki67/DAPI. (A) The percentage of proliferating malignant plasma cells was analyzed by flow cytometry using ki67+/DAPI+ staining, in bone marrow samples from 11 patients with MGUS, 49 newly diagnosed patients and 18 patients at relapse. (B) A percentage of proliferating MM cells defined by ki67+/DAPI+ staining >1.1% could predict for shorter event free survival. Patients of a cohort of 31 newly diagnosed patients were ranked according to increasing proliferation defined by ki67+/DAPI+ staining and a maximum difference in event free survival (EFS) was obtained using the Maxstat R algorithm.

Accordingly, high risk patients are characterized by a significant enrichment of genes involved in cell cycle and proliferation (CHANG CYCLING GENES, MOLENAAR TARGETS OF CCND1 CDK4, ROSTY CERVICAL CANCER PROLIFERATION CLUSTER and CHIANG LIVER CANCER SUBCLASS PROLIFERATION UP, $P < 0.005$) (Fig. 1C). In contrast, MM patients with low risk proliferation assessment were characterized by a significant enrichment of genes related to mature bone marrow plasma cells (MOREAUX B LYMPHOCYTE MATURATION BY TACI UP, $P = 0.002$) (Fig. 1D). Altogether, these data revealed that combination of MFC with BrdU incorporation or KI67/DAPI staining is a practical method for MM cell proliferation assessment. Furthermore, this methodology predicts EFS in newly diagnosed MM patients.

DISCUSSION

During the last years, a significant expansion of MM therapeutic options occurred. This underlines the need of useful biological and prognostic information's to develop rational and targeted therapies. The plasma cell labeling index was shown to be one of the most powerful risk factor in MM (10,11). However, this approach did not separate malignant MM cells from normal PC. In the present study, we have described that combination of multiparameter flow cytometry with BrdU incorporation or ki67 staining allows specific MM cell proliferation assessment. We identified that MM patients with greater than 1.42% proliferating MM cells identified with BrdU/DAPI or greater than 1.1% using ki67/DAPI correlated with significant quicker relapse, in the transplantation settings. The methodology yields important information regarding myeloma prognosis at the time of diagnosis. In this study, as expected, results obtained with BrdU incorporation significantly correlated with ki67/DAPI analyses as described in other cancers (27,28). Both approaches assess myeloma-specific proliferation on routinely collected fresh bone marrow aspirates. Comparing the two methods, BrdU incorporation requires ex vivo labeling of viable PC, in real time, with equipment that could not be available in all clinical laboratories. The Ki67/DAPI methodology could be used in any laboratories with equipment for clinical multiparametric flow cytometry analyses. BrdU incorporation allows detection of S-phase cells only. Ki67 is found in multiple phases of the cell cycle including S, G2, M (27,28) and could provide more information relative to quiescence (29).

Several groups have shown the value of minimal residual disease (MRD) analysis over previous response criteria in MM patients (3–5). Our methodology is also suitable for MRD with low population of tumor cells. Combining the high clinical sensitivity of MFC to the detection of a proliferative contingent of MM cells will enable the identification of patients with active disease during the follow-up of MRD.

Furthermore, in the context of new effective myeloma treatment options, assessment of MM cell proliferation may be valuable, in clinical trials, to identify novel agents that could significantly affect the small proliferative compartment of MM cells.

In conclusion, combination of MFC with BrdU incorporation or ki67/DAPI staining could become a standard for the determination of the fraction of proliferating malignant plasma cells.

ACKNOWLEDGEMENTS

This work was supported by grants from French INCA (Institut National du Cancer) Institute (2012-109/087437 and PLBIO15-256), Languedoc Roussillon CRLR (R14026FF), LR-FEDER Hemodiag, ITMO Cancer (MM&TT) and AF3M.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest to declare.

LITERATURE CITED

1. Raab MS, Podar K, Breitkreutz I, Richardson PG, Anderson KC. Multiple myeloma. Lancet. 2009;374:324–339.
2. Rajkumar SV. Treatment of multiple myeloma. Nat Rev Clin Oncol. 2011;8:479–491.
3. Landgren O, Owen RG. Better therapy requires better response evaluation: Paving the way for minimal residual disease testing for every myeloma patient. Cytometry B Clin Cytom. 2016;90B:14–20.
4. Paiva B, Almeida J, Perez-Andres M, Mateo G, Lopez A, Rasillo A, Vidriales MB, Lopez-Berges MC, Miguel JF, Orfao A. Utility of flow cytometry immunophenotyping in multiple myeloma and other clonal plasma cell-related disorders. Cytometry B Clin Cytom. 2010;78B:239–252.
5. Rawstron AC, Child JA, de Tute RM, Davies FE, Gregory WM, Bell SE, Szubert AJ, Navarro-Coy N, Drayson MT, Feyler S and others. Minimal residual disease assessed by multiparameter flow cytometry in multiple myeloma: Impact on outcome in the Medical Research Council Myeloma IX Study. J Clin Oncol 2013;31:2540–2547.
6. Maurer HR. Potential pitfalls of [3H]thymidine techniques to measure cell proliferation. Cell Tissue Kinet. 1981;14:111–120.
7. Neckers LM, Funkhouser WK, Trepel JB, Cossman J, Gratzner HG. Significant non-S-phase DNA synthesis visualized by flow cytometry in activated and in malignant human lymphoid cells. Exp Cell Res. 1985;156:429–438.
8. Falini B, Canino S, Sacchi S, Ciani C, Martelli MF, Gerdes J, Stein H, Pileri S, Gobbi M, Fagioli M, et al. Immunocytochemical evaluation of the percentage of proliferating cells in pathological bone marrow and peripheral blood samples with the Ki-67 and anti-bromo-deoxyuridine monoclonal antibodies. Br J Haematol. 1988;69:311–320.
9. San Miguel JF, Garcia-Sanz R, Gonzalez M, Moro MJ, Hernandez JM, Ortega F, Borrego D, Carnero M, Casanova F, Jimenez R, et al. A new staging system for multiple myeloma based on the number of S-phase plasma cells. Blood. 1995;85:448–455.
10. Greipp PR, Katzmann JA, O'Fallon WM, Kyle RA. Value of beta 2-microglobulin level and plasma cell labeling indices as prognostic factors in patients with newly diagnosed myeloma. Blood. 1988;72:219–223.
11. Larsen JT, Chee CE, Lust JA, Greipp PR, Rajkumar SV. Reduction in plasma cell proliferation after initial therapy in newly diagnosed multiple myeloma measures treatment response and predicts improved survival. Blood. 2011;118:2702–2707.
12. Alexandrakis MG, Passam FH, Kyriakou DS, Dambaki K, Niniraki M, Stathopoulos E. Ki-67 proliferation index: Correlation with prognostic parameters and outcome in multiple myeloma. Am J Clin Oncol. 2004;27:8–13.
13. Alaterre E, Raimbault S, Garcia JM, Reme T, Requirand G, Klein B, Moreaux J. Automated and simplified identification of normal and abnormal plasma cells in Multiple Myeloma by flow cytometry. Cytometry B Clin Cytom. 2017;94B:484–492.
14. Hose D, Reme T, Meissner T, Moreaux J, Seckinger A, Lewis J, Benes V, Benner A, Hundemer M, Hielscher T and others. Inhibition of aurora kinases for tailored risk-adapted treatment of multiple myeloma. Blood 2009;113:4331–4340.
15. Kassambara A, Reme T, Jourdan M, Fest T, Hose D, Tarte K, Klein B. GenomicScape: An easy-to-use web tool for gene expression data analysis. Application to investigate the molecular events in the differentiation of B cells into plasma cells. PLoS Comput Biol. 2015;11:e1004077.
16. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, et al. Bioconductor: Open software development for computational biology and bioinformatics. Genome Biol. 2004;5:R80.
17. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES and others. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005;102:15545–15550.
18. Hothorn T, Lausen B. On the exact distribution of maximally selected rank statistics. Comput Stat Data Anal. 2003;43:121–137.
19. Bruyer A, Maes K, Herviou L, Kassambara A, Seckinger A, Cartron G, Reme T, Robert N, Requirand G, Boireau S and others. DNMTi/HDACi combined epigenetic targeted treatment induces reprogramming of myeloma cells in the direction of normal plasma cells. Br J Cancer 2018; 118:1062–1073.
20. Kassambara A, Hose D, Moreaux J, Walker BA, Protopopov A, Reme T, Pellestor F, Pantesco V, Jauch A, Morgan G, et al. Genes with a spike expression are clustered in chromosome (sub)bands and spike (sub)bands have a powerful prognostic value in patients with multiple myeloma. Haematologica. 2012;97:622–630.
21. Viziteu E, Klein B, Basbous J, Lin YL, Hirtz C, Gourzones C, Tiers L, Bruyer A, Vincent L, Grandmougin C, et al. RECQ1 helicase is involved in replication stress survival and drug resistance in multiple myeloma. Leukemia. 2017;10:2104–2113.
22. Zhan F, Huang Y, Colla S, Stewart JP, Hanamura I, Gupta S, Epstein J, Yaccoby S, Sawyer J, Burington B, et al. The molecular classification of multiple myeloma. Blood. 2006;108:2020–2028.
23. Hose D, Reme T, Hielscher T, Moreaux J, Messner T, Seckinger A, Benner A, Shaughnessy JD Jr, Barlogie B, Zhou Y, et al. Proliferation is a central independent prognostic factor and target for personalized and risk-adapted treatment in multiple myeloma. Haematologica. 2011;96:87–95.
24. Shaughnessy JD Jr, Zhan F, Burington BE, Huang Y, Colla S, Hanamura I, Stewart JP, Kordsmeier B, Randolph C, Williams DR, et al. A validated gene expression model of high-risk multiple myeloma is defined by deregulated expression of genes mapping to chromosome 1. Blood. 2007;109:2276–2284.
25. Reme T, Hose D, Theillet C, Klein B. Modeling risk stratification in human cancer. Bioinformatics. 2013;29:1149–1157.
26. Decaux O, Lode L, Magrangeas F, Charbonnel C, Gouraud W, Jezequel P, Attal M, Harousseau JL, Moreau P, Bataille R, et al. Prediction of survival in multiple myeloma based on gene expression profiles reveals cell cycle and chromosomal instability signatures in high-risk patients and hyperdiploid signatures in low-risk patients: A study of the Intergroupe Francophone du Myelome. J Clin Oncol. 2008;26:4798–4805.
27. Goodson WH, Moore DH, Waldman FM. Ki-67 correlates with in vivo bromodeoxyuridine labeling index in operable breast cancer. J Clin Oncol. 2006;24:3809 author reply 3809.
28. Gasparri F, Wang N, Skog S, Galvani A, Eriksson S. Thymidine kinase 1 expression defines an activated G1 state of the cell cycle as revealed with site-specific antibodies and ArrayScan assays. Eur J Cell Biol. 2009;88:779–785.
29. Yao G. Modelling mammalian cellular quiescence. Interface Focus. 2014;4:20130074.