Received: 28 July 2023 | Accepted: 7 November 2023

DOI: 10.1002/hem3.30

HemaSphere EHA EUROPEAN HEMATOLOGY ASSOCIATION

LETTER

Robust identification of conventional and leukemic nonnodal mantle cell lymphomas using epigenetic biomarkers

Marco M. Bühler[1,2,*] | Marta Kulis[1,*] | Martí Duran-Ferrer[1] | Cristina López[1,3,4] | Guillem Clot[1,3,4] | Ferran Nadeu[1,3] | Mònica Romo[1] | Eva Giné[1,5] | Armando López-Guillermo[1,3,5] | Sílvia Beà[1,3,4,6] | Elías Campo[1,3,4,6] | José Ignacio Martín-Subero[1,4,7]

Correspondence: Elías Campo (ECAMPO@clinic.cat); José Ignacio Martín-Subero (IMARTINS@recerca.clinic.cat)

Mantle cell lymphoma (MCL) is a B-cell lymphoma whose heterogeneous clinical presentation and evolution seem to be mediated by differences in cell of origin as well as in genetic, epigenetic, and transcriptional profiles.[1–4] MCL is currently categorized into two distinct molecular subtypes. Conventional MCLs (cMCLs), usually clinically aggressive, are derived from germinal center-inexperienced B cells and show a high burden of molecular alterations. In contrast, leukemic nonnodal MCLs (nnMCLs) frequently present with a more indolent disease originating from germinal center-experienced cells and show an overall lower genetic complexity.[1]

Current methods to discern the two MCL molecular subtypes include expression of SOX11, IGHV mutational status, and gene expression profiling (16 gene panel L-MCL16[2]). However, the differential diagnosis of cMCL and nnMCL poses several challenges as no method can unequivocally differentiate the two subtypes in fresh, frozen, or paraffin-embedded formalin-fixed (FFPE) material in the most affected tissues, for example, lymph node (LN), peripheral blood (PB), and bone marrow (BM). For instance, SOX11 expression determined by immunohistochemistry might be only partial with unclear significance or difficult to stain in BM samples, and the L-MCL16 gene expression signature has been designed for PB samples. In this context, the use of DNA methylation biomarkers may represent an alternative strategy, as DNA methylation is comparably more stable than gene or protein expression levels and can be measured in DNA obtained from different sample types. In MCL, a comprehensive analysis of the DNA methylome revealed a signature differentiating two epigenetic subtypes, termed C1 and C2 MCL. This signature was mostly related to imprints of B cells at different maturation stages, and the C1 subtype was enriched for cases with SOX11 expression, unmutated IGHV status, and showed more genetic alterations and shorter overall survival than C2 MCLs.[3] Based on these findings, C1 showed a strong overlap with cMCL, and C2 with nnMCLs.

The DNA methylation signature differentiating C1 and C2 MCLs[3] can be reduced to only three CpG sites while maintaining the detection accuracy,[5] paving the way to generate locus-specific assays for clinical use. In the present study, we designed DNA methylation assays for these three CpG sites (Illumina CpG cluster IDs: cg23892310, cg07769421, and cg03425785) using bisulfite pyrosequencing (BisPyroSeq),[6] a cost-effective method that shows high performance in a benchmarking study comparing techniques for epigenetic biomarkers.[7] Additionally, as DNA methylation is affected by the tumor cell content of the sample, we also mined previously reported signatures and obtained two CpGs to estimate B-cell purity (Illumina CpG cluster IDs: cg00226923 and cg03860768).[5] See Supporting Information for detailed technical information regarding the assays.

We initially optimized the BisPyroSeq assays for the three MCL-stratifying CpGs using in vitro methylated DNA as well as DNA extracted from two MCL cell lines (Granta-519 and Z-138, both cMCL models) and 30 primary MCLs with available Illumina arrays from previous studies[1,3] and therefore known methylation status, suitable as a control. Methylation values from BisPyroSeq and methylation arrays were highly concordant (cg23892310: $R = 0.91$, $p = 2.6 \times 10^{-12}$; cg07769421: $R = 0.92$, $p = 6.4 \times 10^{-13}$; cg03425785: $R = 0.85$, $p = 3.8 \times 10^{-9}$; Figure 1A and Supporting Information S1: Table 1). Next, we quantified DNA methylation levels in 17 cases of which different sample types were available (three frozen and PB material, eight frozen and FFPE, two with FFPE and PB, one with two different FFPE samples, and three with frozen, PB, and FFPE material). We observed that our DNA methylation assays were very

[1]Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain
[2]Department of Pathology and Molecular Pathology, University Hospital Zurich, Zurich, Switzerland
[3]Centro de Investigación Biomédica en Red de Cáncer (CIBERONC), Madrid, Spain
[4]Department of Basic Clinical Practice, Faculty of Medicine, University of Barcelona, Barcelona, Spain
[5]Hematology Department, Hospital Clínic, Barcelona, Spain
[6]Hematopathology Section, Pathology Department, Hospital Clinic, Barcelona, Spain
[7]Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain
*Marco M. Bühler and Marta Kulis contributed equally to this study.

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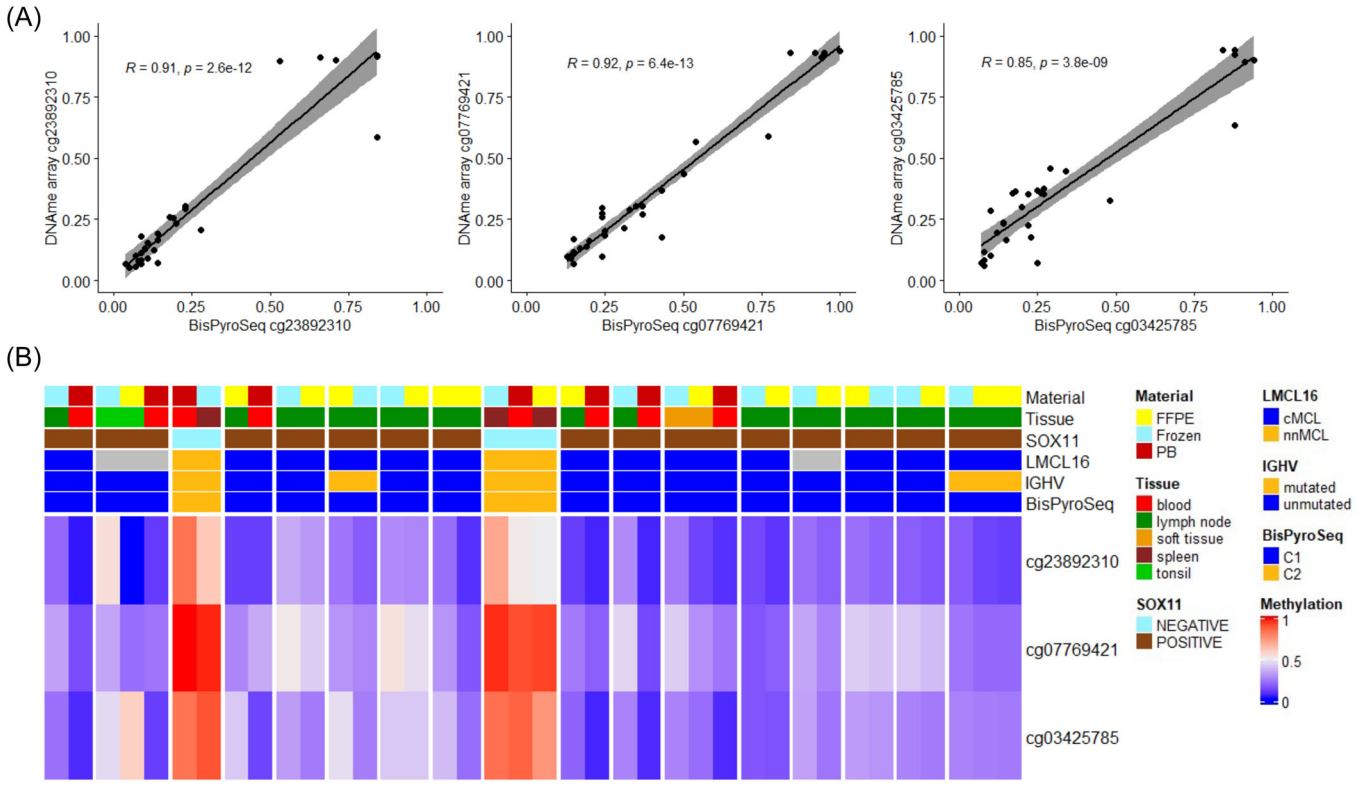

FIGURE 1 Validation of BisPyroSeq MCL classification. (A) Correlation (Spearman's rank correlation coefficient) between DNA methylation measured by BisPyroSeq (*x*-axis) and Illumina DNA methylation arrays (*y*-axis) for the three CpGs (cg23892310, cg07769421, and cg03425785). (B) Heatmap of DNA methylation of the three CpGs in 17 cases with different types of samples/material. Top 5 rows list type of material (FFPE, frozen, or peripheral blood), tissue (blood, lymph node, soft tissue, spleen, and tonsil), and stratification features related to each case, performed on the material adequate for each method, that is, immunohistochemical SOX11 expression (positive or negative), L-MCL16 classification (cMCL or nnMCL; assays performed on peripheral blood samples), and IGHV status using a 98% cutoff (mutated or unmutated). The final, sixth row, represents the classification of specific sample/material with BisPyroSeq (C1 or C2). BisPyroSeq, bisulfite pyrosequencing; cMCL, conventional mantle cell lymphoma; FFPE, paraffin-embedded formalin-fixed; nnMCL, nonnodal MCL.

highly reproducible in different tissues and sample types, in all three measured CpGs, leading to identical classification in all cases (Figure 1B and Supporting Information S1: Table 2). Therefore, we could prove the reliability of our DNA methylation assay in the most common types of clinical samples of MCL. In addition, we also validated the two CpGs to infer the tumor content by BisPyroSeq. We evaluated their accuracy in 50 MCL cases, by comparing the methylation-based estimates of tumor purity with other available measurements from the same samples (i.e., 50 cases based on flow cytometry, 38 based on cancer cell fractions estimated from genomic sequencing data, and 11 based on previously published DNA methylation microarray data[1,3]). The BisPyroSeq estimates of purity were highly concordant with those obtained with molecular methods such as DNA methylation arrays[3] (median difference between methods of 4.5%; interquartile range [IQR] = 3.0%) or whole-genome sequencing[1] (median difference between methods of 3.4%; IQR = 6.2%) as well as standard flow cytometry measurements (median difference between methods of 5.2%; IQR = 4.7%) (Supporting Information S1: Table 3).

Next, we explored the potential clinical application of our DNA methylation assays. For that aim, we extended our series of patients to reach 115 MCL cases with sufficient material to apply our DNA methylation assays. DNA methylation quantification of three MCL biomarkers CpGs could be performed in 110/115, while one CpG, cg03425785, failed in five cases. Tumor content could be determined in 87 cases (due to the failure of one of the CpG assays or insufficient DNA in 32 cases in total), with a mean purity of 86.3% (range 54%–97%). Using the *B.cell.tumor.classifier* function[5] with a threshold of probability of subtype higher than 0.6, we could classify 98 cases as C1 and 14 cases as C2, while three could not be unambiguously classified (Supporting Information S1: Table 4). After excluding the unclassified cases, molecular features were analyzed (Supporting Information S1: Table 5). SOX11 expression was available for 96 cases: all C2 cases with available SOX11 data were negative (13/13), while only 2.4% of C1 were SOX11 negative (2/83) (Supporting Information S1: Tables 5 and 6). Comparison of methylation subtypes and IGHV mutational status ($n = 67/115$) show that all C2 MCL ($n = 12$) have a mutated IGHV status using a 98% cutoff and all but one case when using a 97% cutoff. In the case of C1 MCL ($n = 55$), the average IGHV identity was 98.67%, but seven cases had an IGHV identity lower than 97%. The L-MCL16 classification was available for 81 samples: all C2 MCLs with available L-MCL16 ($n = 12$) were concordantly classified as nnMCLs, whereas in the case of C1s, 94.2% of the cases were classified as cMCL (65/69) and 5.8% could not be determined by L-MCL16 assay (unclassified, 4/69). In addition to molecular features, cases were classified according to the clinical presentation: cases diagnosed in LN or extramedullary biopsies were classified as nodal (N), whereas cases with leukemic disease and absence of significant lymphadenopathies or other tissue involvement (except spleen) in imaging studies were defined as having leukemic

nonnodal (LNN) presentation. We observed that, as expected, 92% of C1 cases had N presentation (76/83), while 89% of C2 cases had LNN presentation (8/9). Overall, these findings indicate that although the C1/C2 classification based on epigenetic biomarkers, the IGHV status, and the L-MCL16 expression classifier are highly concordant and represent good markers to categorize MCL into two entities, approximately 5% of the cases show discrepant results (Supporting Information S1: Table 6). Specifically, four cases were found to have discordant results across methods. One C2/SOX11-negative/IGHV-mutated case (biologically nnMCL) did show a nodal clinical presentation. Two cases classified as C1 were SOX11 negative by immunohistochemistry, raising the possibility of SOX11 false negativity, supported by nodal clinical presentation in one case and unmutated IGHV status (with 98% identity cutoff) in both cases. This finding underscores the problem of relying solely on immunohistochemical SOX11 expression for MCL subtype classification, which might originate from failed staining (especially on decalcified bone marrow biopsies) or ambiguous partial expression.[8] Finally, one C1 case was found to have the leukemic presentation and mutated IGHV status but was classified as cMCL by DNA methylation, SOX11 expression, and L-MCL16 gene expression signature.

Finally, we performed a clinicopathological analysis of C1 and C2 cases identified with the new methylation assay (Figure 2B and Supporting Information S1: Tables 5 and 6). Sufficient clinical data for survival analysis was available for 112 patients with BisPyroSeq classification. As expected, C1 cases showed a trend toward shorter overall survival than C2 cases, although the log-rank test did not reach statistical significance, most likely due to the small sample size of the C2 group ($n = 14$) (Figure 2B, left panel). In line with this finding, no significant differences in overall survival were observed in our series when cases were stratified by other commonly used methods, such as SOX11 expression ($n = 99$) or L-MCL16 ($n = 79$) (Figure 2B, middle and right panel).

The present work demonstrates that microarray-derived DNA methylation subclassification of MCL can be assessed by only quantifying DNA methylation of three CpGs and, importantly, can be performed on any type of clinical specimen that has a sufficient tumor cell content (ideally above 60%). The DNA methylation-based classification of MCL is highly concordant with other available methods (L-MCL16, SOX11 expression, IGHV mutational status) and we could show that in two cases SOX11 immunohistochemistry was negative, despite classification as C1/cMCL by at least two other methods. With the few

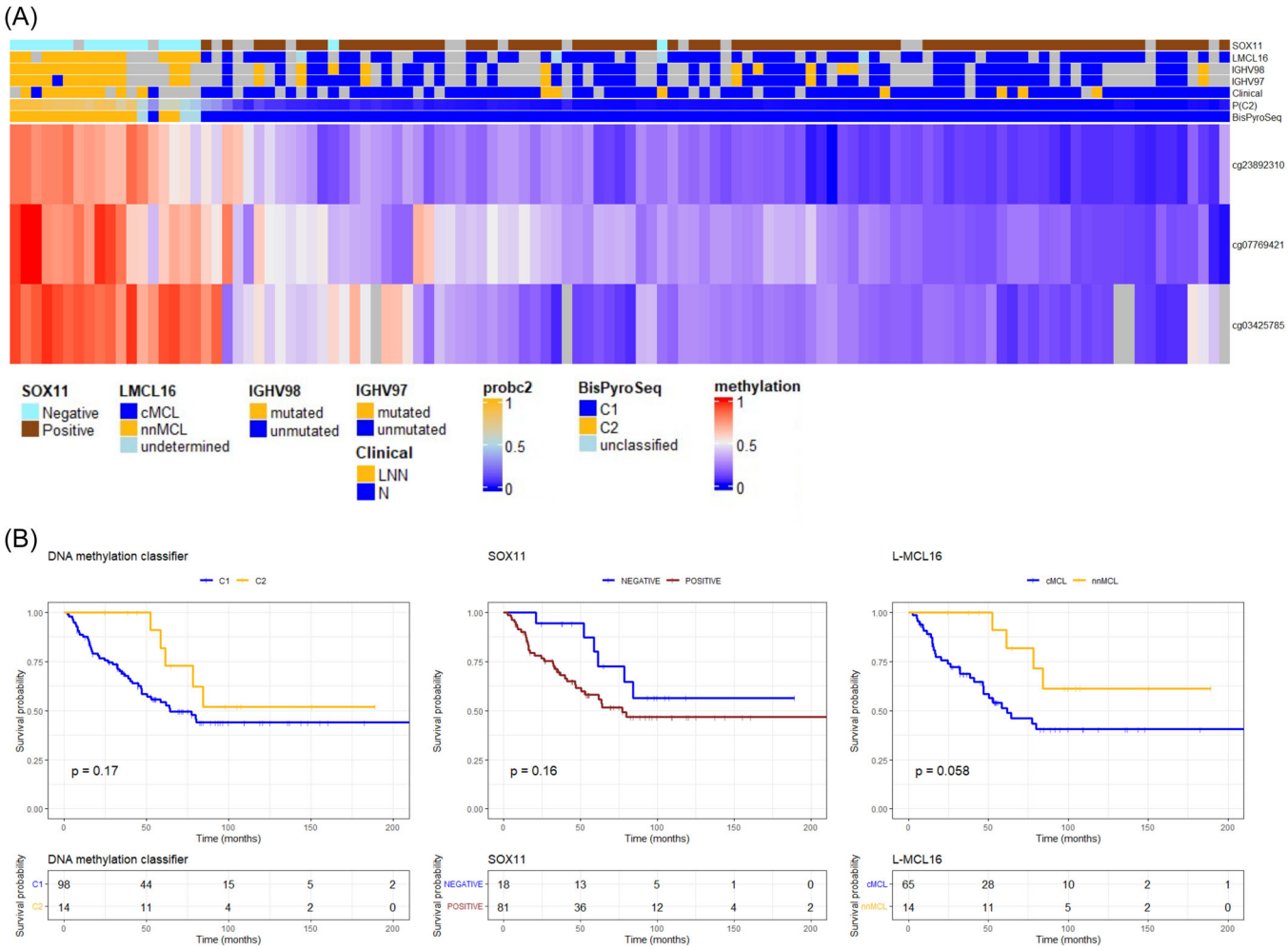

FIGURE 2 Results of MCL cohort and survival analysis. (A) Heatmap showing DNA methylation of three CpGs in all analyzed cases ($n = 115$), annotation on top lists SOX11 expression, L-MCL16 classification, IGHV mutational status using 98% and 97% cutoffs, clinical presentation, probability of C2 classification, and final classification by BisPyroSeq. (B) Kaplan–Meier curves, stratified by DNA methylation classification, SOX11 expression, and L-MCL16 classification. BisPyroSeq, bisulfite pyrosequencing; LNN, leukemic nonnodal presentation; MCL, mantle cell lymphoma; N, nodal presentation.

C2 cases identified, we could not demonstrate a statistically significant difference in overall survival between C1 and C2 MCL, but the observed pattern was largely consistent with previous studies.[2] However, an interesting finding was that more than half of C2 MCL were managed with watch and wait alone, highlighting the more indolent nature of this disease subtype. In conclusion, we show that DNA methylation-based MCL subclassification is a highly robust method, which can be determined by analyzing only three CpGs and can technically be performed on any type of clinical sample with sufficient tumor cell content.

ACKNOWLEDGMENTS

This work was developed at the Centro Esther Koplowitz (CEK, Barcelona, Spain). The authors are indebted to the Genomic Core Facility of the IDIBAPS as well as G. Frigola and O. Balagué from the Hematopathology Unit of Hospital Clinic (Barcelona, Spain).

AUTHOR CONTRIBUTIONS

Marco M. Bühler and Marta Kulis performed research, analyzed and interpreted data, performed statistical analysis, and wrote the manuscript. Martí Duran-Ferrer, Guillem Clot, and Ferran Nadeu performed research and contributed analytical tools. Mònica Romo performed research. Cristina López and Sílvia Bea collected data and provided samples. Eva Giné and Armando López-Guillermo collected data, analyzed, and interpreted data. Elías Campo and José Ignacio Martín-Subero designed and supervised the research and wrote the manuscript. All authors reviewed the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the Supporting Information of this article.

FUNDING

This work was supported by research funding from the National Institutes of Health, National Cancer Institute “Molecular Diagnosis, Prognosis, and Therapeutic Targets in Mantle Cell Lymphoma” (P01CA229100 to E. C.) as well as PID2020-118167RB-I00 (to J. I. M.-S.) and PID2021-123054OB-I00 (to E. C.) funded by MCIN/AEI/10.13039/501100011033 and, as appropriate, by “ERDF A way of making Europe”, by the “European Union” or by the “European Union NextGenerationEU/PRTR.” Furthermore, the authors would like to thank the support of the Generalitat de Catalunya Suport Grups de Recerca AGAUR (2021-SGR-1343 to J. I. M.-S.; 2021-SGR-01172 to E. C.; and 2021-SGR-01293 to S. B.) and Centro de Investigación Biomédica en Red de Cáncer (CIBERONC) (CB16/12/00225 and CB16/12/00334). M. M. B. is supported by the Swiss Cancer League (BIL KLS-5130-08-2020) and the Nuovo-Soldati Foundation for Cancer Research. C. L. is supported by postdoctoral Beatriu de Pinós grant, Secretaria d'Universitats I Recerca del Departament d'Empresa i Coneixement de la Generalitat de Catalunya, and by Marie Sklodowska-Curie COFUND program from H2020 (2018-BP-00055). M. D.-F. is supported by a postdoctoral grant from the Spanish Association Against Cancer (AECC). E. C. is an Academic researcher at the Catalan Institution for Research and Advanced Studies (ICREA).

ORCID

Marco M. Bühler https://orcid.org/0000-0003-0850-3326
José Ignacio Martín-Subero http://orcid.org/0000-0001-8809-5195

SUPPORTING INFORMATION

Additional supporting information can be found in the online version of this article.

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