New Molecular Assay for the Proliferation Signature in Mantle Cell Lymphoma Applicable to Formalin-Fixed Paraffin-Embedded Biopsies

David W. Scott, Pau Abrisqueta, George W. Wright, Graham W. Slack, Anja Mottok, Diego Villa, Pedro Jares, Hilka Rauert-Wunderlich, Cristina Royo, Guillem Clot, Magda Pinyol, Merrill Boyle, Fong Chun Chan, Rita M. Brazier, Wing C. Chan, Dennis D. Weisenburger, James R. Cook, Timothy C. Greiner, Kai Fu, German Ott, Jan Delabie, Erlend B. Smeland, Harald Holte, Elaine S. Jaffe, Christian Steidl, Joseph M. Connors, Randy D. Gascoyne, Andreas Rosenwald, Louis M. Staudt, Elias Campo, and Lisa M. Rimsza, for the Lymphoma/Leukemia Molecular Profiling Project

Author affiliations and support information (if applicable) appear at the end of this article.

Published at jco.org on March 14, 2017.

D.W.S., P.A., E.C., and L.M.R. contributed equally to this work.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Corresponding author: David W. Scott, MBChB, PhD, BC Cancer Research Centre, 675 W 10th Ave, Vancouver, BC V5Z 1L3, Canada; e-mail: dscott8@bccancer.bc.ca.

© 2017 by American Society of Clinical Oncology

0732-183X/17/3515w-1668w/\$20.00

ABSTRACT

Purpose

Mantle cell lymphoma is an aggressive B-cell neoplasm that displays heterogeneous outcomes after treatment. In 2003, the Lymphoma/Leukemia Molecular Profiling Project described a powerful biomarker—the proliferation signature—using gene expression in fresh frozen material. Herein, we describe the training and validation of a new assay that measures the proliferation signature in RNA derived from routinely available formalin-fixed paraffin-embedded (FFPE) biopsies.

Methods

Forty-seven FFPE biopsies were used to train an assay on the NanoString platform, using microarray gene expression data of matched fresh frozen biopsies as a gold standard. The locked assay was applied to pretreatment FFPE lymph node biopsies from an independent cohort of 110 patients uniformly treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone. Seventeen biopsies were tested across three laboratories to assess assay reproducibility.

Results

The MCL35 assay, which contained a 17-gene proliferation signature, yielded gene expression of sufficient quality to assign an assay score and risk group in 108 (98%) of 110 archival FFPE biopsies. The MCL35 assay assigned patients to high-risk (26%), standard-risk (29%), and low-risk (45%) groups, with different lengths of overall survival (OS): a median of 1.1, 2.6, and 8.6 years, respectively (log-rank for trend, $P < .001$). In multivariable analysis, these risk groups and the Mantle Cell Lymphoma International Prognostic Index were independently associated with OS ($P < .001$ for both variables). Concordance of risk assignment across the three independent laboratories was 100%.

Conclusion

The newly developed and validated MCL35 assay for FFPE biopsies uses the proliferation signature to define groups of patients with significantly different OS independent of the Mantle Cell Lymphoma International Prognostic Index. Importantly, the analytic and clinical validity of this assay defines it as a reliable biomarker to support risk-adapted clinical trials.

J Clin Oncol 35:1668-1677. © 2017 by American Society of Clinical Oncology

INTRODUCTION

Mantle cell lymphoma (MCL) is an incurable B-cell malignancy with a broad array of clinical and biologic features.^{1,2} The vast majority of cases harbor the t(11;14)(q13;q32) translocation, leading to overexpression of cyclin D1 and dysregulation of the cell cycle. Although most patients have aggressive disease that requires immediate

treatment, there is a group of patients in whom the disease is indolent and can be observed for years without treatment. Recently, it was recognized that MCL encompasses two subtypes, each with distinct biology: conventional MCL and a leukemic non-nodal variant characterized by lymphocytosis, splenomegaly, no (or minimal) lymphadenopathy, and an indolent clinical course.³⁻⁵

There is no universally accepted treatment regimen for MCL at this time. Most centers make

ASSOCIATED CONTENT

Data Supplement

DOI: 10.1200/JCO.2016.70.7901

DOI: 10.1200/JCO.2016.70.7901

treatment decisions on the basis of the patient's age, with intensive regimens offered to younger patients. New therapeutic agents have shown impressive activity and are being incorporated into combination regimens in the frontline and relapse settings (recently reviewed by Cheah et al¹⁶). There is a critical need for reproducible biomarkers that can be incorporated into clinical trial design and ultimately used to guide management decisions.

A number of prognostic tools have been developed for MCL. The most prominent is the MCL International Prognostic Index (MIPI), which combines clinical and laboratory values to assign patients to low-, intermediate-, or high-risk groups. MIPI has been validated in randomized clinical trials.⁷⁻⁹ In 2003, the Lymphoma/Leukemia Molecular Profiling Project performed gene expression profiling on MCL and demonstrated that a coordinated signature of gene expression associated with proliferation was the strongest molecular predictor of survival and integrated the prognostic power of other molecular markers.¹⁰

However, this proliferation signature, requiring fresh frozen (FF) material and using a microarray-based platform, has not penetrated clinical practice. Ki-67 proliferation index (PI), measured using immunohistochemistry (IHC), has been proposed as a surrogate measure of the proliferation signature and has been shown to be prognostic in numerous studies, both alone and in combination with the MIPI.^{7,11-14} However, serious concerns have been raised regarding the analytic validity of the Ki-67 PI in lymphoma and other malignancies, particularly regarding interlaboratory and interobserver variability.¹⁵

Recently, technologies have been developed to reliably quantify gene expression in RNA from formalin-fixed paraffin-embedded (FFPE) tissue, allowing the development of clinically relevant, intermediate-density, gene expression–based assays.¹⁶⁻¹⁸ Herein, we describe the development, analytical validation, and evaluation of the clinical impact of a gene expression–based assay for measuring the proliferation signature in RNA derived from routinely available FFPE biopsies in MCL.

METHODS

Study Design and Patient Population

The overall design of the process for developing and characterizing the new assay for the proliferation signature in MCL is shown in the Data Supplement. The study involved retrospective gene expression profiling of samples from patients with MCL, confirmed by expert pathology consensus review. Biopsies contributing to the training of the new assay included 80 biopsies described in Rosenwald et al,¹⁰ along with an additional 51 biopsies gathered from the clinical sites of the Lymphoma/Leukemia Molecular Profiling Project. These biopsies, with tumor content $\geq 60\%$, were obtained from patients who subsequently received a broad range of treatment regimens.

The assay was validated using 110 pretreatment biopsies from an independent cohort of patients treated at the BC Cancer Agency (BCCA; Table 1; Fig 1). Patients diagnosed with MCL at the BCCA between 2003 and 2012 were identified using the BCCA Lymphoid Cancer Database. Inclusion in the validation cohort required a diagnostic excisional FFPE biopsy of a lymph node with tumor content $\geq 60\%$ and treatment with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) within 3 months of the diagnostic biopsy. Biopsies with a predominantly mantle zone involvement by lymphoma cells were excluded.

All biopsies were centrally reviewed to confirm a diagnosis of conventional MCL and were positive for cyclin D1 by IHC.¹ The BCCA policy

during this era was to treat MCL using the R-CHOP regimen with a planned consolidative autologous stem-cell transplantation (ASCT) for appropriate patients ≤ 65 years of age. A policy to provide maintenance rituximab (375 mg/m² intravenously every 3 months for 2 years) to patients who did not receive a consolidative ASCT was introduced in 2011. The study was approved by the University of British Columbia–BCCA Research Ethics Board.

Gene Expression Profiling

Gene expression profiling of RNA extracted from FF biopsies used in the training of the assay was performed on Affymetrix U133 plus 2.0 (Thermo Fisher Scientific, Waltham, MA) microarrays.¹⁹

Nucleic acids were extracted from 10- μ m sections of FFPE biopsies using the QIAGEN AllPrep DNA/RNA FFPE Kit (QIAGEN, Hilden, Germany) after deparaffinization according to the manufacturer's instructions. Gene expression was quantitated in 200 ng of RNA on the NanoString platform (NanoString Technologies, Seattle, WA), using the "high sensitivity" setting on the nCounter Prep Station and 490 fields of view on the nCounter analyzer (generation 2) or 1,155 fields of view when a generation 1 analyzer was used. Normalization for RNA loading was performed using the geometric mean of 18 housekeeping genes. Samples in which this geometric mean was > 10 -fold below the median were deemed to have failed. Probes to exon 3 and the 3' untranslated region (UTR) of *CCND1* were used to assess the status of the *CCND1* 3' UTR (Data Supplement).

IHC and the MIPI

Ki-67 IHC (MIB-1) was performed on whole-tissue sections on a Ventana BenchMark platform (Ventana Medical Systems, Tucson, AZ) and scored by counting 200 cells per biopsy, according to the recommendations of Klapper et al.²⁰ The Ki-67 PI was defined as the proportion of positive tumor cells. TP53 IHC (clone DO-7) was performed on tissue microarrays comprising duplicate 0.6-mm cores from FFPE blocks of the biopsies, with positivity defined as strong uniform nuclear staining of tumor cells; all positive biopsies had staining in $> 30\%$ of tumor cells. The MIPI was calculated per Hoster et al.⁷

Statistical Analysis

The statistical analysis plan was specified before the evaluation of gene expression from the validation cohort. Fisher's exact and Kruskal-Wallis exact tests were used to examine the significance of differences in patient and pathology characteristics between groups. The median follow-up was estimated using the reverse censoring method.²¹ The primary end point of the study was overall survival (OS), which was calculated from the date of diagnosis to date of death from any cause. OS was estimated using the Kaplan-Meier method. A planned subgroup analysis was performed, which was limited to patients for whom there was a per-policy intention-to-treat with a consolidative ASCT.

Univariable analyses using Cox models were implemented to examine the relationship between continuous variables and OS. Log-rank tests were used to test the relationship between discrete variables and OS. Cox proportional hazards regression model score tests were used to test the association of variables with OS in combination with other variables. It was prespecified that one-sided *P* values $< .05$ would be considered significant.

RESULTS

Development of the MCL35 Assay

The proliferation signature was originally described using gene expression defined on the basis of RNA derived from 92 FF tissue biopsies on custom Lymphochip microarrays.¹⁰ In a first step toward producing a new assay, gene expression analysis was performed on the 80 available samples from the original 92 FF

Table 1. Patient Demographic Data and Disease Characteristics

Variable	Total Cohort	MCL35 Categories			P*
		Low-Risk Group	Standard-Risk Group	High-Risk Group	
Patients					
Assessable patients	108	49 (45)	31 (29)	28 (26)	
Male	86 (80)	39 (80)	26 (84)	21 (75)	.75
Female	22 (20)	10 (20)	5 (16)	7 (25)	
Age in years, median (range)	62 (41-84)	60 (41-84)	64 (45-74)	68 (41-81)	.18
> 65	39 (36)	12 (24)	12 (39)	15 (54)	.04
Clinical features					
ECOG performance status					.20
0-1	75 (76)	39 (83)	20 (74)	16 (64)	
2-4	24 (24)	8 (17)	7 (26)	9 (36)	
Missing	9	2	4	3	
White cell count, median (range)	6.9 (1.7-79.2)	6.4 (2.7-12.7)	8.7 (1.7-41.4)	7.9 (2.3-79.2)	.02
LDH					.007
Normal	54 (57)	30 (68)	17 (63)	7 (29)	
> ULN	41 (43)	14 (32)	10 (27)	17 (71)	
Missing	13	5	4	4	
MIPI					.001
Low (< 5.7)	38 (41)	27 (61)	8 (31)	3 (13)	
Intermediate (5.7-6.2)	20 (22)	7 (16)	8 (31)	5 (22)	
High (≥ 6.2)	35 (38)	10 (23)	10 (38)	15 (65)	
Missing	15	5	5	5	
Pathology					
Morphology					< .001
Classic	95 (88)	49 (100)	29 (94)	17 (61)	
Pleomorphic	3 (3)	0	0	3 (11)	
Blastoid	10 (9)	0	2 (6)	8 (29)	
Ki-67 proliferation index (%)					< .001
< 30	53 (49)	45 (92)	6 (19)	2 (7)	
≥ 30	55 (51)	4 (8)	25 (81)	26 (93)	
TP53 immunohistochemistry					< .001
Negative	93 (87)	49 (100)	26 (87)	18 (64)	
Positive	14 (13)	0	4 (13)	10 (36)	
Fail	1	0	1	0	
CCND1 3' UTR					< .001
Wildtype	89 (82)	49 (100)	27 (87)	13 (46)	
Truncated	19 (18)	0	4 (13)	15 (54)	
Treatment					
R-CHOP	108 (100)	49 (100)	31 (100)	28 (100)	
Consolidative ASCT					.96†
Per-protocol intention-to-treat‡	58 (84)	31 (84)	17 (89)	10 (77)	
Received transplantation per protocol§	42 (72)	24 (77)	12 (71)	6 (60)	
Received transplantation outside protocol	1	0	1	0	
Median follow-up, months	78	98	68	75	

NOTE. All values are expressed as the number (%) unless indicated otherwise.

Abbreviations: ASCT, autologous stem-cell transplantation; ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase; MIPI, mantle cell lymphoma International Prognostic Index; R-CHOP, rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone; ULN, upper level of normal; UTR, untranslated region.

*P values are for comparisons across the three risk groups determined by the MCL35 score.

†Comparison (across groups) of the number of patients who received an ASCT with the number of patients for whom there was an intention to consolidate with an ASCT.

‡Percentage of patients ≤ 65 years of age.

§Percentage of patients for whom there was an intention to consolidate with an ASCT.

RNA samples using Affymetrix U133 plus 2.0 microarrays because these arrays provide broader coverage of the coding genome. Comparison of the correlation of expression of individual genes and the proliferation signature with the relationship between gene expression and overall survival, expressed as the Z-score from univariable Cox models, is shown in Figure 2. The strong association observed ($r^2 = 0.82$) suggests that the proliferation signature encompasses much of the prognostic information present in gene expression in MCL.

Furthermore, whereas the original proliferation signature solely contained genes that were overexpressed in biopsies with a high proliferation score, it is evident that a number of genes are underexpressed in these biopsies, allowing the design of a “balanced” gene expression model. Sixty-nine genes of interest, along with 30 potential housekeeping genes, were selected for further assay development, on the basis of this analysis and other published studies that have described the relationship between gene expression and outcomes in MCL^{22,23} (Data Supplement).

Fig 1. Patient flow for the validation cohort. B+R, bendamustine plus rituximab; CLB, chlorambucil; MCL: mantle cell lymphoma; R, rituximab; R-CHOP, rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone; R+CP, rituximab plus cyclophosphamide and prednisone; R+CVP, rituximab plus cyclophosphamide, vincristine, and prednisone.

Digital gene expression was performed to quantitate these 99 genes in RNA extracted from 47 FFPE biopsies, including all 39 suitable biopsies with matched Affymetrix gene expression data on RNA from FF biopsies. Seventeen genes were selected to replicate the proliferation signature based on the following criteria: being highly correlated across the NanoString (FFPE) and Affymetrix (FF) platforms, being moderately to highly expressed on the NanoString platform, and having high variance across the samples. Eighteen housekeeping genes were also selected on the basis of

Fig 2. Gene expression data in the training cohort. The correlation of the expression of individual genes to the proliferation signature calculated in Rosenwald et al¹⁰ plotted against the Wald test Z-score for overall survival (OS) for that gene. The data are from gene expression profiling of 80 fresh frozen biopsies from Rosenwald et al¹⁰ using Affymetrix U133 plus 2.0 microarrays. Gold and gray dots represent genes that were included in the NanoString gene set, which was used to select genes to replicate the proliferation signature. Gray dots represent genes that were selected for the MCL35 assay.

having low variance across the samples and moderate to high expression levels. Digital gene expression was then performed on the same 47 FFPE RNA samples using a smaller code set containing these 35 genes.

After normalization with the 18 housekeeping genes, a model was developed using expression of the 17 proliferation genes to replicate the proliferation signature score described by Rosenwald et al¹⁰ (Fig 3A). Optimal thresholds for defining three groups with distinct outcomes (ie, OS) were determined using Affymetrix data from 123 FF biopsies, including the 80 biopsies from Rosenwald et al¹⁰ (Fig 3B). The final model, named the MCL35 assay, including the gene coefficients and thresholds, was then locked and validated in an independent cohort of patients. Details of the model building are presented in the Data Supplement.

MCL35 Assay Is Prognostic in Patients Treated With R-CHOP

The MCL35 assay was then applied to pretreatment FFPE lymph node biopsies from 110 patients treated with R-CHOP, with or without ASCT, at the BCCA (Table 1; Fig 1). Adequate gene expression was obtained in 108 (98%) of the biopsies. As a continuous variable, the MCL35 score was significantly associated with OS (univariate $P < .001$; Harrell's C-index, 0.74 [95% CI, 0.66 to 0.82]). The assay assigned 28 (26%) patients to the high-risk group, 31 (29%) to the standard-risk group, and 49 (45%) to the low-risk group (Fig 4A). The outcomes were significantly different among these three groups, with median OS of 1.1, 2.6, and 8.6 years in the high-, standard-, and low-risk groups, respectively, (log-rank for trend, $P < .001$; Fig 4B).

Recognized high-risk MCL features were more frequently encountered in the high-risk group, including morphologic characteristics (pleomorphic and blastoid variants^{24,25}), TP53

Fig 3. The gene expression-based model for the proliferation signature in the training cohort. (A) The MCL35 assay is shown in the form of a heat map, with the 17 informative genes shown as rows and the 47 patient biopsies shown as columns. The three patient groups identified by the assay are shown below the heat map. (B) Kaplan-Meier curves of the overall survival (OS) of the three patient groups identified by the MCL35 assay. Outcome data were available for 44 of the 47 patients.

positivity by IHC,²⁶ and the presence of *CCND1* mRNA with truncated 3' UTRs¹⁰ (Table 1; Fig 4A; Data Supplement). In a planned subgroup analysis, the assay also defined groups with significantly different OS in patients ≤ 65 years of age for whom there was intention-to-treat with R-CHOP followed by a consolidative ASCT. In this group, the median OS was 1.4 years, 5.9 years, and not reached in the high-, standard-, and low-risk groups, respectively, (log-rank for trend, $P < .001$; Fig 4C).

The MIPI also identified groups of patients with significantly different OS in the total validation cohort (log-rank for trend, $P < .001$; Harrell's C-index, 0.74 [95% CI, 0.66 to 0.82]). In multivariable analyses, both the MCL35 and the MIPI independently contributed to OS ($P < .001$ for both variables) whether the variables were continuous or grouped (Data Supplement).

There was a significant positive correlation between the Ki-67 PI and the MCL35 score ($r^2 = 0.72$; Data Supplement). As a continuous variable, the Ki-67 PI was significantly associated with OS (univariable $P < .001$; Harrell's C-index, 0.69 [95% CI, 0.61 to

0.77]). Applying previously published thresholds,¹⁴ 55 (50%) of the biopsies had a Ki-67 PI $\geq 30\%$, 38 (35%) had a Ki-67 PI of 10% to 29%, and 17 (15%) had a Ki-67 PI $< 10\%$. A Ki-67 PI $\geq 30\%$ was associated with inferior OS (median, 2.2 years; log-rank ν Ki-67 PI 10% to 29%, $P < .001$), whereas the lengths of OS when the Ki-67 PI was 10% to 29% and $< 10\%$ were not significantly different from one another (median, 6 and 7.2 years, respectively; log-rank $P = .75$; Data Supplement). In multivariable Cox models, the Ki-67 PI ($P = .36$) did not contribute prognostically when adjusted for the MCL35 assay results, whereas the MCL35 did contribute ($P < .001$) when adjusted for the Ki-67 PI, whether the variables were continuous or grouped (Ki-67 PI groups: 0% to 29% and $\geq 30\%$; Data Supplement).

Analytic Validity of the MCL35 Assay

Experiments were then performed to determine the intra- and interlaboratory reproducibility of the MCL35 assay. Seventeen biopsies were selected on the basis that the MCL35 scores were

Fig 4. The gene expression-based model for the proliferation signature in the validation cohort. (A) The MCL35 assay is shown in the form of a heat map, with the 17 informative genes shown as rows and the 108 patient biopsies shown as columns. The three patient groups identified by the assay are shown below the heat map. Shown below are the Ki-67 proliferation index (PI), pathologic characteristics, and the mantle cell lymphoma International Prognostic Index (MIPI). (B) Kaplan-Meier curves of the overall survival (OS) of the three patient groups in the validation cohort identified by the MCL35 assay. Hazard ratios (HR) are reported with the standard-risk group used as the reference. (C) Kaplan-Meier curves of the overall survival of the three patient groups within the subgroup of patients for whom there was an intention to consolidate response with an autologous stem-cell transplantation (ASCT). HRs are reported with the standard-risk group used as the reference. IHC, immunohistochemistry; UTR, untranslated region.

equally distributed across the population (Fig 5A) and thus representative of the distribution of MCL35 scores in the validation cohort. For intralaboratory comparison, the RNA from each of these biopsies was run on the MCL35 assay in triplicate, with each run performed on a different aliquot of RNA and on different NanoString cartridges. The results showed 100% concordance of risk group assignment (Fig 5B) across the triplicates.

One outlier result was observed, where the gene expression was disparate from the other replicates. This outlier result was

removed from further analyses. The standard deviation of the intralaboratory error was four points, compared with a range of scores across the validation cohort of 586 points. For inter-laboratory comparison, scrolls of tissue from the 17 biopsies were distributed to two independent laboratories in Barcelona, Spain and Würzburg, Germany, where RNA was extracted and run on the MCL35 assay (Fig 5C). There was 100% concordance of risk group assignment and no significant bias was seen compared with the mean of the triplicate results from the laboratory in Vancouver,

Fig 5. Studies of the analytic validity of the MCL35 assay. (A) MCL35 scores are shown in ascending order, left to right, in the validation cohort. Gold dots represent the scores of the 17 biopsies (equally spread across the spectrum of scores) selected for the analytic validation studies. Blue dots represent the scores of the biopsies not selected. (B) MCL35 scores of RNA from the 17 biopsies identified in (A) run in triplicate (y-axis) plotted against the average of the three scores (continued on next page)

British Columbia, Canada (95% CIs of bias: Barcelona, -6.1 to 0.6 ; Würzburg, -3.7 to 3.0 points).

The standard deviation of the interlaboratory error was three points, giving a standard deviation of the total (intra- plus interlaboratory) error of five points. Given that the examination of a small number of samples provides an imprecise estimate of concordance over a population, the distribution of the MCL35 score in this study and the calculated distributions of error were used to estimate concordance of risk group assignment between laboratories over a large population (Data Supplement). This model estimated that 1.2% of biopsies would change risk group assignment between laboratories. The Data Supplement contains these analyses if the outlier result was retained.

Finally, to determine the lower limit of RNA input for the MCL35 assay, RNA from the same 17 biopsies was run on the assay with input of 100 ng, 50 ng (in duplicate), and 25 ng (Figs 5D–F). No significant bias was observed at 100 and 50 ng compared with the mean of the triplicates at 200 ng. However, at 25 ng, there was a consistent trend toward higher MCL35 scores.

DISCUSSION

To our knowledge, this is the first description of a molecular assay that translates the research-derived proliferation signature in MCL into a test applicable to routinely available FFPE biopsies. The clinical validity of the MCL35 assay, identifying patient groups at significantly different risk of death, was demonstrated in an independent cohort of uniformly treated patients. The assay was demonstrated to be a powerful prognostic biomarker in patients treated with R-CHOP, identifying sizeable groups of patients with dismal or excellent outcomes. Furthermore, the prognostic power of the assay was maintained in younger patients for whom there was a plan to consolidate with an ASCT.

Similar to the original proliferation signature, the assay summates established high-risk disease features, including blastoid and pleomorphic morphology, TP53 overexpression, and truncation of the 3' UTR of *CCND1* mRNA transcripts. In addition, the prognostic power of the assay was independent of the MIPI.

This initial study was restricted to lymph node biopsies with a tumor content $\geq 60\%$, which encompasses the vast majority of patients with conventional MCL. Further studies are required to establish the clinical validity of the assay in biopsies that have low tumor content or are from extranodal sites. Similarly, this study exclusively used biopsies fixed in formalin, which is the methodology used by the vast majority of clinical laboratories. Further study would be required to determine whether the performance of the assay is affected by alternative fixation methodologies.

Proliferation of MCL cells in peripheral blood is typically, but not universally, lower than in matched lymph-node infiltrates; this effect is thought to reflect activation of the NF- κ B pathway in the

malignant cells by the tumor microenvironment, which dissipates upon exit from the lymph node.²⁷ This inconsistent relationship of proliferation between different tumor compartments would require alteration of the assay parameters and may affect the clinical validity of the MCL35 assay in peripheral blood samples. Similarly, it is also not known whether the assay will have clinical validity in the rare leukemic non-nodal subtype of the disease.

The analytic validity of the assay was demonstrated by examining both intra- and interlaboratory variability, showing a low estimated 1.2% rate of discordance across laboratories. This reproducibility sharply contrasts with the published literature regarding the Ki-67 PI as a surrogate marker for the proliferation signature, which has high interlaboratory and interobserver variability in lymphoma.^{15,20} This study was not designed or powered to directly compare the clinical validity of the new assay with this surrogate marker, but the MCL35 assay subsumed the prognostic power of the Ki-67 PI in pairwise multivariable analyses. Finally, the demonstration that there is no appreciable bias with RNA loading down to 50 ng will allow the assay to be applied to the majority of tissue biopsies, including core needle biopsies.

Clinical utility, as defined by improving patient outcomes, relies on the ability of the biomarker to guide clinical management. It is appreciated that the design of this study does not establish the assay as a predictive biomarker because it was tested in a homogeneously treated population. To establish the MCL35 assay as a predictive biomarker, it will need to be applied to prospectively collected samples from clinical trials testing the efficacy of modern treatment regimens. The recognition of highly variable treatment outcomes in this disease, along with the increasing range of efficacious treatment options, makes risk-stratified approaches attractive whereby toxic and/or expensive therapies are provided to patients in whom the most benefit will be accrued.²⁸

In conclusion, the newly developed and validated MCL35 assay for FFPE biopsies uses the proliferation signature to define groups of patients with significantly different OS independent of the MIPI. The analytic and clinical validity of this assay make it the ideal candidate to support future trials of risk-adapted therapeutic strategies.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Disclosures provided by the authors are available with this article at jco.org.

AUTHOR CONTRIBUTIONS

Conception and design: David W. Scott, Pau Abrisqueta, George W. Wright, Randy D. Gascoyne, Andreas Rosenwald, Louis M. Staudt, Elias Campo, Lisa M. Rimsza
Financial support: Elias Campo, Lisa M. Rimsza
Administrative support: Joseph M. Connors

(continued) (x-axis). The gold dot represents an outlier score. (C) MCL35 scores of RNA from the 17 biopsies that were extracted and run at two independent laboratories in Würzburg, Germany and Barcelona, Spain (y-axis) plotted against the original MCL35 score (x-axis) generated at the BC Cancer Agency, Vancouver, BC, Canada. Calculation of the MCL35 scores was performed centrally. (D) MCL35 scores from 100 ng of RNA from the 17 biopsies (y-axis) plotted against the score when 200 ng was loaded. The solid line represents the line-of-best-fit. (E) MCL35 scores from 50 ng of RNA run in duplicate from the 17 biopsies (y-axis) plotted against the score when 200 ng was loaded. The solid line represents the line-of-best-fit. (F) MCL35 scores from 25 ng of RNA from the 17 biopsies (y-axis) plotted against the score when 200 ng was loaded. The solid line represents the line-of-best-fit.

Provision of study materials or patients: Graham W. Slack, Rita M. Braziel, Wing C. Chan, Dennis D. Weisenburger, James R. Cook, Timothy C. Greiner, Kai Fu, German Ott, Jan Delabie, Erlend B. Smeland, Harald Holte, Elaine S. Jaffe, Joseph M. Connors, Randy D. Gascoyne, Andreas Rosenwald, Elias Campo, Lisa M. Rimsza
Collection and assembly of data: David W. Scott, Pau Abrisqueta, George W. Wright, Graham W. Slack, Anja Mottok, Diego Villa, Pedro Jares, Hilka Rauert-Wunderlich, Cristina Royo, Magda Pinyol, Merrill Boyle, Rita M. Braziel, Wing C. Chan, Dennis D. Weisenburger, James R. Cook, Timothy C. Greiner, Kai Fu, German Ott, Jan Delabie, Erlend B. Smeland, Harald Holte, Elaine S. Jaffe, Christian Steidl, Joseph M.

Connors, Randy D. Gascoyne, Andreas Rosenwald, Elias Campo, Lisa M. Rimsza
Data analysis and interpretation: David W. Scott, Pau Abrisqueta, George W. Wright, Graham W. Slack, Anja Mottok, Diego Villa, Guillem Clot, Fong Chun Chan, Rita M. Braziel, Wing C. Chan, Dennis D. Weisenburger, James R. Cook, Timothy C. Greiner, Kai Fu, German Ott, Jan Delabie, Erlend B. Smeland, Elaine S. Jaffe, Christian Steidl, Joseph M. Connors, Randy D. Gascoyne, Andreas Rosenwald, Elias Campo, Lisa M. Rimsza
Manuscript writing: All authors
Final approval of manuscript: All authors
Accountable for all aspects of the work: All authors

REFERENCES

1. Swerdlow SH, Campo E, Harris NL, et al. World Health Organization Classification of Tumours of Haematopoietic and Lymphoid Tissues, 4th Ed. IARC Press, Lyon. 2008.
2. Jares P, Colomer D, Campo E: Molecular pathogenesis of mantle cell lymphoma. *J Clin Invest* 122:3416-3423, 2012
3. Orchard J, Garand R, Davis Z, et al: A subset of t(11;14) lymphoma with mantle cell features displays mutated IgVH genes and includes patients with good prognosis, nonnodal disease. *Blood* 101:4975-4981, 2003
4. Fernández V, Salameo O, Espinet B, et al: Genomic and gene expression profiling defines indolent forms of mantle cell lymphoma. *Cancer Res* 70:1408-1418, 2010
5. Swerdlow SH, Campo E, Pileri SA, et al: The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood* 127:2375-2390, 2016
6. Cheah CY, Seymour JF, Wang ML: Mantle cell lymphoma. *J Clin Oncol* 34:1256-1269, 2016
7. Hoster E, Dreyling M, Klapper W, et al: A new prognostic index (MIPI) for patients with advanced-stage mantle cell lymphoma. *Blood* 111:558-565, 2008
8. Hoster E, Klapper W, Hermine O, et al: Confirmation of the mantle-cell lymphoma International Prognostic Index in randomized trials of the European Mantle-Cell Lymphoma Network. *J Clin Oncol* 32:1338-1346, 2014
9. Geisler CH, Kolstad A, Laurell A, et al: Nordic MCL2 trial update: Six-year follow-up after intensive immunochemotherapy for untreated mantle cell lymphoma followed by BEAM or BEAC + autologous stem-cell support: Still very long survival but late relapses do occur. *Br J Haematol* 158:355-362, 2012
10. Rosenwald A, Wright G, Wiestner A, et al: The proliferation gene expression signature is a quantitative integrator of oncogenic events that predicts survival in mantle cell lymphoma. *Cancer Cell* 3:185-197, 2003
11. Tiemann M, Schrader C, Klapper W, et al: Histopathology, cell proliferation indices and clinical outcome in 304 patients with mantle cell lymphoma (MCL): A clinicopathological study from the European MCL Network. *Br J Haematol* 131:29-38, 2005
12. Katzenberger T, Petzoldt C, Höller S, et al: The Ki67 proliferation index is a quantitative indicator of clinical risk in mantle cell lymphoma. *Blood* 107:3407, 2006
13. Hoster E, Rosenwald A, Berger F, et al: Prognostic value of Ki-67 index, cytology, and growth pattern in mantle-cell lymphoma: Results from randomized trials of the European Mantle Cell Lymphoma Network. *J Clin Oncol* 34:1386-1394, 2016
14. Determann O, Hoster E, Ott G, et al: Ki-67 predicts outcome in advanced-stage mantle cell lymphoma patients treated with anti-CD20 immunochemotherapy: Results from randomized trials of the European MCL Network and the German Low Grade Lymphoma Study Group. *Blood* 111:2385-2387, 2008
15. de Jong D, Rosenwald A, Chhanabhai M, et al: Immunohistochemical prognostic markers in diffuse large B-cell lymphoma: Validation of tissue microarray as a prerequisite for broad clinical applications—A study from the Lunenburg Lymphoma Biomarker Consortium. *J Clin Oncol* 25:805-812, 2007
16. Geiss GK, Bumgarner RE, Birditt B, et al: Direct multiplexed measurement of gene expression with color-coded probe pairs. *Nat Biotechnol* 26:317-325, 2008
17. Filipits M, Nielsen TO, Rudas M, et al: The PAM50 risk-of-recurrence score predicts risk for late distant recurrence after endocrine therapy in postmenopausal women with endocrine-responsive early breast cancer. *Clin Cancer Res* 20:1298-1305, 2014
18. Scott DW, Wright GW, Williams PM, et al: Determining cell-of-origin subtypes of diffuse large B-cell lymphoma using gene expression in formalin-fixed paraffin-embedded tissue. *Blood* 123:1214-1217, 2014
19. National Center for Biotechnology Information: Gene Expression Omnibus, GEO Accession: GSE93291. <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi>
20. Klapper W, Hoster E, Determann O, et al: Ki-67 as a prognostic marker in mantle cell lymphoma—Consensus guidelines of the pathology panel of the European MCL Network. *J Hematop* 2:103-111, 2009
21. Schemper M, Smith TL: A note on quantifying follow-up in studies of failure time. *Control Clin Trials* 17:343-346, 1996
22. Kienle D, Katzenberger T, Ott G, et al: Quantitative gene expression deregulation in mantle-cell lymphoma: Correlation with clinical and biologic factors. *J Clin Oncol* 25:2770-2777, 2007
23. Hartmann E, Fernández V, Moreno V, et al: Five-gene model to predict survival in mantle-cell lymphoma using frozen or formalin-fixed, paraffin-embedded tissue. *J Clin Oncol* 26:4966-4972, 2008
24. Lardelli P, Bookman MA, Sundeen J, et al: Lymphocytic lymphoma of intermediate differentiation. Morphologic and immunophenotypic spectrum and clinical correlations. *Am J Surg Pathol* 14:752-763, 1990
25. Argatoff LH, Connors JM, Klasa RJ, et al: Mantle cell lymphoma: A clinicopathologic study of 80 cases. *Blood* 89:2067-2078, 1997
26. Nordström L, Sembo S, Eden P, et al: SOX11 and TP53 add prognostic information to MIPI in a homogeneously treated cohort of mantle cell lymphoma—A Nordic Lymphoma Group study. *Br J Haematol* 166:98-108, 2014
27. Saba NS, Liu D, Herman SEM, et al: Pathogenic role of B-cell receptor signaling and canonical NF- κ B activation in mantle cell lymphoma. *Blood* 128:82-92, 2016
28. Dreyling M, Ferrero S, Vogt N, et al: New paradigms in mantle cell lymphoma: Is it time to risk-stratify treatment based on the proliferative signature? *Clin Cancer Res* 20:5194-5206, 2014

Affiliations

David W. Scott, Pau Abrisqueta, Graham W. Slack, Anja Mottok, Diego Villa, Merrill Boyle, Fong Chun Chan, Christian Steidl, Joseph M. Connors, and Randy D. Gascoyne, BC Cancer Agency; Anja Mottok, Christian Steidl, and Randy D. Gascoyne, University of British Columbia, Vancouver, British Columbia; Jan Delabie, University of Toronto, Toronto, Ontario, Canada; Pau Abrisqueta, Vall d'Hebron University Hospital; Pedro Jares, Cristina Royo, Guillem Clot, Magda Pinyol, and Elias Campo, Universitat de Barcelona, Institut d'Investigacions Biomèdiques August Pi i Sunyer, Barcelona, Spain; George W. Wright, Elaine S. Jaffe, and Louis M. Staudt, National Institutes of Health, Bethesda, MD; Hilka Rauert-Wunderlich and Andreas Rosenwald, University of Würzburg, Würzburg; German Ott, Robert Bosch Hospital and Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart, Germany; Rita M. Braziel, Oregon Health & Sciences University, Portland, OR; Wing C. Chan and Dennis D. Weisenburger, City of Hope, Duarte, CA; James R. Cook, Pathology and Laboratory Medicine Institute, Cleveland Clinic, Cleveland, OH; Timothy C. Greiner and Kai Fu,

University of Nebraska Medical Center, Omaha, NE; **Erlend B. Smeland** and **Harald Holte**, Oslo University Hospital, Oslo, Norway; and **Lisa M. Rimsza**, Mayo Clinic, Phoenix, AZ.

Support

Supported by a National Cancer Institute Strategic Partnering to Evaluate Cancer Signatures (SPECS II) grant (5U01CA157581-05) and in part by the Leukemia and Lymphoma Society of Canada and the Terry Fox Research Institute (Grant No. 1061). The BC Cancer Agency Centre for Lymphoid Cancer clinical database is supported by Roche. The research at the City of Hope was supported in part by the National Cancer Institute (award No. P30CA033572). The research at the University of Nebraska Medical Center was supported by the Fred & Pamela Buffett Cancer Center's National Cancer Institute Cancer Center Support Grant (P30CA036727). Research at Oslo University Hospital was supported by The Research Council of Norway's Centres of Excellence Scheme Research Council (Project No. 179571). D.W.S. is supported by the BC Cancer Agency. P.A.'s fellowship at the BC Cancer Agency was supported by funds from Vall d'Hebron University Hospital, Barcelona, Spain. G.W.W. is supported by the Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, MD. A.M. is supported by fellowship awards from the Mildred-Scheel-Cancer Foundation, the Michael Smith Foundation for Health Research, and Lymphoma Canada. G.O. is supported by the Robert-Bosch-Foundation, Stuttgart, Germany. H.H. is supported by the Norwegian Cancer Society. E.S.J. and L.M.S. are supported by the Center for Cancer Research, National Cancer Institute, Bethesda, MD. C.S. is the recipient of a Career Investigator Award from the Michael Smith Foundation for Health Research. J.M.C. received research funding support from the Terry Fox Research Institute, Genome Canada, Genome British Columbia, the Canadian Institutes for Health Research, and the BC Cancer Foundation. E.C. was supported in part by the Ministerio de Economía y Competitividad Grant No. SAF2015-64885-R, Generalitat de Catalunya Suport Grups de Recerca 2014-SGR-795, and Institució Catalana de Recerca i Estudis Avançats. L.M.R.'s laboratory infrastructure and tissue banking are supported by awards from the National Cancer Institute Specialized Program in Research Excellence (5P50CA097274) and the Mayo Comprehensive Cancer Center (5P30CA015083).

Prior Presentation

Presented in part as an oral abstract at the Annual Meeting of the American Society of Clinical Oncology, Chicago, IL, June 3-7, 2016.

Participate in ASCO's Practice Guidelines Implementation Network and Influence Cancer Care

ASCO members are invited to serve in the society's Practice Guidelines Implementation Network (PGIN), a network of oncology professionals who raise awareness of ASCO's evidence-based recommendations on cancer care.

Participation in PGIN provides an opportunity for members to positively influence the way that clinical oncology is delivered now and in the future. PGIN members have the opportunity to:

- Participate in guideline panels and advisory groups
- Aid in developing and reviewing guidelines and guideline clinical tools and resources
- Serve as an "ambassador" to state societies
- Better implement guidelines

To learn how you can participate, visit asco.org/guidelines, or contact PGIN@asco.org.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

New Molecular Assay for the Proliferation Signature in Mantle Cell Lymphoma Applicable to Formalin-Fixed Paraffin-Embedded Biopsies

The following represents disclosure information provided by authors of this manuscript. All relationships are considered compensated. Relationships are self-held unless noted. I = Immediate Family Member, Inst = My Institution. Relationships may not relate to the subject matter of this manuscript. For more information about ASCO's conflict of interest policy, please refer to www.asco.org/rwc or ascopubs.org/jco/site/ifu.

David W. Scott

Honoraria: Roche

Consulting or Advisory Role: Celgene, Janssen

Patents, Royalties, Other Intellectual Property: Named inventor on a pending patent describing gene expression profiling in prognostication in classical Hodgkin lymphoma (Inst); named inventor on a patent: "Methods for selecting and treating lymphoma types" licensed to NanoString Technologies (Inst); named inventor on a patent: "Evaluation of mantle cell lymphoma and methods related thereof" (Inst).

Pau Abrisqueta

Honoraria: Janssen, Roche

Consulting or Advisory Role: Roche, Janssen, Gilead

Patents, Royalties, Other Intellectual Property: Named inventor on a patent: "Evaluation of mantle cell lymphoma and methods related thereof."

Travel, Accommodations, Expenses: Janssen

George W. Wright

Patents, Royalties, Other Intellectual Property: Named inventor on a patent: "Methods for selecting and treating lymphoma types"; named inventor on a patent: "Evaluation of mantle cell lymphoma and methods related thereof."

Graham W. Slack

Consulting or Advisory Role: Seattle Genetics

Patents, Royalties, Other Intellectual Property: Named inventor on a patent: "Evaluation of mantle cell lymphoma and methods related thereof."

Anja Mottok

Patents, Royalties, Other Intellectual Property: Named inventor on a patent: "Evaluation of mantle cell lymphoma and methods related thereof" (Inst).

Diego Villa

Honoraria: Roche, Lundbeck, Seattle Genetics, Celgene, Amgen

Consulting or Advisory Role: Roche, Lundbeck, Seattle Genetics, Celgene, Amgen

Research Funding: Roche; research funding to support the Centre for Lymphoid Cancer Database (Inst)

Patents, Royalties, Other Intellectual Property: Named inventor on a patent: "Evaluation of mantle cell lymphoma and methods related thereof."

Travel, Accommodations, Expenses: Lundbeck, Roche, AbbVie

Pedro Jares

Patents, Royalties, Other Intellectual Property: Named inventor on a patent: "Evaluation of mantle cell lymphoma and methods related thereof."

Hilka Rauert-Wunderlich

No relationship to disclose

Cristina Royo

No relationship to disclose

Guillem Clot

No relationship to disclose

Magda Pinyol

No relationship to disclose

Merrill Boyle

No relationship to disclose

Fong Chun Chan

No relationship to disclose

Rita M. Brazil

Patents, Royalties, Other Intellectual Property: Named inventor on a patent: "Evaluation of mantle cell lymphoma and methods related thereof"; named inventor on a patent: "Methods for selecting and treating lymphoma types" licensed to NanoString Technologies.

Wing C. Chan

Patents, Royalties, Other Intellectual Property: Named inventor on a patent: "Evaluation of mantle cell lymphoma and methods related thereof"; named inventor on a patent: "Methods for selecting and treating lymphoma types" licensed to NanoString Technologies.

Dennis D. Weisenburger

Honoraria: Seattle Genetics

Consulting or Advisory Role: Seattle Genetics

Speakers' Bureau: Seattle Genetics

Patents, Royalties, Other Intellectual Property: Named inventor on a patent: "Evaluation of mantle cell lymphoma and methods related thereof"; named inventor on a patent: "Methods for selecting and treating lymphoma types" licensed to NanoString Technologies.

Travel, Accommodations, Expenses: Seattle Genetics

James R. Cook

Patents, Royalties, Other Intellectual Property: US Provisional Patent Application No. 61/900,553, filed November 6, 2013. Title: Methods for selecting and treating lymphoma types. Role: Co-Inventor. Named inventor on a patent: "Evaluation of mantle cell lymphoma and methods related thereof."

Timothy C. Greiner

Patents, Royalties, Other Intellectual Property: Named inventor on a patent: "Methods for selecting and treating lymphoma types" licensed to NanoString Technologies; named inventor on a patent: "Evaluation of mantle cell lymphoma and methods related thereof."

Kai Fu

Patents, Royalties, Other Intellectual Property: Named inventor on a patent: "Methods for selecting and treating lymphoma types" licensed to NanoString Technologies; named inventor on a patent: "Evaluation of mantle cell lymphoma and methods related thereof"; named inventor on a patent: "Methods for selecting and treating lymphoma types" licensed to NanoString Technologies (Inst); named inventor on a patent: "Evaluation of mantle cell lymphoma and methods related thereof" (Inst).

German Ott

Patents, Royalties, Other Intellectual Property: Named inventor on a patent: "Methods for selecting and treating lymphoma types" licensed to NanoString Technologies; named inventor on a patent: "Evaluation of mantle cell lymphoma and methods related thereof."

Jan Delabie

Patents, Royalties, Other Intellectual Property: Named inventor on a patent: "Evaluation of mantle cell lymphoma and methods related thereof"; named inventor on a patent: "Methods for selecting and treating lymphoma types" licensed to NanoString Technologies.

Erlend B. Smeland

Stock or Other Ownership: Nordic Nanovector

Patents, Royalties, Other Intellectual Property: Named inventor on a patent: “Methods for selecting and treating lymphoma types” licensed to NanoString Technologies; named inventor on a patent: “Evaluation of mantle cell lymphoma and methods related thereof.”

Harald Holte

No relationship to disclose

Elaine S. Jaffe

Patents, Royalties, Other Intellectual Property: Named inventor on a patent: “Methods for selecting and treating lymphoma types” licensed to NanoString Technologies; named inventor on a patent: “Evaluation of mantle cell lymphoma and methods related thereof.”

Christian Steidl

Consulting or Advisory Role: Affimed Therapeutics

Expert Testimony: Juno Therapeutics

Joseph M. Connors

Research Funding: Seattle Genetics (Inst), Roche (Inst), Millennium (Inst), Bristol-Myers Squibb (Inst)

Patents, Royalties, Other Intellectual Property: “Evaluation of mantle cell lymphoma and methods related thereof” (Inst); “Methods for selecting and treating lymphoma types” (Inst).

Randy D. Gascoyne

Honoraria: Seattle Genetics, Celgene

Consulting or Advisory Role: Celgene, Seattle Genetics

Speakers’ Bureau: Seattle Genetics

Patents, Royalties, Other Intellectual Property: Named inventor on a patent: “Evaluation of mantle cell lymphoma and methods related thereof”; named inventor on a patent: “Methods for selecting and treating lymphoma types” licensed to NanoString Technologies.

Andreas Rosenwald

Consulting or Advisory Role: Roche, Celgene, MorphoSys, Janssen Pharmaceuticals

Patents, Royalties, Other Intellectual Property: Named inventor on a patent: “Methods for selecting and treating lymphoma types” licensed to NanoString Technologies; named inventor on a patent: “Evaluation of mantle cell lymphoma and methods related thereof.”

Travel, Accommodations, Expenses: Roche, Janssen Pharmaceuticals

Louis M. Staudt

Patents, Royalties, Other Intellectual Property: Named inventor on a patent: “Evaluation of mantle cell lymphoma and methods related thereof.”

Elias Campo

Consulting or Advisory Role: Bayer, Gilead

Research Funding: Gilead

Patents, Royalties, Other Intellectual Property: Named inventor on a patent: “Methods for selecting and treating lymphoma types” licensed to NanoString Technologies; named inventor on a patent: “Evaluation of mantle cell lymphoma and methods related thereof.”

Expert Testimony: Gilead

Travel, Accommodations, Expenses: Celgene

Lisa M. Rimsza

Consulting or Advisory Role: Celgene

Patents, Royalties, Other Intellectual Property: Filed with the National Cancer Institute.