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The MCL35 gene expression proliferation assay predicts high-risk MCL patients in a Norwegian cohort of younger patients given intensive first line therapy

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Summary

Mantle cell lymphomas have generally a dismal prognosis. Intensified induction treatment with rituximab and high dose cytarabine, and consolidation with high-dose therapy with autologous stem cell support has resulted in 10 year overall survival (OS) higher than 60%. However, the clinical course varies. Diagnostic tools capable of stratifying patients include the Mantle Cell Lymphoma International Prognostic Index (MIPI), gene expression-based proliferation signature,

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Conflict of interest statement: Andreas Rosenwald, Lisa M. Rimsza, Erlend B Smeland and David W. Scott are named inventors on two patents filed by the National Cancer Institute: “Methods for selecting and treating lymphoma types” licensed to NanoString Technologies, and “Evaluation of mantle cell lymphoma and methods related thereof.” Harald Holte is named inventor on one patent filed by the National Cancer Institute: “Methods for selecting and treating lymphoma types” licensed to NanoString Technologies.

Ki-67 proliferation index or tumor cell morphology. Here, we tested the performance of a newly developed Nanostring-based RNA expression **-based proliferation** assay (MCL35) on formalin-fixed paraffin-embedded tumor tissue from younger patients recruited in or treated according to Nordic MCL protocols compared to the prognosticators listed above. Seventy-four patients were included and the assay performed well in all cases except four with inadequate RNA quality. The patients were evenly distributed in the MCL35 low-, intermediate- and high risk categories. MCL35 low- and intermediate- risk groups had overlapping progression-free survival, while patients in the high-risk category had significantly inferior progression-free survival. **Combining MCL35 with MIPI or MIPI-C (MIPI with the additional score for Ki67 score if ≥30%) showed a better discrimination than either assessment alone**. In conclusion, the MCL35 assay alone or combined with MIPI or MIPI – C scores can identify patients **who still have a dismal outcome despite intensified treatment**.

Keywords

Mantle cell lymphoma; gene expression; prediction; therapy; FFPE

Introduction

Mantle cell lymphoma (MCL) is an aggressive disease with poor overall survival (OS). Although new intensive treatment regimens have improved survival, there is no indication that the disease can be cured with standard treatments (Dreyling *et al*, 2014, Geisler *et al*, 2012, Kolstad *et al*, 2014, Abrahamsson *et al*, 2014, Kluin-Nelemans *et al*, 2012). The outcome is variable, but clinical factors can discern between prognostic groups based on age, performance status, lactate dehydrogenase (LDH), and leukocyte count, when combined as the Mantle Cell lymphoma International Prognostic Index (MIPI) (Hoster *et al*, 2008).

Microarray RNA expression analyses have expanded the diagnostic accuracy in malignant lymphomas and have identified a proliferation signature as a strong predictor of survival in MCL (Rosenwald *et al*, 2003). These analyses were pioneered from the National Cancer Institute and the Leukemia and Lymphoma Molecular Profiling Project (LLMPP). However, gene expression analyses were performed on fresh frozen tissue which is not readily available from the majority of patients. To translate these findings to clinical practice, tumor cell proliferation index, based on Ki-67 staining by immunohistochemistry (IHC) has been tested and recognized as a strong adverse prognostic factor (Hoster *et al*, 2014, Hoster *et al*, 2016, Katzenberger *et al*, 2006, Raty *et al*, 2002, Tiemann *et al*, 2005, Determann *et al*, 2008), and was the only biological factor at diagnosis with predictive power in multivariate analysis in a Nordic prospective phase II study (Geisler *et al*, 2012). **However, several studies on IHC analysis in lymphoma, including MCL, have shown large inter-observer variability, which poses a challenge for comparison of results between labs (de Jong *et al*, 2007, Klapper *et al*, 2009, Sander *et al*, 2014). Therefore, a standardization of IHC evaluation of Ki-67 index in MCL is recommended (Klapper *et al*, 2009, Dreyling *et al*, 2013)**.

An assay using Nanostring® technology to measure RNA expression of a selected number of genes extracted from formalin-fixed, paraffin-embedded tissue (FFPE) has been developed (Roberts *et al*, 2007). This technology has been applied to discern between the two major subtypes of diffuse large B-cell lymphomas, Activated B-cell like and Germinal Centre B-cell like (Scott *et al*, 2014). An assay which applies the same technology has been developed for MCL by selecting 35 different genes, of which 17 were associated with proliferation and 18 were housekeeping genes. The MCL35 assay stratified patients into high-, standard- and low risk in a training set of 47 biopsies and was validated in a separate cohort of 110 patients (Scott *et al*, 2017). The validation cohort originated from British Columbia and consisted of patients who received rituximab-CHOP (cyclophosphamide, doxorubicin, vincristine, prednisolone) chemoimmunotherapy. Of note, in approximately half the patients, there was an intention to consolidate with high dose chemotherapy and autologous stem cell support (HD-ASCT). In this study, the pre-treatment biopsies contained at least 60% tumor cells and were excisional nodal biopsies.

For the above assay to be recommended in routine clinical practice, it should also be validated in a separate cohort of patients who have been treated with a regimen that now is considered to be standard for younger patients: Alternating rituximab-CHOP with a rituximab-high dose cytarabine – containing regimen (total of 6 courses) followed by HD-ASCT. We have, therefore, tested the MCL35 assay on a cohort of Norwegian patients, recruited in two Nordic MCL2 and MCL3 clinical trials (Geisler *et al*, 2012, Kolstad *et al*, 2014) or treated accordingly after closure of the trials.

Methods

Patients and Material

All Norwegian patients diagnosed with MCL with available FFPE diagnostic material and who had been treated in two consecutive Nordic phase II trials (MCL2 and MCL3) or according to the MCL2 protocol were eligible for this study - in total 153 cases. **Danish and Swedish patients were not included**. Further selection of patients was performed according to the Consort diagram (Supplementary Figure 1). Only surgical material from lymph nodes or Waldeyer's ring and with at least 60% tumor infiltration was considered suitable. Thus, out of 153 cases, 78 were deemed suitable for the MCL35 assay.

The study was approved by Regional Committee for Medical and Health Research Ethics South East (reg. no. 2016/1459) and by the Protection Officer at Oslo University Hospital.

RNA extraction and gene expression profiling

Unstained sections from FFPE tissue (1–2 slides if the surface area was > 1 cm^2, 5 slides for 0.1–1 cm^2 and 10 slides for < 0.1 cm^2) were sent to Vancouver for deparaffinization with the QIAGEN Deparaffinization Solution (catalogue number 19093) and RNA extraction with the QIAGEN AllPrep DNA/RNA FFPE Kit (catalogue number 80234, QIAGEN GmbH, Hilden, Germany). The RNA was quantified with spectrophotometry (NanoDrop, Thermo Scientific, DE). The MCL35 assay was performed on a minimum of 200 ng of RNA on

NanoString technology (NanoString Technologies, Seattle, WA) as previously described (Scott *et al*, 2014).

Pathology

Biopsies from patients included in the Nordic MCL2 and MCL3 studies or treated after closure of the Nordic MCL studies according to the MCL2 protocol were reviewed. Only biopsy samples originating from a lymph node or Waldeyer's ring, fulfilling the histological and immunohistochemical criteria established by the WHO (2017) for the diagnosis of MCL and containing ≥ 60% tumor cells were selected for this study. Following WHO criteria, tumors were subclassified into those with blastoid/pleomorphic (referred to as blastic) and those with clasical histology. **Ki-67 score was calculated as an average percentage of positive cells after counting immunohistochemically positive cells in representative tumor areas by an experienced pathologist**.

Statistics

The chi square or Fisher tests were applied to examine differences in patient characteristics between MCL35 risk categories. The primary endpoint was OS, which was calculated from date of diagnosis or inclusion in prospective studies until date of death from any cause. Secondary end point was progression free survival (PFS), calculated from date of diagnosis or study until date of progression or death from any cause (Cheson *et al*, 2007). OS and PFS were estimated using the Kaplan-Meier method. Log-rank test was used to examine the relationship between categorical variables and OS or PFS. Univariable analyses using Cox models were used to examine the relationship between defined variables and OS, including continuous variables. Cox proportional hazards regression tests were used to test the association of variables with OS in combination with other variables (adjusted analysis in Table 2). Two-sided p values < .05 were considered significant.

Results

Description of patient material

The MCL35 assay was applied to the biopsies of 78 patients, with 74 (95%) passing quality control. The assay assigned 27 (36%), 29 (39%) and 18 (24%) of the patients to low-, standard- and high-risk MCL35 categories, **(thresholds −143 and −28)** respectively. **The median MCL35 value was −113.05 (range −303.6 – +129.9)**. The demographic data, disease characteristics and treatments of all 74 MCL patients with MCL35 score (Scott *et al*, 2017) are shown in Table 1. There was a male predominance; the median age was 59 years, most patients had ECOG performance status 0–1 and bone marrow infiltration, and the distribution of MIPI and MIPI-C was as expected. There was an even distribution of Ki-67 score less than or greater than 30%. A quarter of the biopsies were of blastic (blastoid or pleomorphic) morphology. Of note, the majority of patients completed the intended treatment with alternating R-Maxi CHOP/R-HD cytarabine and consolidation with HD-ASCT. The median observation time was 5.3 years, there were no missing data in the final cohort and no patients were lost to follow up.

Distribution of patient and tumor characteristics across MCL35 risk groups

Figure 1 shows the heat map of RNA expression of the 35 genes (rows) for the 74 cases included in the analyses (columns). Underneath is depicted the corresponding Ki-67dichotomized score, MIPI score and histology (blastic versus classical). Table 1 shows the baseline distribution of disease characteristics across the three MCL35 risk groups. There were significant correlations between MCL35 high-risk and elevated LDH ($p = 0.043$), blastic histology ($p < 0.001$) and Ki-67 score > 30% ($p < 0.001$) (Table 1 and Figure 2A and 2B), but no significant correlations to MIPI (Table 1, Figure 2C) MIPI-B (MIPI with the addition of Ki67 score <10%, ≥10%−29% or ≥ 30%, Hoster *et al*, 2014) or MIPI-C (MIPI with the addition of binary Ki67 score +/−30%, Hoster *et al*, 2016) (Table 1).

Survival prediction for risk factors

The median observation time for all patients was 5.2 years (range 0.5–13.7). The estimated **5-year OS and PFS was 77% and 60%, respectively**, and at 10 years 60% and 48% (Figure 3A and 3B). Patients with Ki-67 ≥30% and blastic morphology (Figures 3C and 3D) had an inferior survival. Compared with data in the original manuscript[16], low- and standard-risk groups did not have significantly different outcomes, while the high-risk group had inferior outcome (Figure 3E and 3F). MIPI and MIPI-C discriminated well between the different risk categories (Figures 4A – 4D). In Cox unadjusted analysis, LDH (normal versus elevated), WHO performance status (0–1 versus 2–4), Ki-67 5% incremental **up to 30%**, Ki-67 +/− 30%, MIPI Score, MIPI-C score and MCL35 score predicted OS and PFS (albeit MCL35 score only of borderline significance for PFS (Table 2). In Cox adjusted multivariate analysis including Ki-67% with 5% increments and MCL35, only Ki-67 was significant for OS and PFS. However, when Ki-67 was included as a dichotomized variable (≥ versus < 30%), neither were associated with survival (Table 2). When MIPI and MCL35 were included in multivariable analysis, both variables were associated with OS, but with the MIPI score showing a better survival prediction (Table 2). Lastly, in multivariable analysis including MCL35, Ki-67 and MIPI, the two latter variables were significantly associated with OS and PFS. Unadjusted analyses of MCL35 score (standard versus low and high versus low) and adjusted multivariate analysis of the same variables, including Ki-67 and MIPI score, is shown in Supplementary Table 1. As MCL35 score did not correlate with the MIPI scores, there is a rationale for combining the MCL35 scores with MIPI scores in an exploratory analysis. When combining high risk MCL35 with high risk MIPI, we identify six patients with a very high risk (Figure 4E), and when combining with high or high intermediate MIPI-C, we identify 12 patients with substantially inferior survival (Figure 4F). Unadjusted and **adjusted analyses of MCL35 and MIPI for Ki67** are shown in Supplementary Table 2.

Discussion

The intention of the current analysis was to test the ability of the MCL35 assay to predict survival in a cohort of MCL patients younger than 70 years treated with alternate R-MaxiCHOP/R-HD Cytarabine and HD-ASCT (BEAM). The estimated **5-year OS of 77%** was comparable to what has been reported from two Nordic MCL studies where the patients received a similar regimen (Geisler *et al*, 2012, Kolstad *et al*, 2014) and with no plateaus in

the survival curves. The overall outcome was superior to that shown in the original report on the MCL35 assay (Scott *et al*, 2017), where the patients received R-CHOP and only around half of them were consolidated with HD-ASCT. Outcome for our total cohort was comparable to that of the MCL35 low risk group in the original report. This justifies our evaluation of the performance of the MCL35 assay in a patient cohort of younger patients treated with, what is now considered to be, standard of care. While in the original publication there was a very clear discrimination among three risk groups, the MCL35 assay did not separate the standard-risk group from the low-risk group in our cohort. However, the MCL35 assay was able to identify a subgroup with inferior outcome despite treatment with intensified chemo-immunotherapy and HD-ASCT therapy. This indicates that survival was improved, especially for patients in the MCL35 standard-risk upon introduction of current intensified treatment regimens.

When comparing the predictive value of the assay relative to MIPI and MIPI-C in our cohort, the MIPI scores provided improved stratification compared to the MCL35 assay. Similarly, Ki-67 score (≥ versus < 30%) alone and histology (blastic versus classical) stratified patient outcome better than the MCL35 assay. However, there is still a rationale to use the MCL35 assay in risk stratification: the MIPI score identifies clinical risk factors only, and was not correlated to MCL35 scores. In addition, the Ki-67 score included in the MIPI-C prognostic index is difficult to assess in **routine** clinical practice and has shown considerable inter-observer variability (de Jong et al. 2007, Sander et al 2014 Klapper *et al*, 2009), **although the reproducibility can be improved by standardizing the IHC evaluation** (Klapper *et al 2009*, Dreyling *et al* 2013). **In comparison, the MCL35 assay has been shown to have a good reproducibility across different centers** (Scott *et al*, 2017). **The data suggest that the prognostic effect of the MCL35 assay is independent of clinical factors, but it remains unclear whether its prognostic value is comparable to the Ki-67 index. A future comparative analysis of MCL35 and Ki-67 index using a larger patient cohort is warranted to clarify whether the presumed higher reproducibility of the MCL35 score is at the expense of lower prognostic value. Further, there is a need for validating our findings in a separate and larger, but similarly treated patient cohort**.

A combination of MIPI score and MCL35 score is plausible for taking into account both clinical and biological risk factors. When combining high-risk MCL35 with high-risk MIPI score, we identified a smaller subgroup with dismal prognosis. Such patients should probably not receive standard treatment but rather be recruited to up-front clinical trials with novel investigational drugs like ibrutinib**, lenalidomide** and venetoclax as single agents or in combination with other drugs (Wang *et al*, 2013, Wang *et al*, 2016, Davids *et al*, 2017, Trneny *et al*, 2016, Dreyling *et al*, 2016). Indeed, both clinical and biological risk factors have to be assessed when new therapeutic approaches are introduced.

Novel biological risk factors have been identified during recent years like non-functional TP53 through genetic loss of p17 or mutations (Eskelund *et al*, 2017, Aukema *et al*, 2018) for identifying patients with inferior outcome. Hence, a combination of MIPI and TP53 alterations has been proposed as a new predictive marker.

Strengths of this study include that patients were uniformly treated and the fact that there were no missing values of any of the variables. Furthermore, the MCL35 analysis was performed by the group who developed the assay. For two patients, ultimately excluded from the study, results of the MCL35 assay did not fit with what was expected for a MCL biopsy. A second review of the diagnosis in these cases revealed that MCL could not be confirmed in one patient and for the other patient the sample was massively necrotic (data not shown) resulting in a viable tumor content of < 60%. Thus, the assay, although developed for predictive purposes, might also be of diagnostic value.

A weakness of this study is the limited sample size which reduces the power of the analyses. Additionally, the assay was developed for surgical biopsies from lymph nodes with tumor content of at least 60%, excluding samples from bone marrow and non-lymphoid tissue. **However, OS and PFS at 5 and 10 years are nearly identical to those published for the whole cohort of Nordic patients included in the MCL2 and MCL3 studies (Kolstad A et al. 2014). Nevertheless**, the MCL35 assay remains to be validated in samples that do not meet these criteria and, this so far reduces the applicability of the assay.

In conclusion, the MCL35 assay was able to identify a group of MCL patients with inferior outcome after intensive first-line treatment, including HD-ASCT, but standard- and low-risk groups had similar outcomes. By combining MIPI or MIPI-C and MCL35, patients with dismal prognosis were identified, and they should be considered for novel strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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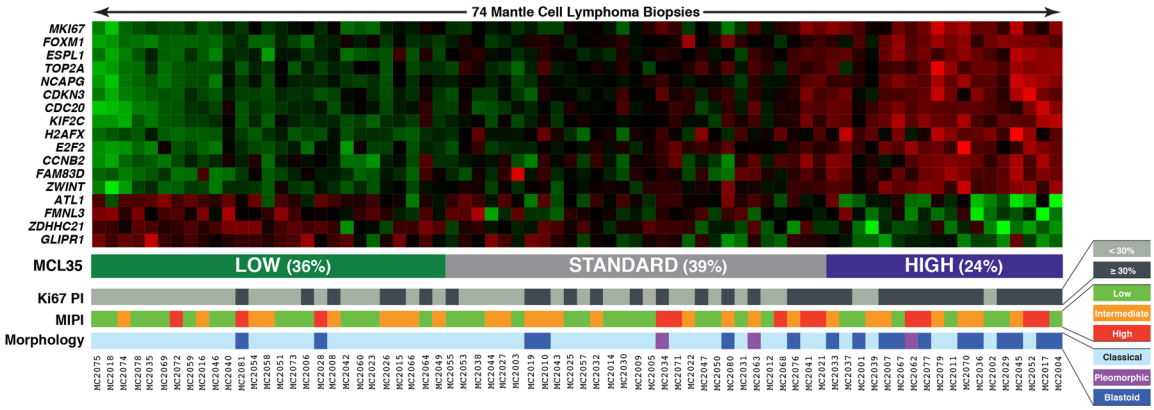

Figure 1.

Heatmap of RNA expression for the 35 genes included (rows) for the 74 individual biopsies included (columns). The total score indicate the three categories (low, standard, high). Underneath is depicted the corresponding Ki-67 score dichotomized into +/−30%, the MIPI score and whether the biopsies had a blastic or classical morphology type.

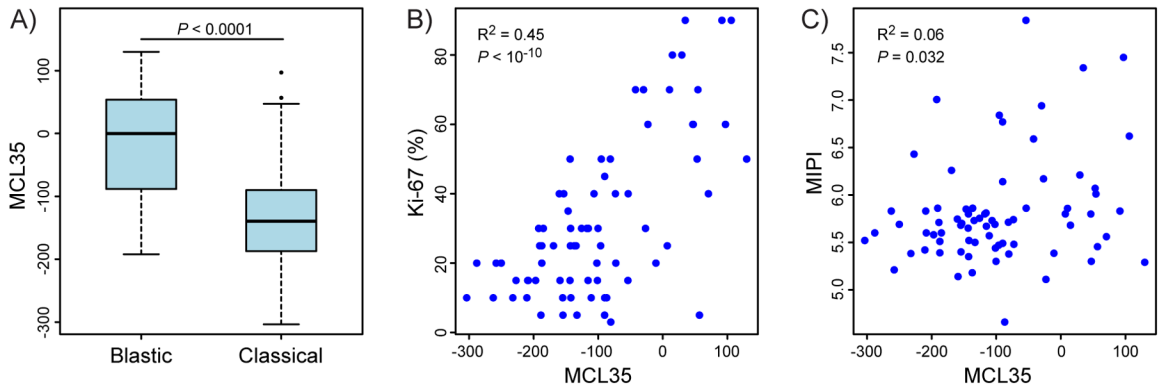

Figure 2.

A. Box plot of MCL35 score in blastic versus classical MCL type. Statistics: Welch's unequal variances t-test. B. MCL35 score versus Ki-67 score. Correlation by Spearman rank test. C. MCL35 score versus MIPI score. Correlation by Spearman rank test.

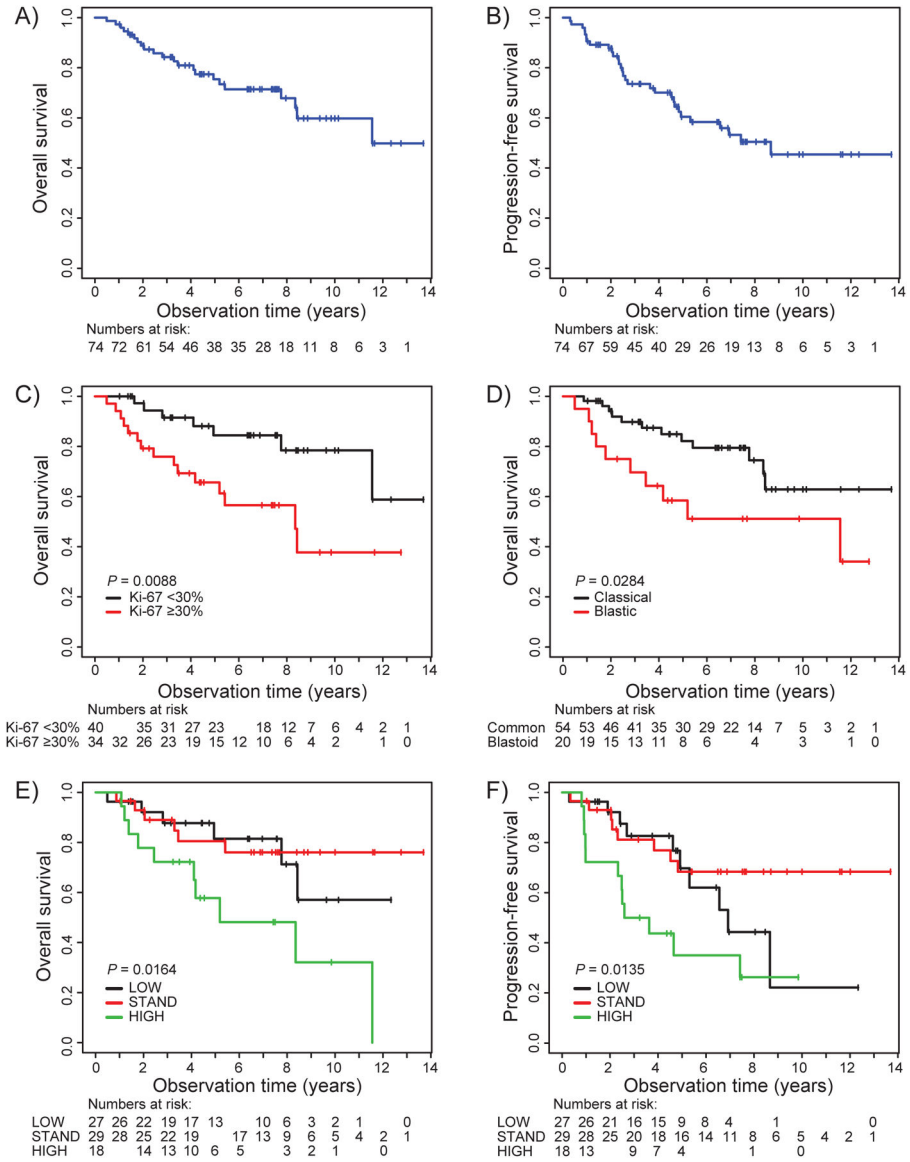

Figure 3.

Kaplan Meier survival plots. A. OS, all patients. B. PFS, all patients. C. OS for Ki-67 score categorized as ≥ or < 30%. D. OS for blastic (blastoid or pleomorphic) versus classical / small cell MCL histology. E. OS for MCL35 risk groups. F. PFS for MCL35 risk groups.

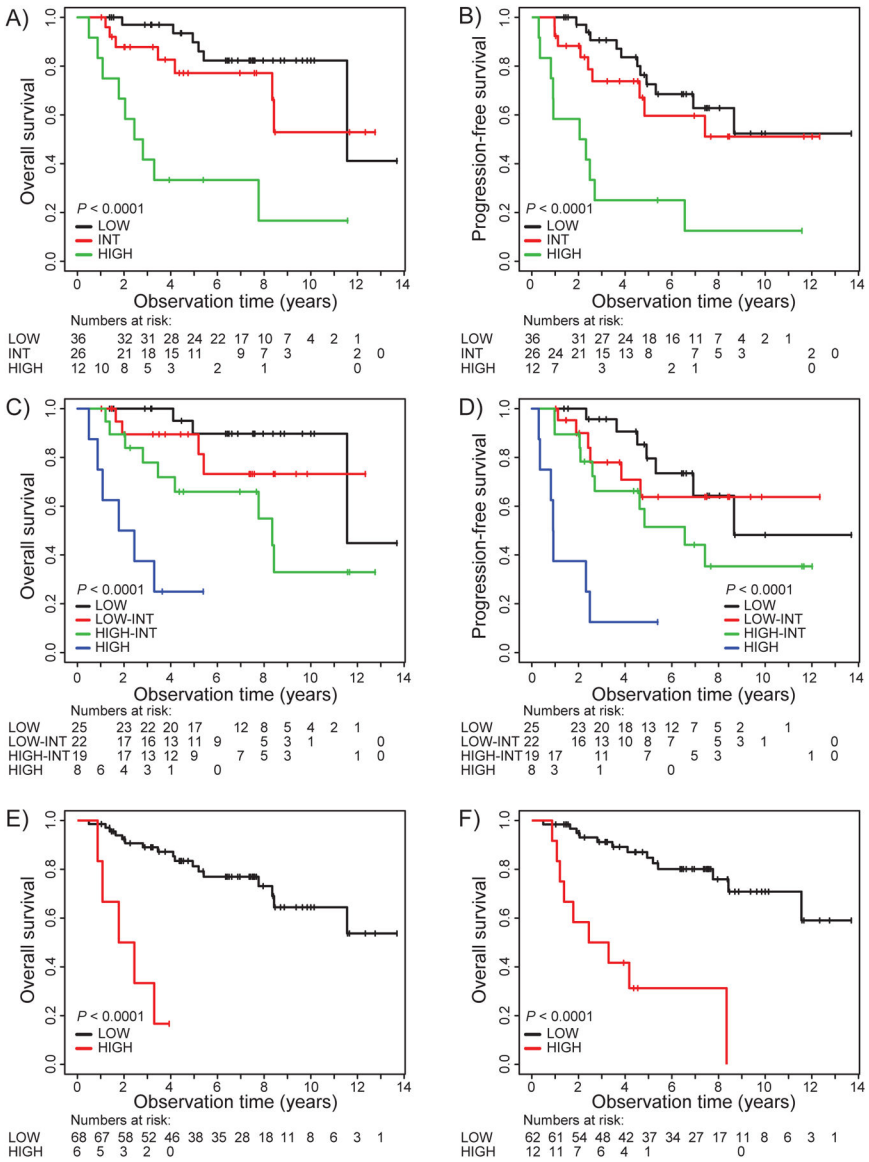

Figure 4.

Kaplan-Meier survival plots demonstrate stratification of MCL35 assay alone or in combination with MIPI or MIPI-C score. A. OS for MIPI risk groups. B. PFS for MIPI risk groups. C. OS for MIPI-C risk groups. D. PFS for MIPI-C risk groups. E. OS for MCL35 high risk combined with MIPI high risk versus **others.**. F. OS for MCL35 high risk combined with MIPI-C high/high-intermediate versus **others.**

Table 1:

Demographic data and disease characteristics distributed among the MCL35 risk groups

Patient Demographic Data and Disease Characteristics					
Variable	**Total Cohort, *n* (%)**	**Low Risk Group *n* (%)**	**Standard-Risk Group *n* (%)**	**High-Risk Group *n* (%)**	**P**
Assessable patients	74	27 (36)	29 (39)	18 (24)	1
Male	64 (86)	23 (85)	25 (86)	16 (89)	
Female	10 (14)	4 (15)	4 –(14)	–2 (11)	
Median age, years (range)	59 (41–67)	60 (45–67)	58 (41–67)	60.5 (43–65)	
> 65	2	1	1	0	
Clinical features					
ECOG performance stat					
0–1	68 (92)	26 (96)	27 (93)	15 (83)	0.32
2–4	6 (8)	1 (4)	2 (7)	3 (7)	
Missing					
White cell count, median (range)	6.8 (3.4–56.2)	6,3 (4.2–20.5)	7,2 (3.4–56.2)	8,4 (4.4–18.2)	0.31
Bone marrow infiltration	59 (80)	24 (89)	19 (66)	16 (89)	0.051
LDH					
Normal	35 (47)	16 (59)	15 (52)	4 (25)	0.043
>ULN	39 (53)	11 (41)	14 (48)	14 (75)	
MIPI					
Low < 5.7	36 (49)	15 (56)	14 (48)	7 (39)	0.81
Intermediate (5.7–6.2	26 (35)	9 (33)	10 (35)	7 (39)	
High (>= 6.2)	12 (16)	3 (11)	5 (17)	4 (27)	
MIPI – c					
Low	25 (34)	13 (48)	10 (35)	2 (11)	0.16
Low-intermediate	22 (30)	8 (30)	8 (28)	6 (33)	
High-intermediate	19 (26)	5 (19)	8 (28)	6 (33)	
High	8 (11)	1 (4)	3 (10)	4 (22)	
Ki67 proliferation index					
< 30	40 (54)	21 (78)	16 (55)	3 (17)	<0.001
>=30	34 (46)	6 (12)	13 (45)	15 (83)	
MIPI-B					
Low < 5.7	9 (12)	5 (19)	3 (10)	1 (6)	0.064
Intermediate 5.7–6.5	37 (50)	15 (56)	17 (59)	5 (28)	
High >= 6.5	28 (38)	7 (26)	9 (31)	12 (67)	
Pathology					
Classic	54 (73)	25 (93)	23 (80)	6 (33)	<0.001
Blastic (pleomorphic, blastoid)	20 (27)	2 (7)	6 (21)	12 (67)	
Given HDT with BEAM	68 (92)	24 (89)	27 (93)	17 (94)	

Patient Demographic Data and Disease Characteristics					
Variable	**Total Cohort, *n* (%)**	**Low Risk Group *n* (%)**	**Standard-Risk Group *n* (%)**	**High-Risk Group *n* (%)**	**P**
Reason not given HDT					
Toxicity	1			1	
Harvest failure	2	1	1		
Stage I	1	1			
Age	1		1		
No response induction	1	1			
CR	66 (90)	24 (90)	26 (90)	16 (90)	0.92
CR / PR	72 (98)	26 (97)	28 (97)	18 (100)	

Table 2.

Prognostic value of clinical and tumor proliferation associated variables including MCL35 raw score for overall and progression free survival

		OS		PFS	
type	**variable**	**Pval**	**HR**	**Pval**	**HR**
Unadjusted	Age (>60 vs <=60)	0.20	1.7496	0.62	1.2171
Unadjusted	Gender (Female vs Male)	0.60	1.3595	0.	1.6261
Unadjusted	LDH (High vs Normal)	0.0041	4.9138	0.0043	3.2600
Unadjusted	WHO (2–4 vs 0–1)	0.0248	3.5401	0.0025	4.4789
Unadjusted	Ki67 index (+5%)	0.0001	1.4268	0.0002	1.3502
Unadjusted	Ki67 index (>=30%)	0.0129	3.1364	0.0315	2.2314
Unadjusted	Histology (Blastic vs Classic)	0.0339	2.5004	0.1073	1.8436
Unadjusted	MIPI (+1)	0.0009	2.3200	0.0059	1.9666
Unadjusted	MIPI (3 cat)	0.0002	2.9070	0.0005	2.2900
Unadjusted	MIPI-C (4 cat)	0.0001	2.7082	0.0002	2.0987
Unadjusted	MCL35 score (+100)	0.0101	1.7152	0.0441	1.4297
Adjusted	MCL35 score (+100)	0.0316	1.6252	0.0710	1.4068
	MIPI score (+1)	0.0044	2.0499	0.0111	1.8536
Adjusted	MCL35 score (+100)	0.94	1.0234	0.48	0.8329
	Ki67 index (+5%)	0.0064	1.1903	0.0025	1.1982
Adjusted	MCL35 score (+100)	0.96	0.9839	0.50	0.8350
	Ki67 index (+5%)	0.0101	1.1727	0.0033	1.1833
	MIPI score (+1)	0.0088	2.1322	0.0178	1.9553
Adjusted	MCL35 score (+100)	0.14	1.4243	0.35	1.2223
	Ki67 index (>=30%)	0.13	2.2188	0.20	1.7778
Adjusted	MCL35 score (+100)	0.36	1.2734	0.50	1.1642
	Ki67 index (>=30%)	0.0710	2.6588	0.12	1.9893
	MIPI score (+1)	0.0029	2.3238	0.0074	2.0288