Yang *et al. Diagnostic Pathology* (2024) 19:149
https://doi.org/10.1186/s13000-024-01577-z

Diagnostic Pathology

RESEARCH Open Access

Clinical significance of Cyclin D1 by complete quantification detection in mantle cell lymphoma: positive indicator in prognosis

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Abstract

Objectives The positive expression of Cyclin D1 in immunohistochemical (IHC) staining serves as the cornerstone for diagnosing mantle cell lymphoma (MCL). However, existing literature does not conclusively establish whether the expression ratio and staining intensity significantly influence diagnostic outcomes or patient prognosis. In this retrospective study, the correlation between comprehensive Cyclin D1 quantification and the prognosis of MCL patients was studied.

Methods The Cyclin D1 protein level was assessed in 120 formalin-fixed paraffin-embedded samples from MCL patients using the quantitative dot blot (QDB) analysis technique. R language software was employed for statistical analysis to determine the optimal threshold with statistical significance. Additionally, Kaplan-Meier method was utilized to evaluate the relationship between the absolute level of Cyclin D1 protein and overall survival (OS) of patients. Furthermore, the Chi-square test was applied to analyze the causes of single and multiple fractures, with a significance level of $p < 0.05$. Finally, the Log-rank test was used to compare two survival curves, where a significance level of $p < 0.05$ was considered statistically significant.

Results At the optimized cutoff of 0.46 nmol/g, univariate analysis revealed a positive correlation between Cyclin D1 protein level and patient survival (OS). Specifically, in the subgroup with complete quantification of Cyclin D1 higher than the cutoff, the 5-year OS was 18%, whereas in the subgroup with complete quantification of Cyclin D1 lower than the cutoff, the 5-year OS was 4.8% (Log-rank test, $P = 0.017$). This indicates that patients with Cyclin D1 levels above the cutoff had significantly better overall survival compared to those below the cutoff. Additionally, in the Pearson distribution test, Ki-67 emerged as an independent prognostic factor for the complete quantification of Cyclin D1. Notably, Cyclin D1 complete quantification results remained unaffected by factors such as gender, age, LDH (Lactate Dehydrogenase) level, Ann Arbor stage(AAS), Ki-67, IPI(International prognostic index), MIPI(Mantle International prognostic index), and MIPI-c (MIPI Combined with Ki-67 Proliferation Index Chi-square test, $p > 0.05$).

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Conclusions Comprehensive Cyclin D1 quantification, especially above a threshold, significantly correlates with better overall survival in MCL. This highlights its prognostic importance in MCL management. Full quantification of CyclinD1 aids MCL prognosis, while QDB technology for biomarker quantification supports precise clinical prognostic stratification.

Keywords Cyclin D1, QDB, MCL, Protein quantitation, Prognosis

Introduction

Mantle cell lymphoma (MCL) is an uncommon subtype of non-Hodgkin lymphoma (NHL) originating from B cells, accounting for about 3–10% of NHL cases [1]. Its defining biological feature is the chromosomal translocation *(11;14) (q13;q32)*, leading to the overexpression of Cyclin D1 [2]. This translocation results in a G1/S phase disorder, which forms the molecular basis of MCL. Most patients are diagnosed at an advanced disease stage (Ann Arbor stage III or IV), often with bone marrow or peripheral blood involvement [3]. The clinical heterogeneity of MCL presents a spectrum that includes both aggressive forms with slow progression, poor prognosis, and challenging treatment outcomes.

Cyclin D1 is a crucial positive regulator of the cell cycle and a recognized proto-oncogene [4]. It shows positive expression in various solid malignancies [5–15] and has a significant correlation with tumor prognosis [9, 10], playing a role in promoting tumor progression [11] and potentially serving as a therapeutic target in various cancers [12–15].

The Ehinger team [16] studied 231 cases of CD5-negative diffuse large B-cell lymphoma (DLBCL) and found a 4.3% positive expression rate of Cyclin D1 in the absence of chromosomal translocation. Lymphomas such as follicular center lymphoma (FCL), diffuse large cell lymphoma (DLCL), anaplastic large cell lymphoma (ALCL), and peripheral T-cell lymphoma (PTCL) exhibit nonspecific expression of Cyclin D1 [17], which has not been systematically studied in these lymphomas, leaving the mechanisms and prognostic implications unclear.

To date, no studies have reported on the relationship between Cyclin D1 expression and prognosis in MCL patients. IHC can detect target proteins through localization and qualitative and semi-quantitative methods but does not achieve complete quantification. Traditional immunoblotting methods are also relatively quantitative. Recently, in light of the limitations of immunohistochemical staining, Yantai Zestern Biotechnique Co., LTD China developed a new immunoblotting technique for absolute quantification of protein biomarkers in formalin-fixed and FFPE- QDB [18]. This technique represents the first immunoassay method for absolute and complete quantification detection of protein biomarkers in FFPE tissues [19]. Based on the specific binding of antigens and antibodies, this method allows for the complete quantification of proteins and the analysis of many samples, thus facilitating the detection of protein expression levels across multiple parameters. QDB analysis uses 96-well plates with nitrocellulose membranes and requires only a small amount of sample cracking solution, saving significant amounts of working time and research resources, and effectively improving work efficiency. This method has been evaluated at both the cell and tissue levels, yielding good experimental results [20] and addressing the shortcomings of traditional immunoblotting methods by ensuring the objectivity of experimental results.

This study aims to detect Cyclin D1 protein in 120 FFPE samples from MCL patients using the QDB technique, analyze and evaluate the predictive value of Cyclin D1 protein for the prognosis of MCL patients, investigate its correlation with other clinical parameters, and explore its potential as a new molecular biomarker for prognostic assessment and clinical treatment decision-making in MCL.

Materials and methods

Patient selection

This retrospective study included 120 cases of MCL diagnosed by the pathology departments of Yantai Yuhuangding Hospital, Shandong Provincial Hospital, Shandong University Qilu Hospital, and the Affiliated Hospital of Southwest Medical University from 2008 to 2020.

Methods

All patients received treatment with Rituximab. The survival status of the patients was updated through telephone follow-ups. The last follow-up date was September 18, 2020. The overall survival period was calculated from the disease diagnosis date to either the date of death or the last follow-up date for the patient. The study received approval from the ethics committee of Yantai Yuhuangding Hospital (approval No.: Yantai Medical Ethics Review 2021-041).

QDB analysis

In this study, 0.5 μg of MCL FFPE lysate (adjusted to a concentration of 0.25 μg/μl) and 0.02 μg of BT474 lysate were added to the QDB plate. Simultaneously, serially diluted recombinant Cyclin D1 protein was added as the protein standard and dried at room temperature for 1 h. Primary antibodies were diluted in 4% non-fat milk blocking buffer to the desired concentrations (ranging from 1:500 to 1:5000) and incubated for 1 h at room

temperature, with 100 μL per well, followed by overnight incubation at 4 °C. The QDB plate was rinsed with TBST (TBS+Tween) solution for 3 sets of 5-minute washes.

Next, the secondary antibody was diluted to the required concentration (ranging from 1:1000 to 1:50000) and incubated for 4 h at room temperature. The QDB plate was rinsed again, and ECL(enhanced chemiluminescence) substrates were added to the 96-well microplate evenly. The QDB plates were then inserted into the 96-well microplate for 2 min, removed, placed on the microplate, and the luminous intensity was detected using a microplate reader. Each sample was tested three times, and the average value of these three experiments was used for analysis.

The absolute level of Cyclin D1 in the BT474 cell lysate was detected using the QDB method with BT474 cell lysate serving as an internal control, and the results were recorded for further analysis.

Statistical analysis

The Survival and Survminer packages were used in R to analyze patient survival and prognosis in risk groups. Specifically, the "survminer" package version 4.1.2 in R was employed to calculate the optimal cut-off value. Cyclin D1 levels, measured by the QDB method, were dichotomized for OS analysis, and survival curves were generated accordingly.

To analyze the relationship between the complete quantification of Cyclin D1 and patient prognosis and survival, we employed SPSS software version 25.0. Kaplan-Meier analysis was used for univariate analysis of relevant clinicopathological parameters and prognosis. Univariate Cox proportional hazard models were fitted to estimate risk ratios (RR) and corresponding 95% confidence intervals (CI), and R packages were used to plot univariate survival curves for comparing survival differences.

The correlation between Cyclin D1 complete quantification values and each clinicopathological parameter was assessed using the chi-square test. Additionally, multivariate Cox proportional hazards regression analysis was conducted to analyze the influence of clinicopathological parameters on patient prognosis.

Additionally, the correlation between the complete quantification value of Cyclin D1 and the IHC positive expression rate of Ki-67 in MCL patients was investigated using Pearson's correlation coefficient. A p-value of less than 0.05 was considered statistically significant in all analyses.

Results

Patients characteristics

As of September 18, 2020, the follow-up duration for the study cohort was 116 months. The age of onset among patients ranged from 35 to 86 years, with a median age of 63 years. Among the patients with available follow-up data (n=72), the median follow-up time was 48 months. The median survival time observed was 24 months, with a stable survival rate extending to 41 months.

Among these patients, 42 individuals succumbed to the disease, accounting for 58.3% (42/72) of the cohort, while 30 patients survived, representing 41.7% (30/72) of the cohort. The calculated 3-year OS rate was 31.9%, and the 5-year OS rate was 13.9%. These figures provide insight into the prognosis and survival outcomes observed in this cohort of patients with mantle cell lymphoma.(Fig. 1).

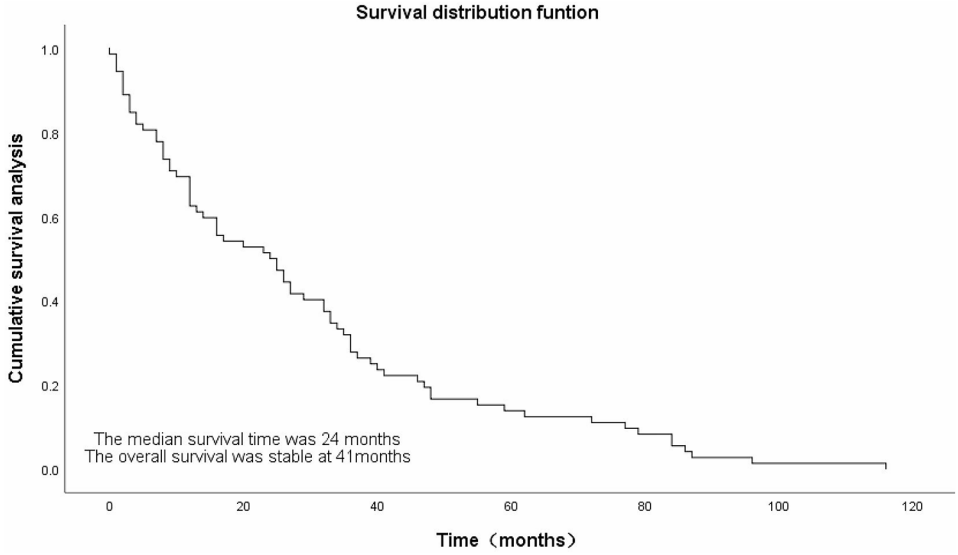

Fig. 1 The median survival time for 72 MCL patients was 24 months (with corresponding 3-year and 5-year overall survival rates of 31.9% and 13.9%, respectively)

Cyclin D1 complete quantification and optimal cut-off value

Cyclin D1 Quantification and Determination of Optimal Cut-off Value in MCL Samples: Cyclin D1 was quantified in 120 MCL samples. One case was excluded due to insufficient sample volume, leaving 119 samples for analysis. The quantification ranged from 0.081325936 nmol/g to 21.2259093 nmol/g, with an average of 1.9004718900794 nmol/g. Using the 'survminer' R package for statistical analysis, the optimal cut-off value for Cyclin D1 in OS analysis was determined to be 0.46 nmol/g, which showed statistical significance (as illustrated in Fig. 2). Patients were categorized into two subgroups based on this threshold: a Cyclin D1-high (>0.46 nmol/g) subgroup and a Cyclin D1-low (≤0.46 nmol/g) subgroup.

Correlation between complete quantification of Cyclin D1 and prognosis of patients

Based on the threshold classification data analyzed above, the analysis of the difference in prognosis and survival between the two subgroups was conducted using the R package. Among the 72 patients with follow-up results, the FFPE samples of one patient failed to pass the QDB test (n=71). The Cyclin D1-h subgroup comprised 50 patients (70.4%, 50/71), whereas the Cyclin D1-l subgroup included 21 patients (29.6%, 21/71). Statistical analysis revealed a significant correlation between Cyclin D1 complete quantification and the survival prognosis of MCL patients (p=0.017). This statistical significance is pertinent to patients' OS prognosis.(Fig. 3).

Correlation between complete quantification of Cyclin D1 in MCL and various clinicopathological parameters

The relationship between complete quantification of Cyclin D1 and various clinicopathological parameters was analyzed using the chi-square test and exact Fisher test. However, no significant correlation was found between the absolute levels of Cyclin D1 protein and each clinicopathological parameter, as summarized in Table 1. The parameters assessed included age (P=0.804), gender (P=0.176), LDH level (P=0.169), AAS (P=0.72), IPI (P=1.000), MIPI (P=0.329), Ki67 (P=0.729), and MIPI-c (P=0.474). It is important to note that a p-value exceeding 0.05 was not considered statistically significant in this analysis.(Table 1).

Relationship between clinicopathological parameters and prognosis of patients

Following Kaplan-Meier analysis and log-rank test, the univariate Cox proportional hazards model was employed to fit OS data. This model was used to evaluate hazard ratios (HR) and corresponding 95% CI. This analysis revealed statistically significant correlations between Cyclin D1 complete quantification, age, Ki-67, MIPI, and AAS with patient prognosis and OS (P: 0.017, 0.040, 0.015, 0.048, 0.016, respectively). However, gender, MIPI-c, IPI, and LDH levels did not exhibit statistical significance in this context (P: 0.058, 0.066, 0.107, 0.347, respectively).(Table 2).

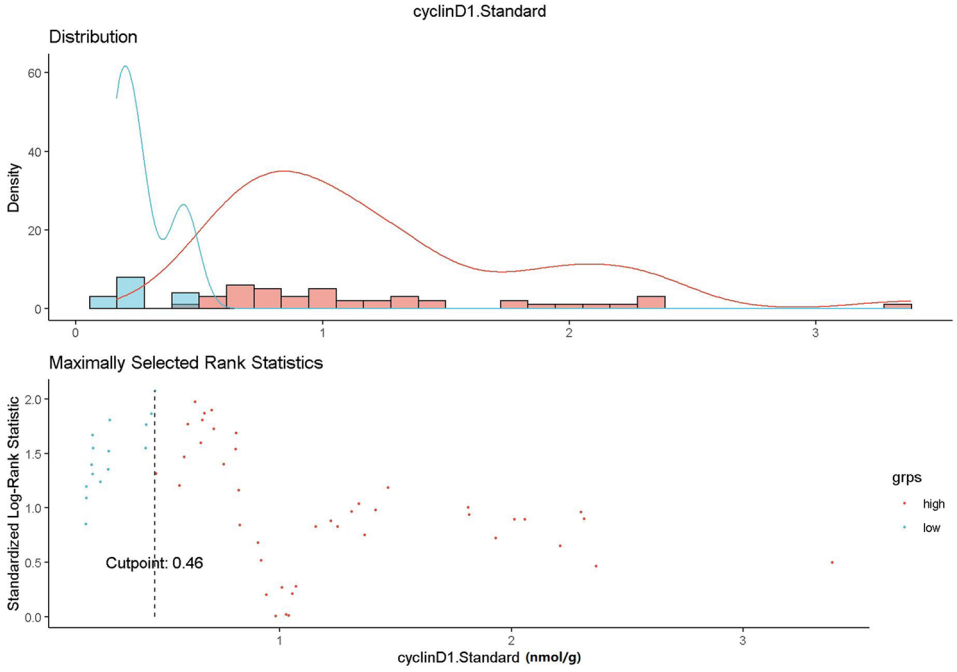

Fig. 2 Cyclin D1 absolute quantification via R software identified 0.46nmol/g as the optimal threshold for significantly predicting patient prognosis

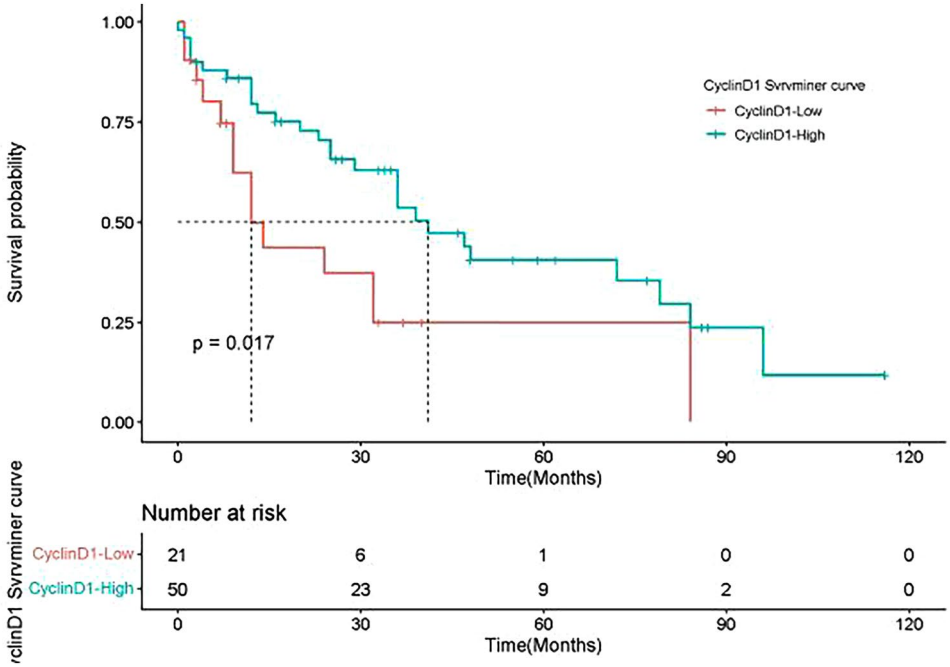

Fig. 3 Patients were stratified by Cyclin D1 levels (0.46nmol/g threshold), using "survminer" to assess prognosis. Cyclin D1-high patients (>0.46nmol/g) showed significantly better survival outcomes ($p=0.017$), with longer median survival and higher survival rates than Cyclin D1-low (≤0.46nmol/g) patients

Table 1 Relationship between Cyclin D1 and clinicopathological parameters in MCL

clinicopathological parameters	classification	Cyclin D1 ≤ 0.46nmol/g	Cyclin D1>0.46nmol/g	x^2	*P*
Age	≤60	12	31	0.062	0.804
	>60	17	49		
Gender	female	5	26	2	0.176
	male	25	63		
LDH	normal	14	27	1.89	0.169
	rise	7	28		
AAS	I and II	1	1	0.131	0.721
	III and IV	9	27		
IPI	0、1、2、3	5	13	0.000	1.000
	4、5、6	4	12		
Ki-67	≤30%	5	18	0.12	0.729
	>30%	11	32		
MIPI-c	I	1	3	2.507	0.474
	II	2	10		
	III	2	11		
	IV	7	12		
MIPI	I	2	6	2.226	0.329

Results of multivariate prognostic analysis

The Cox proportional hazards model was utilized to analyze the prognosis of patients, considering statistically significant single parameters and multivariate prognosis. The omnibus test yielded a p-value of 0.012. However, upon analysis, the effects of Cyclin D1, age, Ki-67, AAS, and MIPI on the survival time of patients were not statistically significant ($P>0.05$). Consequently, no independent prognostic parameters affecting MCL were identified.(Table 3).

Correlation between complete quantification of Cyclin D1 and positive rate of Ki-67 in MCL

Both the Ki-67 index and Cyclin D1 are markers associated with cell proliferation. However, after conducting Spearman correlation analysis, it was found that the complete quantification of Cyclin D1 and the positive rate of Ki-67 index IHC staining were not correlated ($p>0.05$). (Table 4)

Table 2 Relationship between clinicopathological parameters and prognosis of patients

Clinicopathological parameters	Number of cases	95%CI		*p*	HR
		lower limit	upper limit		
Cyclin D1					
≤0.46nmol/g	21	5.659	18.341	0.017	0.463
>0.46nmol/g	50	25.949	56.051		
Gender					
male	58	21.299	42.701	0.058	0.344
female	14	-	-		
Age					
≤60	23	23.805	134.195	0.040	2.069
>60	46	16.617	33.383		
MIPI					
I	7	77.456	88.544	0.048	1.788
II	18	21.014	60.986		
III	22	0.000	42.900		
Ki-67					
≤30%	35	27.031	54.969	0.015	2.143
>30%	32	0.906	39.094		
MIPI-c					
I	4	-	-	0.066	1.788
II	11	5.006	76.994		
III	11	10.313	39.687		
IV	20	0.000	21.164		
IPI score					
0、1、2	16	-	0.000	0.11	0.433
3、4、5	23	14.066	49.934		
Ann Arbor stage					
II	2	-	-	0.016	0.134
III	12	24.341	57.659		
IV	22	22.860	71.140		
LDH level					
normal	32	27.243	54.757	0.35	1.004
rise	27	16.841	47.159		

Disscussion

In this pioneering study, we utilized the QDB method for the first time to achieve a comprehensive quantification of Cyclin D1 in MCL FFPE samples. Our findings provide initial evidence that the absolute and exhaustive quantification of Cyclin D1 is intricately linked to patient prognosis, exhibiting a favorable correlation with survival duration and OS, albeit not as an autonomous determinant.

Table 4 Correlation between complete quantitative of Cyclin D1 and positive rate of Ki-67 in MCL

Clinicopathological parameters		Cyclin D1	Ki-67
Cyclin D1	Correlation coefficient	1.00	0.029
	P-value		0.760
Ki-67	Correlation coefficient	0.029	1.00
	P-value	0.760	

Given the intricate biological underpinnings of MCL onset and progression, coupled with the myriad factors influencing patient survival and prognosis, developing an optimal prognostic model remains a formidable challenge, particularly in light of MCL's low incidence and inherent heterogeneity [21]. Prior investigations into prognostic factors in MCL patients have lamented suboptimal treatment outcomes [22]. While MCL characteristically expresses Cyclin D1 through IHC staining, this approach is inherently subjective and prone to variability [23]. To address these limitations, we adopted the QDB method [24], which not only circumvents the shortcomings of IHC but also uncovers a significant association between Cyclin D1 overexpression and MCL patient survival.

Using the R package, the optimal cut-off value of Cyclin D1 was determined to be 0.46 nmol/g. Patients were subsequently divided into two groups and subjected to Kaplan-Meier univariate survival analysis. This rigorous analysis underscores the pivotal role of Cyclin D1, age, MIPI, Ki-67, and AAS as statistically significant prognostic factors in MCL ($P<0.05$). Furthermore, the R package facilitated the generation of prognostic survival curves for each factor, offering profound insights into their individual contributions to patient outcomes.(Fig. 4).

The median survival time of the Cyclin D1-h group (41 months) was significantly longer than that of the Cyclin D1-l group (12 months). At 30 months after diagnosis, the OS rate for patients in the Cyclin D1-h group was 46% (23/50), whereas it was approximately 28.6% (6/21) for patients in the Cyclin D1-l group. By the 60-month mark, the 5-year OS rate for the Cyclin D1-h group dropped to 18% (9/50), and for the Cyclin D1-l group, it was 4.8% (1/21). At 90 months, the OS rate for the Cyclin D1-h group was 4% (2/50), while it was 0% (0/21) for the Cyclin D1-l group. As of the follow-up date, one patient

Table 3 Relationship between multivariate analysis and prognosis of MCL patients

Clinicopathological parameters	Grouping category	Number of cases	HR	95%CI		*p*
				lower limit	upper limit	
Cyclin D1	>0.46vs≤0.46	71	2.739	0.783	9.584	0.115
Age	>60 vs.≤60	69	1.802	0.466	6.974	0.393
Ki-67	>30 vs.≤30	67	0.565	0.111	2.881	0.492
AAS	1、2、3、4	38	0.262	0.044	1.556	0.141
MIPI	1、2、3	50	1.228	0.349	4.319	0.749

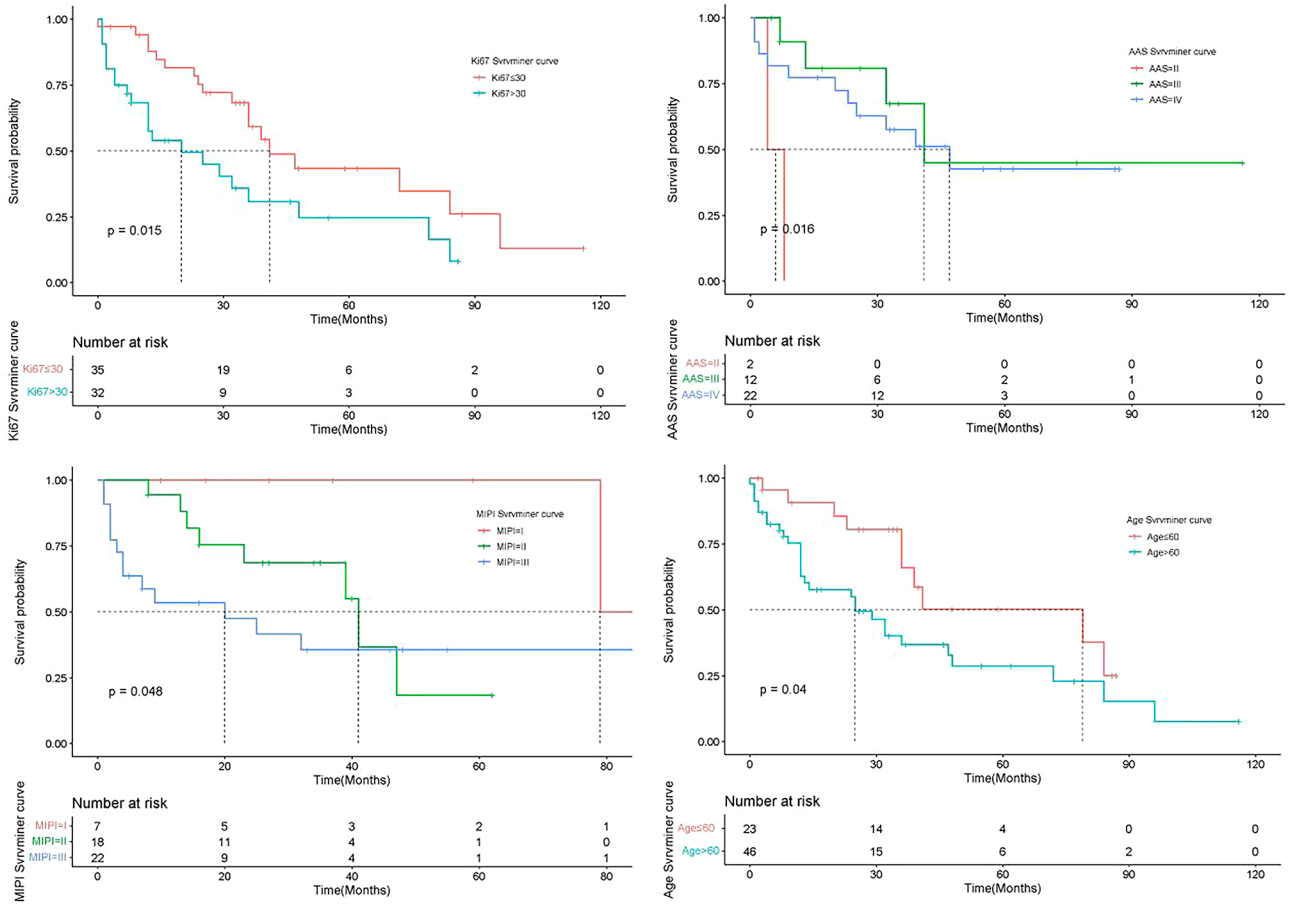

Fig. 4 Univariate prognostic analysis of MCL patients showed that Ki-67, AAS, MIPI, and Age were significant for the prognosis of patients

in the Cyclin D1-h group was still alive, with a survival time of 116 months (refer to Fig. 4). Cyclin D1 emerged as a significant prognostic factor for patients with MCL.

A Cox proportional hazards regression multivariate analysis indicated that no independent factors significantly affected MCL prognosis. Cyclin D1, along with other factors, were all identified as risk factors for MCL occurrence. Notably, Cyclin D1 did not independently influence prognosis ($p>0.05$), aligning with existing literature [25].

This study revealed that the complete quantification of Cyclin D1 was not correlated with patients' age, MIPI, AAS, Ki-67, MIPI-c, sex, or LDH level (chi-square test, $p>0.05$, see Table 1). Therefore, the absolute level of Cyclin D1 in MCL remained unaffected by other clinicopathological factors.

Both Ki-67 and Cyclin D1 serve as markers of cell proliferation and are associated with MCL prognosis. However, bivariate Pearson analysis demonstrated their independence from each other in the context of MCL (r = -0.048, $p=0.621$, $p>0.05$). The *t (11; 14) (q13; q32)* chromosomal translocation leading to Cyclin D1 overexpression promotes abnormal cell proliferation and is considered an initial factor in MCL. Meanwhile, Ki-67 reflects tumor cell proliferative activity independently.

The complete quantification of Cyclin D1 may emerge as a novel biomarker for evaluating MCL prognosis, OS, and survival rates. However, this study focused solely on patients who received rituximab treatment and had follow-up results ($n=72$). Therefore, the applicability of the determined threshold requires confirmation in future studies. Larger-scale studies are advocated to validate the application of this biomarker in routine clinical practice.

Conclusions

In conclusion, we advocate for incorporating Cyclin D1 into MCL prognostic assessments, underscoring its autonomy from other risk factors and positive survival correlation. The pivotal prognostic threshold of 0.46 nmol/g for MCL underscores its clinical significance. Looking ahead, QDB is an emerging technology for the fully quantitative detection of biomarkers. In the past, our research group has conducted in-depth studies on important biomarkers of breast cancer using this technology and achieved certain research results. This article represents the first attempt to apply QDB technology in

clinical lymphoma research, focusing solely on the fully quantitative study of CyclinD1 protein, which has significant diagnostic value in mantle cell lymphoma (MCL). Other important markers such as P53, Ki67, and even T-lymphocytes in the tumor microenvironment will be included in subsequent research. We hope that this work can provide assistance in the precise diagnosis, prognostic stratification, and selection of treatment strategies for patients with mantle cell lymphoma.

Abbreviations

Cyclin D1	G1/S-specific Cyclin-D1
IHC	Immunohistochemical
MCL	Mantle cell lymphoma
QDB	Quantitative dot blot
OS	Overall survival
LDH	Lactate Dehydrogenase
AAS	Ann Arbor stage
IPI	International prognostic index
MIPI	Mantle International prognostic index
MIPI-c	MIPI Combined with Ki-67 Proliferation Index
NHL	Non-Hodgkin lymphoma
DLBCL	Diffuse large B-cell lymphoma
FCL	Follicular center lymphoma
DLCL	Diffuse large cell lymphoma
ALCL	Anaplastic large cell lymphoma
PTCL	Peripheral T-cell lymphoma
FFPE	Formalin-fixed and paraffin-embedded tissues
TBST	TBS + Tween
ECL	Enhanced chemiluminescence
RR	Risk ratios
CI	Confidence intervals
HR	Hazard ratios

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13000-024-01577-z.

Supplementary Material 1

Acknowledgements

This project received financial support from the Shandong Natural Science Foundation under the general project (No. ZR2022MH297) and from the Yantai Science and Technology Plan under project (No. 2021MSGY043). We express our gratitude to Xingwang Sun from the Department of Pathology, Southwest Medical University; Cuijuan Zhang from the Department of Pathology, Cheeloo College of Medicine, Shandong University; and Xiaoyan Lin from the Department of Pathology, Shandong Provincial Hospital affiliated with Shandong First Medical University, for their invaluable assistance in providing relevant case information. We also extend our appreciation to the patients' relatives and families for granting us permission to share their medical history and clinical course.

Author contributions

※Y. Yang and L. Song made qual contribution to this research.*Corresponding author: Y. Zhang (404269654@qq.com) and G. Yu (guohuayu@qdu.edu.cn). This project received financial support from the Shandong Natural Science Foundation under the general project (No. ZR2022MH297) and from the Yantai Science and Technology Plan under project (No. 2021MSGY043). We express our gratitude to Xingwang Sun from the Department of Pathology, Southwest Medical University; Cuijuan Zhang from the Department of Pathology, Cheeloo College of Medicine, Shandong University; and Xiaoyan Lin from the Department of Pathology, Shandong Provincial Hospital affiliated with Shandong First Medical University, for their invaluable assistance in providing relevant case information. We also extend our appreciation to the patients' relatives and families for granting us permission to share their medical history and clinical course.

Funding

This project is supported by the general project ZR2022MH297 of Shandong Natural Science Foundation and the project 2021MSGY043 of Yantai Science and Technology Plan.

Data availability

No datasets were generated or analysed during the current study.

Declarations

Competing interests

The authors declare no competing interests.

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Received: 12 April 2024 / Accepted: 7 November 2024
Published online: 21 November 2024

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