High frequency and prognostic value of *MYD88* L265P mutation in diffuse large B-cell lymphoma with R-CHOP treatment

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Abstract. The aim of this study was to analyze the prevalence and prognostic value of myeloid differentiation factor 88 (*MYD88*) L265P in diffuse large B-cell lymphoma (DLBCL) patients treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP). We assessed the *MYD88* L265P mutation using an allele-specific semi-nested polymerase chain reaction method in 53 DLBCL patients treated with R-CHOP. The *MYD88* L265P mutation was detected in 16 of 53 DLBCL (30.19%) samples from patients treated with R-CHOP. Age and location were statistically significantly associated with *MYD88* L265P (P=0.025, 0.033, respectively), while treatment response and tumor recurrence were not. Univariate analysis showed that B symptoms (P=0.004) and Ki-67 (P=0.03) were significantly associated with progression-free survival (PFS), while *MYD88* L265P showed no significant association with overall survival and PFS. Multivariate analysis showed that B symptoms were significantly associated with PFS. Our study suggests that the prognostic value of *MYD88* L265P in DLBCL patients with R-CHOP requires further research.

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Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common type of malignant lymphoma in adults, accounting for 31% of all non-Hodgkin's lymphoma (NHL) in western countries (1). DLBCL can be divided into two main subtypes, germinal center B-cell-like (GCB) and activated B-cell-like (non-GCB), based on evaluation of the cell of origin using gene expression profiling (2). Non-GCB DLBCL tends to have an inferior prognosis compared to GCB DLBCL, with a 3-year progression-free survival (PFS) rate of 40% compared to 75% in GCB DLBCL (3).

The combination chemotherapy regimen with cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) plus rituximab (R-CHOP) is the new standard in first-line therapy for DLBCL, which can significantly improve overall survival (OS) in both GCB and non-GCB DLBCL. Previous studies have shown that 76% of DLBCL patients acquire complete response (CR) with R-CHOP, while ~40% of patients will have an initial response followed by refractory or relapsed disease and most of these patients will eventually succumb to disease (4,5). Therefore, researchers are studying the molecular biology and genetics of tumor cells in order to discover novel biomarkers, provide new therapeutic targets, and develop new ideas to improve prognosis.

Myeloid differentiation factor 88 (*MYD88*) is the first identified member of the Toll-interleukin-1 (IL-1) receptor (TIR) family, an adaptor protein that mediates toll and interleukin receptor signaling and activates nuclear factor-κB (NF-κB) pathways (6). The constitutive activation of NF-κB pathways is a distinguishing feature of non-GCB DLBCL (7,8). Ngo *et al* identified that the *MYD88* signaling pathway is essential for the pathogenesis of non-GCB DLBCL. Among mutations affecting

this pathway, the *MYD88* L265P mutation is the most frequent and has the most severe oncogenic effects through its alteration of NF-κB signaling pathways (9). This mutation was identified in 29% of non-GCB DLBCL but is rare in GCB DLBCL (9).

To our best knowledge, there are seven studies investigated the prognosis value of *MYD88* L265P in DLBCL. Three studies reported that the *MYD88* L265P mutation was not a significant prognostic indicator for DLBCL and primary breast diffuse large B-cell lymphoma (PBDLBCL) (10-12). Nevertheless, the other four studies found that MYD88 L265P mutation was associated with poor prognosis of DLBCL, primary cutaneous diffuse large B-cell lymphoma, and primary central nervous system lymphoma (13-16). Researchers have not reached a consensus regarding the role of *MYD88* L265P as a prognostic factor for this subset of DLBCL patients.

With the arrival of various targeted therapeutic agents acting on NF-κB pathways, mutational analysis of a limited number of genes in these pathways could help in selecting an optimal treatment strategy in DLBCL (17,18). The majority of non-GCB DLBCL patients treated with the R-CHOP regimen have poor outcomes, which raises concerns regarding the *MYD88* L265P mutation. To the best of our knowledge, there has been no analysis regarding the association between treatment response to R-CHOP and the *MYD88* L265P mutation in DLBCL patients. Therefore, in our study we investigated the prevalence of the *MYD88* L265P mutation in patients with DLBCL and evaluated its association with the response to R-CHOP and other clinicopathologic characteristics, including patient outcome.

Materials and methods

Patients and sample collection. This study was retrospective in nature and included 53 patients who were newly diagnosed with DLBCL between January 2007 and January 2015 in the Sichuan Cancer Hospital based on the current World Health Organization classifications (19). Inclusion criteria were as follows: i) Available clinical and follow-up data; ii) CD20-positive; iii) undergoing R-CHOP chemotherapy for at least 3 continuous cycles; and iv) tumor samples available at diagnosis for DNA analysis. Classification into the GCB/non-GCB subgroups by immunohistochemistry followed the algorithm of Hans (20). Overall survival (OS) was defined as the period from clear diagnosis to death, lost follow-up or deadline. Progression-free survival (PFS) was defined as the period from clear diagnosis of the tumor to first tumor progression, death, lost follow-up or deadline.

DNA was extracted from 4% formalin-fixed paraffin-embedded tissues with the QIAamp DNA FFPE Tissue kit (Qiagen, Ltd., Sussex, UK) following the manufacturer's instructions. The L265P mutant of *MYD88* was prepared by PCR with site-directed mutagenic primers using DNA from a healthy individual as a positive control, and the wild-type *MYD88* allele from a healthy person was used as a reference (Table I). L265P mutant DNA and wild-type DNA was validated by examination of agarose gels and Sanger sequencing (Fig. 1). The standards for *MYD88* L265P were generated by a serial dilution of the mutant DNA with the wild-type DNA (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12}, 10^{-13}). All primers were designed using Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA, USA). Primer synthesis and Sanger sequencing were conducted by Tsingke (Chengdu, China).

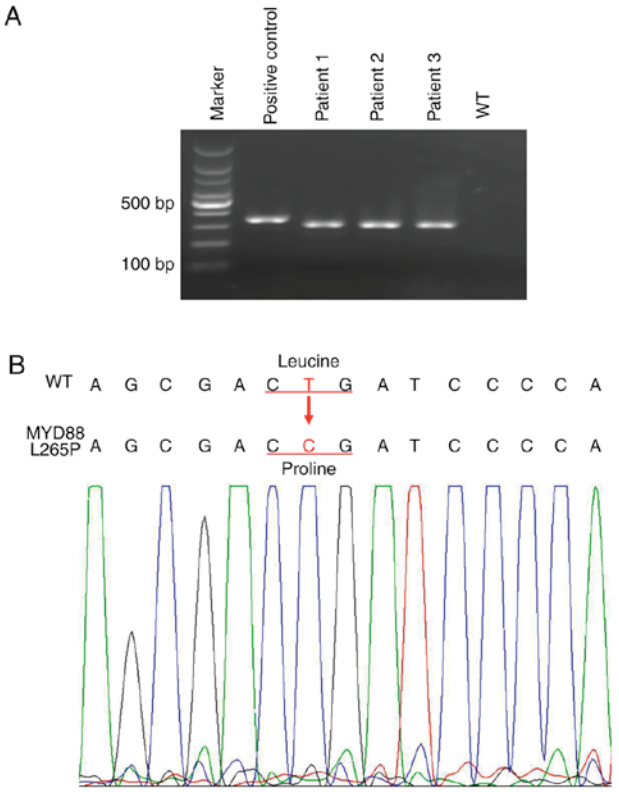

Figure 1. Result of site-directed mutagenesis polymerase chain reaction. (A) Agarose gel electrophoresis of PCR products. The size of 'Positive control' is 340 bp and was prepared by PCR with site-directed mutagenic primers from the DNA of a healthy person. 'Patient 1', 'Patient 2', and 'Patient 3' represent the enrolled patients harboring the MYD88 L265P mutation. Fragment size in these cases is 304 bp, which is the size of the product from the second round of PCR. (B) Sequence analysis of PCR products. PCR, polymerase chain reaction; MYD88, Myeloid differentiation factor 88.

The study was performed after patients signed informed consent, and it was approved by the Ethics Committee of the Sichuan Cancer Hospital in accordance with the Declaration of Helsinki.

Development of allele-specific semi-nested PCR (ASSN-PCR) assay for MYD88 L265P assessment. The ASSN-PCR method included two steps of PCR. The first round was a conventional AS-PCR assay. We designed two reverse primers to separate the mutant and wild-type alleles of *MYD88* L265P and one common primer to amplify large fragments to improve the sensitivity of the ASSN-PCR. To increase the specificity of the ASSN-PCR, we introduced two internal mismatches in the second and third positions from the 3'-end in the reverse primer (Table I) (21).

PCR was performed in a total reaction volume of 25 ml, including 50 nM of each primer, 15 ng DNA and 2X master mix (Tsingke). Thermal cycling conditions consisted of the following: Five minutes of preheating at 95˚C, followed by 40 cycles of 30 sec at 95˚C, 45 sec at 56˚C, and 1 min at 72˚C. The final step was an extension step for 5 min at 72˚C.

The second round was a quantitative AS-(q)PCR assay for the assessment of *MYD88* L265P. After the first round of conventional PCR, we obtained two PCR products for each specimen (wild-type products, W; likely mutation products, M).

Table I. Primers of PCR in this study.

Variable	Primers
Site-directed mutagenesis primers (340 bp)	F: 5'-CAGCCTCTCTCCAGGTAAGCTCAACC-3' R: 5'-ATTGCCTTGTACTTGATGGGGATCGGTCGCTTCTG-3' (containing mutation L265P base)
First round general PCR (604 bp)	F: 5'-CAGCCTCTCTCCAGGTAAGCTCAACC-3' RW: 5'-ATTGCCTTGTACTTGATGGGGATCA-3' RM: 5'-CCTTGTACTTGATGGGGAAGG-3' (two internal mismatches in the 2nd and 3rd position from the 3'-end)
Second real-time PCR (304 bp)	F: 5'-GGCAAGAGAATGAGGGAATGTG-3' RW: 5'-GCCTTGTACTTGATGGGGAACA-3' RM: 5'-CCTTGTACTTGATGGGGAACG-3' (an internal mismatch in the 3rd position from the 3'-end)

F, forward; RW, reverse wild-type primer; RM, reverse mutation primer.

To optimize the real-time PCR, we diluted W and M to 10^{-8} and 10^{-4}, respectively. Then, the second round of real-time AS-PCR was developed using specific primers (Table I) with diluted W, diluted M and standards as templates. Power SYBR Green PCR Master Mix was applied following the manufacturer's instructions and reactions were run on the ABI Prism 7500 Sequence Detection system (Applied Biosystems, Foster City, CA, USA). The contents of the PCR reactions were the same as in the first round of AS-PCR. Thermal cycling conditions were: Two minutes of preheating at 95˚C, followed by 40 cycles of 30 sec at 95˚C, 45 sec at 62˚C, and routine melt curve cycling conditions. The products of the second round of real-time AS-PCR were confirmed by Sanger sequencing.

Interpretation of AS-qPCR results. The $C_{T\ (MYD88\ L265P)}$ represents the amount of mutated *MYD88* L265P within the sample, while the $C_{T\ (wild\text{-}type)}$ reflects the total amount of *MYD88* allelic template in the sample. ΔC_T cut-off value was measured using the formula below:

$$\Delta C_T = C_{T(MY88\ L265P)} - C_{T(wild\text{-}type)}$$

$$\Delta C_T \text{ cut-off} = C_{T(10^{-8})} - C_{T(AR\text{-}W)}$$

where $C_{T\ (10^{-8})}$ is the average $C_{T\ (MYD88\ L265P)}$ value of the 10^{-8} dilution of positive control template mixed into a normal DNA template, and $C_{T(AR\text{-}W)}$ is the average $C_{T(wild\text{-}type)}$ value of allelic reference.

A positive result for the *MYD88* L265P mutation is defined as a mean ΔC_T value less than ΔC_T cut-off value for each sample, while a negative mutation result (i.e., no mutation detected) is defined as a mean ΔC_T exceeding the ΔC_T cut-off value.

Statistical analysis. All statistical analyses were conducted using SPSS version 20.0 (IBM Corp., Armonk, NY, USA). We used a Chi-square or Fisher's exact test to analyze the association between categorical variables and the *MYD88* L265P mutation, and the Mann-Whitney U test to evaluate the association between continuous variables and the *MYD88* L265P mutation. The association between *MYD88* L265P and patient survival (OS and PFS) was evaluated by survival curves using the Kaplan-Meier method and the log-rank (Mantel-Cox test). Cox regression was applied to evaluate the independent factors for OS and PFS. Two-sided P-value <0.05 was considered to indicate a statistically significant difference.

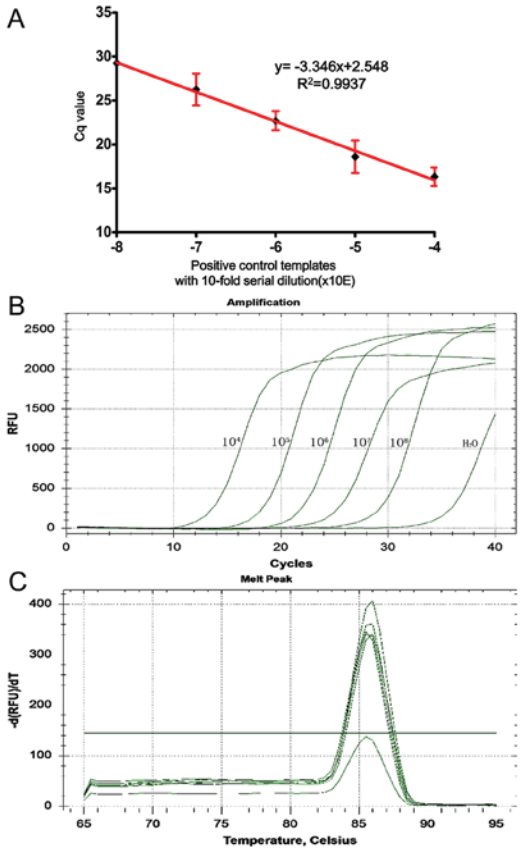

Figure 2. Sensitivity and specificity of AS-PCR assay. (A) Standard curve for AS-qPCR assay. (B) Amplification curves for different dilutions. (C) Dissociation curves for different dilutions. RFU, relative fluorescence units; AS-qPCR, allele-specific quantitative polymerase chain reaction.

Table II. Clinicopathologic characteristics of DLBCL cases.

Clinicopathologic parameters	No. of patients (n=53)	Proportion (%)
Age (years)		
<60	32	56.604
≥60	21	43.396
Sex		
Male	31	58.491
Female	22	41.509
Location		
Nodal	25	47.169
Extranodal	28	52.831
B symptom		
Absent	41	77.358
Present	12	22.642
Clinical stage		
Low (I-II)	26	49.057
High (III-IV)	27	50.943
Subgroup		
GCB	11	20.755
Non-GCB	42	79.245
IPI score		
Low (0-2)	35	66.038
High (3-5)	18	33.962
ECOG score		
Low (0-1)	45	84.906
High (2-4)	8	15.094
LDH		
Normal	24	45.283
High	29	54.717
Ki-67		
≤50	42	79.25
>50	11	20.75
Treatment response		
CR/PR	47	88.679
PD/SD	6	11.321
Recurrence		
Absent	28	52.83
Present	25	47.17

Table III. The association analysis between clinical characters and *MYD88* mutation in DLBCL cases.

Clinicopathologic parameters	No.	*MYD88* mutation (%)		P-value
		WT	L265P	
Age (years)				**0.025**
<60	32	26 (70.03)	6 (37.5)	
≥60	21	11 (29.7)	10 (62.5)	
Sex				0.828
Male	31	22 (59.5)	9 (56.2)	
Female	22	15 (40.5)	7 (43.8)	
Location				**0.033**
Nodal	25	21 (56.8)	4 (25.0)	
Extranodal	28	16 (43.2)	12 (75.0)	
B symptom				0.787
Absent	41	29 (78.4)	12 (75.0)	
Present	12	8 (21.6)	4 (25.0)	
Clinical stage				0.611
Low (I-II)	26	19 (51.4)	7 (43.8)	
High (III-IV)	27	18 (48.6)	9 (56.2)	
Subgroup				0.275
GCB	11	6 (16.2)	5 (31.2)	
Non-GCB	42	31 (83.8)	11 (68.8)	
IPI score				0.322
Low (0-2)	35	26 (70.3)	9 (56.2)	
High (3-5)	18	11 (29.7)	7 (43.8)	
ECOG score				0.729
Low (0-1)	45	31 (83.8)	14 (87.5)	
High (2-4)	8	6 (16.2)	2 (12.5)	
LDH				0.454
Normal	24	18 (48.6)	6 (37.5)	
High	29	19 (51.4)	10 (62.5)	
Ki-67				0.632
<50	5	3 (8.1)	2 (12.5)	
≥50	48	34 (91.1)	14 (87.5)	
Treatment response				0.655
CR/PR	47	32 (86.5)	15 (93.8)	
PD/SD	6	5 (23.5)	1 (6.2)	
Recurrence				0.743
Absent	28	19 (51.4)	9 (56.2)	
Present	25	18 (48.6)	7 (43.8)	

Bold values indicate P<0.05.

Results

Specificity and sensitivity of AS-qPCR assay. We analyzed the sensitivity and specificity of the AS-qPCR assay in detecting the *MYD88* L265P mutation using a standard curve. The standard curve amplification plot and linear regression (the standards diluted from 10^{-4} to 10^{-8}) generated a correlation coefficient of 0.9937, with a y-intercept value of 2.548 and a slope of -3.346. The calculated amplification efficiency was 99% (Fig. 2A and B). This method was determined to be suitable for the detection and quantitative assessment of *MYD88* L265P and is capable of detecting *MYD88* L265P at a lower limit of 10^{-12}. Analysis of the melt curves showed that the PCR assay had good specificity (Fig. 2C). ΔC_T cut-off value has a value of 6.01±0.076. Thus, the sample ΔC_T value of all mutant specimens for each assay ≤6 or >6 was interpreted as positive or negative for the *MYD88* L265P mutation, respectively.

Table IV. The mutation status of enrolled patients.

ID	MYD88	Sex	Age (years)	Extranodal sites (NO.; location)
1	Wide-type	Male	60	1; testis
2	Wide-type	Female	48	3; lung, liver, bone marrow
3	Wide-type	Male	68	0
4	Wide-type	Male	68	2; Liver, CNS
5	L265P	Male	55	0
6	Wide-type	Male	48	2; Oropharynx, stomach
7	Wide-type	Female	58	1; Left frontal lobe
8	Wide-type	Male	26	0
9	Wide-type	Female	41	1; stomach
10	L265P	Female	61	0
11	L265P	Male	70	1; testis
12	Wide-type	Male	25	0
13	L265P	Female	62	3; Bone marrow, iliac, calf skin
14	L265P	Female	40	4; Breast, CNS, spinal cord, pelvic cavity
15	Wide-type	Male	61	1; thyroid
16	Wide-type	Female	61	0
17	Wide-type	Male	27	1; stomach
18	Wide-type	Male	78	1; bone
19	Wide-type	Female	74	1; skin
20	Wide-type	Female	79	1; thyroid
21	Wide-type	Male	49	2; bone marrow, bone
22	Wide-type	Female	53	0
23	L265P	Female	46	1; Bone
24	Wide-type	Female	32	1; Breast
25	Wide-type	Male	73	0
26	Wide-type	Female	20	0
27	Wide-type	Female	56	2; Psoas muscle, vertebral body
28	Wide-type	Female	44	0
29	L265P	Male	57	1; lung
30	L265P	Male	40	1; CNS
31	L265P	Male	34	0
32	Wide-type	Male	56	0
33	Wide-type	Male	54	1; lung
34	L265P	Male	67	0
35	L265P	Male	62	1; thyroid
36	Wide-type	Female	53	0
37	Wide-type	Male	52	0
38	Wide-type	Male	64	0
39	L265P	Female	75	1; thyroid
30	L265P	Male	63	1; lung
41	Wide-type	Male	43	0
42	Wide-type	Male	75	0
43	Wide-type	Female	49	0
44	Wide-type	Male	47	0
45	Wide-type	Female	55	0
46	Wide-type	Male	66	0
47	Wide-type	Female	43	0
48	L265P	Male	72	1; stomach
49	Wide-type	Male	60	0
50	Wide-type	Male	41	0

Table IV. Continued.

ID	MYD88	Sex	Age (years)	Extranodal sites (NO.; location)
51	L265P	Female	66	1; thyroid
52	Wide-type	Male	45	1; stomach
53	L265P	Female	64	2; lung, bone

The unit of age is years. CNS, central nervous system.

Table V. Clinical characters affecting progression-free and overall survival.

	Univariate analysis				Multivariate analysis	
	OS		PFS		PFS	
Clinicopathologic parameters	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
MYD88 (WT vs. L265P)	0.97 (0.31-3.04)	0.952	0.99 (0.41-2.39)	0.981		
Age (<60 vs. ≥60 years)	1.16 (0.43-3.12)	0.766	0.83 (0.37-1.86)	0.645		
Sex (male vs. female)	1.31 (0.48-3.56)	0.596	0.93 (0.42-2.04)	0.848		
Location (nodal vs. extranodal)	0.66 (0.25-1.76)	0.401	1.39 (0.61-3.15)	0.423		
B symptom (absent vs. present)	2.04 (0.69-5.97)	0.184	3.29 (1.39-7.80)	**0.004**	3.08 (1.28-7.41)	**0.012**
Clinical stage (I-II vs. III-IV)	2.50 (0.80-7.77)	0.102	1.45 (0.64-3.30)	0.370		
Subgroup (GCB vs. non-GCB)	3.47 (0.46-26.40)	0.200	2.12 (0.63-7.08)	0.207		
IPI score (0-2 vs. 3-5)	1.67 (0.62-4.46)	0.304	1.14 (0.51-2.55)	0.754		
ECOG score (0-1 vs. 2-4)	2.18 (0.70-6.80)	0.166	2.02 (0.30-5.05)	0.122		
LDH (normal vs. high)	1.50 (0.54-4.15)	0.429	0.93 (0.42-2.04)	0.845		
Ki-67 (<50 vs. ≥50)	2.02 (0.27-15.42)	0.487	0.32 (0.11-0.96)	**0.030**	0.38 (0.12-1.15)	0.085

OS, overall survival; PFS, progression free survival; HR, hazard ratio; CI, confidence interval; Bold values indicate P<0.05.

Correlation between MYD88 L265P status and clinical characteristics. The clinicopathologic characteristics of the 53 DLBCL patients are listed in Table II and associations between clinicopathologic factors and the *MYD88* mutation status are summarized in Table III. Among 53 DLBCL patients, 28 cases presented with extranodal invasion, and mutation statuses of the DLBCL patients are listed in Table IV. Using the ASSN-PCR assay, we detected the *MYD88* L265P mutation in 16 out of 53 R-CHOP-treated DLBCL patients (30.19%). The *MYD88* L265P mutation rate in the central nervous system (CNS) and testicular DLBCLs is 60% (3/5) (Table IV). Further, by excluding the CNS and testicular DLBCLs, the *MYD88* L265P mutation ratio is 27.08% (13/48). We discovered that the *MYD88* L265P mutation was not statistically significantly associated with treatment response or tumor recurrence (P>0.05). However, the *MYD88* L265P mutational status showed a significant association with age (P=0.025) and location (P=0.033).

MYD88 L265P mutation and survival analysis. The median follow-up time across the entire cohort was 18 months (range, 3-80 months), with 3-year OS and PFS rates of 56 and 42%, respectively. Univariate analysis showed that B symptoms (P=0.004) and Ki-67 (P=0.03) were significantly associated with PFS. However, the *MYD88* mutation status and other factors showed no association with OS or PFS (Fig. 3). Cox regression showed that B symptoms remained a significant risk factor for PFS (P=0.012, hazard ratio (HR) = 3.08; 95% CI = 1.28-7.41) (Table V) after controlling for other factors. Further subgroup analysis showed that *MYD88* mutation status is not significantly associated with survival in either the Non-GCB group or the GCB group (all P>0.05; Fig. 4).

Discussion

In our study, we developed the ASSN-PCR to detect the *MYD88* L265P mutation and successfully revealed a high prevalence of the *MYD88* L265P mutation in DLBCL patients undergoing R-CHOP treatment. However, we did not have enough evidence to conclude that there was a significant association between the *MYD88* L265P mutation and treatment response or tumor recurrence. The *MYD88* L265P mutation may not be a significant prognostic factor for DLBCL patients undergoing R-CHOP treatment.

Previous studies have shown that *MYD88* L265P was a key player in the constitutive activation of NF-κB pathways in lymphomagenesis. It was frequently detected in non-GCB type DLBCL (21.6-32.5%), as well as extranodal DLBCL, such as in the central nervous system and testes (50 and 90%,

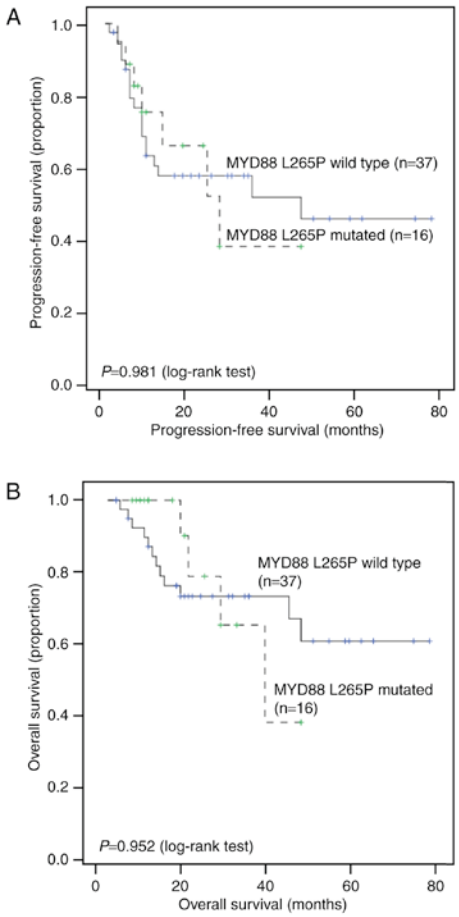

Figure 3. Kaplan-Meier survival curves based on *MYD88* L265P in DLBCL. (A) Progression-free survival of 53 patients with DLBCL. (B) Overall survival of 53 patients with DLBCL. MYD88, myeloid differentiation factor 88; DLBCL, diffuse large B-cell lymphoma.

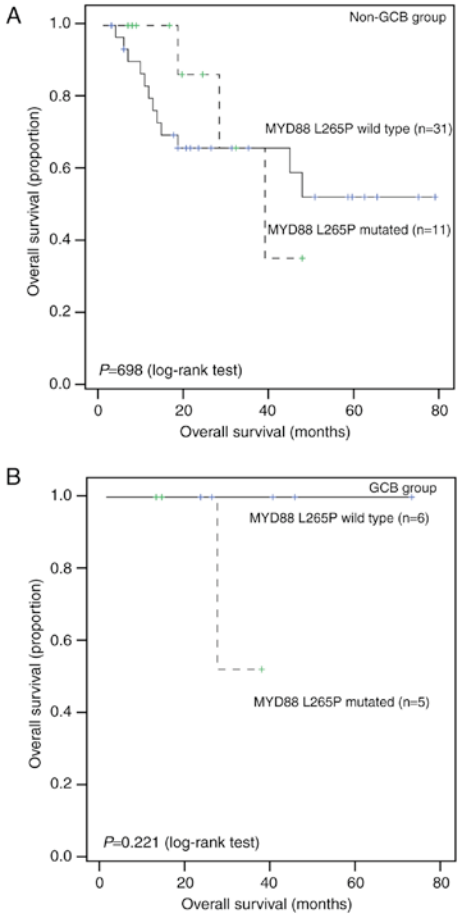

Figure 4. Analysis of the prognostic value of *MYD88* L265P status for overall survival in the (A) non-GCB and (B) GCB subgroups. MYD88, myeloid differentiation factor 88; GCB, germinal center B-cell-like; non-GCB, activated B-cell-like.

respectively) (22-24). In our study, *MYD88* L265P was identified in 30.19% of all DLBCL patients treated with R-CHOP. The *MYD88* L265P mutation was predominantly detected in non-GCB type DLBCL (68.8 vs. 31.2% GCB type), as was previously reported (22,25). It was reported that excessive activation of NF-κB pathways frequently existed in non-GCB type DLBCL, which may explain the predominant existence of *MYD88* L265P in this subtype (21,26).

In our study, 5 out of 7 primary extranodal DLBCL patients harboring *MYD88* L265P were in the advanced stage. This result is consistent with a previous study (27) that suggests that the *MYD88* L265P gene mutation may be an early molecular change in DLBCL tumorigenesis (28). Moreover, we observed a significant association between the *MYD88* L265P mutation and age as well as location, which is consistent with the previous study (15). With increasing age, the incidence of poor prognosis factors, such as various genetic features, non-GCB subtype, and BCL2 expression will increase for DLBCL (29). This may explain the predominance of *MYD88* L265P in elderly DLBCL patients. Meanwhile, we discovered that the *MYD88* L265P mutation was not significantly associated with treatment response or tumor recurrence.

To evaluate the prognostic value of *MYD88* L265P for DLBCL, we conducted univariate analyses and multivariate Cox regression analyses. The presence of B symptoms and Ki-67>50% indicated poor prognosis. After controlling for other factors and conducting the cox regression analysis, Ki-67 lost its prognostic significance. Our results suggest that *MYD88* L265P does not affect the outcome of R-CHOP-treated DLBCL patients. Recent meta-analysis study revealed that the MYD88 L265P mutation was associated with a low survival rate, except for individual studies (30). However, since the study didn't enrolled all published data into pooled analysis, and pathological type and clinical treatment is various, additional studies are required with increased number of patients and differential patient stratification to determine the role of this mutation.

Limitations of our study include that patients had a relatively short period of follow-up and the sample size was relatively small. Therefore, further large-scale, multi-center, prospective studies with longer follow-up periods are warranted. Although there are some limitations, the treatment of enrolled patients was homogeneous; moreover, it is worth noting that we are the first group to use semi-nested PCR to detect the *MYD88* L265P mutation and that the prevalence of detected *MYD88* L265P mutations in our study was 30.19%, which is higher than the prevalence seen when using previously reported methods (13,31). Excluding the CNS and testicular DLBCLs, the *MYD88* L265P mutation ratio is 27.08%, which is higher than the pooled published data (16.5%) (30).

As far as we know, some investigators used general PCR and sequencing to detect MYD88 L265p mutations (10,11,27).

MYD88 L265P mutation rate is low and heterogeneous (6.5-19.3%). Since Sanger sequencing might be unable to detect lower frequency mutations in FFPE samples with fragmented nucleic acids, AS-PCR was applied to detect the MYD88 L265P mutation, which is a highly sensitive and cost-effective (24). Two powerful studies utilized this method and detected relative high rate of MYD88 L265P mutation (22-22.3%) (32,33). Nested PCR is a modification of polymerase chain reaction intended to reduce non-specific binding in products due to the amplification of unexpected primer binding sites. In this study, we combined AS-PCR and semi-nested PCR to assess the *MYD88* L265P status. This method can overcome the issues involved with DNA extraction from paraffin wax, such as poor quality and low concentration, thus improving the sensitivity and specificity of PCR. This method can detect *MYD88* L265P at a lower limit of 10^{-12}, which is more sensitive than the 0.1% previously published for allele-specific oligonucleotide PCR alone (34).

In conclusion, this study indicates that the *MYD88* L265P mutation is not associated with treatment response or tumor recurrence and that *MYD88* L265P does not affect patient outcomes and may not be a prognostic factor for DLBCL patients undergoing R-CHOP treatment. Current data should be validated in further studies.

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