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Research Article

Waldenström macroglobulinemia and non-IgM-type lymphoplasmacytic lymphoma are genetically similar

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Short Title: Genetic similarity between WM and non-IgM-type LPL

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Abstract

Introduction

Waldenström macroglobulinemia (WM) represents a subset of lymphoplasmacytic lymphoma (LPL) with the immunoglobulin (Ig)M paraprotein. *MYD88* L265P and *CXCR4* mutations are common mutations in WM patients, and mutations in *ARID1A* and *KMT2D* (*MLL2*) have also been reported. However, little information has been accumulated on genetic changes in LPL with other paraproteins like IgG.

Methods

We therefore aimed to evaluate genetic differences between WM and LPL with non-IgM paraprotein (non-IgM-type LPL) using targeted next-generation sequencing (NGS) in 20 Japanese patients (10 with WM, 10 with non-IgM-type LPL).

Results

Mutations were detected in *ARID1A* (10%), *CXCR4* (20%), *MYD88* (90%), and *KMT2D* (0%) for WM patients, and in *ARID1A* (10%), *CXCR4* (20%), *MYD88* (70%), and *KMT2D* (10%) for non-IgM-type LPL patients. No significant differences were identified. No mutations were detected in *NOTCH2*, *PRDM1*, *CD274* (*PD-L1*), *PDCD1LG2* (*PD-L2*), *RAG2*, *MYBBP1A*, *TP53*, or *CD79B*.

Discussion

Mutant allele frequency in *MYD88* L265P did not differ significantly between WM and non-IgM-type LPL. Most mutations detected by NGS were subclonal following *MYD88* L265P, although one non-IgM-type LPL patient harbored only *CXCR4* S338X mutation. Our NGS analyses reveal genetic characteristics in LPL patients and suggest genetic similarities between these two subsets of LPL, WM and non-IgM-type.

Introduction

Waldenström macroglobulinemia (WM) represents a subset of lymphoplasmacytic lymphoma (LPL) involving the immunoglobulin (Ig)M paraprotein. One genetic hallmark of WM is the *MYD88* L265P mutation, which is detectable in approximately 90% of WM patients [1-5], but is absent or only rarely present in other patients with overlapping clinicopathological features, including other indolent mature B-cell tumors and myeloma (≤ 10%) [3,5-8]. Mutations in the C-terminal tail of *CXCR4*, which are similar to the autosomal-dominant gain-of-function mutations that cause WHIM (warts, hypogammaglobulinemia, infections, and myelokathexis) syndrome [9], are also reportedly common in WM patients (around 30–40%) [1,2,5,10]. Other genetic changes including mutations in *ARID1A* (3–17%), *NOTCH2* (3–7%), *PRDM1* (4%), *RAG2* (3%), *KMT2D* (*MLL2*) (7–16%), *MYBBP1A* (0–7%), *TP53* (7–8%), and *CD79B* (3–7.8%) have also been reported [1,4,5]. In LPL with other paraproteins like IgG (non-IgM-type LPL), the presence or absence of *MYD88* L265P has only been reported in a small number of patients, and several reports collectively show that 16 of 39 non-IgM-type LPL patients (41%) harboring *MYD88* L265P [5,11-15]. However, comprehensive information on the genetic changes in non-IgM-type LPL remains unclear.
The present study aimed to clarify the patterns of mutations in patients with WM and non-IgM-type LPL using targeted next-generation sequencing (NGS) and to evaluate genetic differences between these two groups. In addition to the 10 genes related to WM mentioned above, we also targeted *CD274* (*PD-L1*) and *PDCD1LG2* (*PD-L2*) on the grounds that these genes are related to the pathogenesis of other B-cell tumors, including primary mediastinal B-cell lymphoma, diffuse large B-cell lymphoma, and classical Hodgkin lymphoma.

Materials and Methods

Patients and sample collection

Patients included in this study had been diagnosed with LPL by at least two pathologists specializing in lymphoma at Gunma University Hospital according to World Health Organization classifications [16]. This study was approved by the institutional review board at Gunma University (approval no. HS2017-112). Supplementary material 1 shows specimen information used in this study. Twenty untreated patients with LPL (WM, n=10; non-IgM-type LPL, n=10) were included in this study. Non-IgM-type LPL includes 8 cases with significantly increased IgG only, IgG and IgM, and IgA and IgM. Serological tests confirmed IgG 731 mg/dl and IgM 820 mg/dl in the IgG/IgM case and IgA 2223 mg/dl and IgM 487 mg/dl in the IgA/IgM case. More male patients were in the WM group (P = 0.019). Informed consent was assumed based on the provision of an opt-out option on the hospital website. DNA was extracted from 8 samples of bone marrow mononuclear cells and 4 samples of peripheral blood mononuclear cells with SepaGene® (Eidia Co., Tokyo, Japan), and 5–8 slices (10 µm) of 8 formalin-fixed paraffin-embedded (FFPE) samples made from bone marrow or lymph nodes with a QIAamp® DNA FFPE Tissue Kit (Qiagen GmbH, Hilden, Germany).

Mutation analysis using NGS

Targeted NGS analysis was performed using an AmpliSeq™ Custom DNA Panel for Illumina® (Illumina, San Diego, CA, USA). We designed primers covering the coding regions of the following 12 genes: *ARID1A*, *NOTCH2*, *CXCR4*, *MYD88*, *PRDM1*, *CD274*, *PDCD1LG2*, *RAG2*, *KMT2D*, *MYBBP1A*, *TP53*, and *CD79B*. Our panel consisted of 585 amplicons, targeting 53,822 bp and covering 47,839 bp, enabling 89% coverage of targeted areas. NGS libraries were prepared from approximately 6.7 ng/µl of DNA and sequencing was performed using NextSeq™ 500 (Illumina). The resulting data were aligned using the DNA Amplicon app (Illumina). Mean coverage depth of amplicons was 20,483 to 53,025, sufficiently satisfying the reliability requirement of amplification using DNA from FFPE samples. The detected mutations were then annotated and filtered using VariantStudio v3.0 software (Illumina) based on GRCh37/hg19 according to the following criteria: read depth < 500; alternative variant frequency < 5% (< 1% on *MYD88* L265P); global frequency > 1% or East Asian population frequency > 1%, based on the 1000 Genomes Project; and synonymous mutations, in-frame mutations, and missense mutations scored as “tolerated” by SIFT or “benign” by PolyPhen.

Analysis to confirm mutations detected by NGS

To confirm the *MYD88* L265P mutation, we designed mutant-specific reverse primers having substitutional base matching the variant nucleotide at the 3' terminal, then performed allele-specific oligonucleotide polymerase chain reaction (ASO-PCR). This method enabled detection of low-frequency mutations [17]. Details about primers are shown in Supplementary material 2. The PCR reaction mixture (25 µl) contained 10 ng of DNA and 5 pmol of each primer. DNA amplification was performed under the following protocol: one cycle at 94°C for 5 min, 40 cycles at 94°C for 20 s, 60°C for 20 s, and 72°C for 20 s. To confirm mutations in *CXCR4*, we designed 18mer peptide nucleic acid (PNA) (FASMAC, Kanagawa, Japan) matched wild-type alleles and performed the PNA PCR

clamp method [18]. The PCR reaction mixture (25 µl) contained 10 ng of DNA, 5 pmol of each primer, and 20 pmol of PNA. The protocol for DNA amplification was: one cycle at 95°C for 10 min, 38 cycles at 94°C for 30 s and 62°C for 1 min. Other mutations in *ARID1A*, *MYD88*, and *KMT2D* were amplified similarly by conventional PCR without using PNA. Details of the primers are shown in Supplementary material 2. The PCR products were cleaned up with ExoSAP-IT™ Express PCR Cleanup Reagents (Thermo Fisher Scientific, Waltham, MA), and sent to Eurofins Genomics K.K. (Tokyo, Japan) for Sanger sequencing. Sequencing data were analyzed using SnapGene version 4.1.8 software (GSL Biotech LLC, Chicago, IL) and identified based on the GenBank database (National Center for Biotechnology Information, Bethesda, MD). To confirm frameshift mutations, TA cloning was performed using pGEM®-T Easy Vector System (Promega Corporation, Madison, WI), DNA Ligation Kit version 2.1 (Takara Bio, Shiga, Japan), and Competent high DH5α (Toyobo Co., Osaka, Japan) before Sanger sequencing.

Analysis of V(D)J recombination

To confirm that samples in which no mutations were detected by NGS did actually contain tumor cells, clonality analysis based on V(D)J recombination was performed by PCR with consensus PCR primers set between framework region 3 and the joining region of the Ig heavy chain gene (Supplementary material 2) [19,20]. The PCR reaction mixture (25 µl) contained 100 ng of DNA and 200 nmol of each primer. The protocol for DNA amplification was: one cycle at 95°C for 10 min, 40 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, one cycle at 72°C for 5 min, followed by heteroduplex analysis [21]. PCR products underwent polyacrylamide gel electrophoresis (PAGE) with a 4–20% Tris-borate-EDTA-PAGE mini (Tefco, Tokyo, Japan).

Statistics

Gender bias and frequencies of patients harboring mutations were compared between WM and non-IgM-type LPL using Pearson's chi-squared test. Mutant allele frequencies of each sample were analyzed using the Mann-Whitney U test. Values of $P<0.05$ were considered statistically significant. Statistical analysis was conducted using IBM® SPSS® Statistics version 25 software (International Business Machines Corporation, Armonk, NY).

Results

Mutations in patients with WM and non-IgM-type LPL detected by NGS

Median age of the patients was slightly lower for non-IgM-type LPL patients than for WM patients. Male sex was more predominant with WM than with non-IgM-type LPL (Supplementary material 1).

Targeted NGS analysis revealed mutations in WM and non-IgM-type LPL (Figure 1). These mutations passed the filter criteria described in the methods. Mutations in *ARID1A* (1 of 10 patients [10%]), *CXCR4* (2 patients [20%]), and *MYD88* (9 patients [90%]) were detected in WM patients. Mutations in *ARID1A* (1 of 10 patients [10%]), *CXCR4* (2 patients [20%]), *MYD88* (7 patients [70%]), and *KMT2D* (1 patient [10%]) were detected in non-IgM-type LPL patients. No mutations were detected in *NOTCH2*, *PRDM1*, *CD274*, *PDCD1LG2*, *RAG2*, *MYBBP1A*, *TP53*, and *CD79B* in either patient group. The three types of mutation detected in the *CXCR4* gene had been previously reported as mutations related to WM [1,2,10]. The detailed data for detected mutations are shown in Table 1. For the number of patients harboring mutations, no significant differences were evident between WM and non-IgM-type LPL patients (1 patient [10%] versus 1 patient [10%], P = 1.0 in *ARID1A*; 2 patients [20%] versus 2 patients [20%], P = 1.0 in *CXCR4*; 9 patients [90%] versus 7 patients [70%], P = 0.26 in *MYD88*; and 0 patients [0%] versus 1 patient [10%], P = 0.31 in *KMT2D*).

Analysis to confirm mutations detected by NGS

ASO-PCR products for patients harboring *MYD88* L265P showed a bright band on 3% agarose gels (Supplementary material 3A). *MYD88* L265P was detected in 9 WM patients and 7 non-IgM-type LPL patients, corresponding those detected by NGS. Sanger sequencing revealed the mutations detected by NGS in the same patients (Supplementary material 3B). All mutations detected by NGS were thus confirmed by both ASO-PCR and Sanger sequencing.

Analysis of V(D)J recombination

The existence of clonal cell populations was shown as sharp bright bands at around 100 bp on 4–20% Tris-borate-EDTA-PAGE gels (Supplementary material 4). These bands appeared in all PCR products, revealing that all samples in which no mutations were detected by NGS did contain tumor cells.

Mutant allele frequency of *MYD88* L265P and other mutations

We analyzed the frequencies of *MYD88* L265P and other mutations in patients. Figure 2A shows the mutant allele frequency of *MYD88* L265P. Patients with no. 7 in WM and nos. 7, 9, and 10 in non-IgM-type LPL were filtered in NGS analysis because the rates were less than 1%. Regardless of whether the patient was diagnosed with WM or non-IgM-type LPL, no significant differences were evident (mean, 15.2% [standard deviation [SD] 17.5] versus

17.6% [SD 16]; median, 7.6% [interquartile range [IQR] 1.7–28.1%] versus 15.7% [IQR 1.2–32.1%]; P = 1.0) (Figure 2B).
We then compared the mutant allele frequency of *MYD88* L265P with mutations that passed the filter criteria in patients with WM and non-IgM-type LPL (Figure 2C,D).
Most mutations were harbored at almost the same frequency as *MYD88* L265P, although one patient with non-IgM-type LPL harbored only a mutation in *CXCR4* (S338X).

Discussion

MYD88 L265P is the most common mutation in WM and is useful for diagnosing WM. Xu et al and Varettoni et al reported that the proportion of WM patients with *MYD88* L265P was higher than that in IgM-MGUS patients or IgG-MGUS patients (WM, 93%; IgM-MGUS, 54%; IgG-MGUS, 0% and WM, 91%; IgM-MGUS, 60%, respectively) [3,4], and median mutant allele frequency was higher in WM patients (15.4%) than in IgM-MGUS patients (1.05%; P < 0.001) [4]. However, information on non-IgM-type LPL patients was scarce. The present study showed no significant differences in the proportion of patients with *MYD88* L265P (90% versus 70%; P = 0.26) or mean mutant allele frequency of *MYD88* L265P (15.2% [SD 17.5%] versus 17.6% [SD 16%]; median, 7.6% [IQR 1.7–28.1%] versus 15.7% [IQR 1.2–32.1%]; P = 1.0) between patients with WM and non-IgM-type LPL, suggesting that no definitive differences exist between the two groups.
One reason for the variation in mutant allele frequency of *MYD88* L265P is considered to be variability in the amount of tumor cells contained in each sample. In addition, Varettoni et al [4] reported that a higher *MYD88* mutant allele frequency was associated with clinical variables including some of the prognostic factors identified in the WM International Prognostic Scoring System (hemoglobin, albumin, β2-microglobulin levels) and was found to offer a good surrogate marker for clinical disease burden in WM patients. Differences in the condition of each patient were also considered to have contributed to the variation in mutant allele frequency.
CXCR4 mutations are also important determinants of WM disease presentation, as well as treatment outcome [2,22]. Those mutations are primarily subclonal, with highly variable clonal distribution among *CXCR4*-mutated WM patients, and almost all *CXCR4* mutations are harbored in *MYD88* L265P [1,2,5,10]. This study also showed that all patients harboring *CXCR4* mutations were also harboring *MYD88* L265P and mutant allele frequencies of those were almost equal except in one non-IgM-type LPL patient harboring *CXCR4* S338X. In non-IgM-type LPL patients, the acquisition of *CXCR4* mutations may not always follow *MYD88* L265P. Further studies are needed to help expand these findings.
We examined mutations in 12 genes related to the pathogenesis of WM or other B-cell tumors, because the relationship between non-IgM-type LPL and those mutations is obscure in terms of the genetic background. No significant difference in the number of patients harboring mutations in each gene was evident between patients with WM and non-IgM-type LPL, suggesting genetic similarity between WM and non-IgM-type LPL (1 patient [10%] versus 1 patient [10%]; P = 1.0 in *ARID1A*, 2 patients [20%] versus 2 patients [20%]; P = 1.0 in *CXCR4*, 9 patients [90%] versus 7 patients [70%]; P = 0.26 in *MYD88*, and 0 patient [0%] versus 1 patient [10%]; P = 0.31 in *KMT2D*). Although we explored mutations in *CD274* and *PDCD1LG2*, which are related to the pathogenesis of some B-cell tumors and classical Hodgkin lymphoma, no mutations were identified. These observations suggest that the mutations in these genes are not founding mutations in non-IgM-type LPL or WM.
This study performed highly sensitive amplification using ASO-PCR and PNA PCR clamp methods to confirm *MYD88* L265P and *CXCR4* mutations. All mutations detected by NGS were confirmed by both ASO-PCR and Sanger sequencing, showing their suitability for detection. Furthermore, V(D)J recombination analysis confirmed that the samples in which no mutations were detected still contained clonal cell populations, ensuring the results were not due to a lack of tumor cells. Our study showed that DNA extracted from FFPE samples is suitable for targeted NGS analysis with high coverage criterion.
This is the first report to reveal comprehensive information on genetic changes in non-IgM-type LPL among Japanese patients. Some previous reports have described mutation profiles of WM in Asia. Shin et al [23] reported that the frequency of *MYD88*/*CXCR4* mutation in Korean and Caucasian patients with WM was similar, but 5 of the 6 *CXCR4* mutations were nonsense, representing a proportion higher than reported in Caucasian individuals. In our data for Japanese WM patients, the proportion of those (*MYD88* L265P, 90%; *CXCR4* mutations, 20%) was similar to previous reports of non-Asian patients (*MYD88* L265P, approximately 90%; *CXCR4* mutations, 30–40%) [1-5,10], and both detected *CXCR4* mutations were frameshift mutations.

One limitation of this study was that the sample size was small. Some mutations that have previously been reported with WM patients were not detected. A small sample size may make the detection of mutations difficult. Further study is needed to elucidate the precise mutation profiles of non-IgM-type LPL.

Statements

Statement of Ethics

Opt-out informed consent protocol was used for this study. This study and the consent procedure were reviewed and approved by the institutional review board at Gunma University, approval number [HS2017-112].

Conflict of Interest Statement

H.H. has received research funding from Celgene, Takeda, Kyowa-Kirin, Chugai, Sanofi, Pfizer, Astellas, Daiichi Sankyo, Eisai, and Shionogi; and honoraria from Celgene, Takeda, Kyowa-Kirin, Chugai, Sanofi, Pfizer, Astellas, Daiichi Sankyo, Eisai, Janssen, and Ono. N.T. has received research funding from Kyowa-Kirin, Chugai, Takeda, Eisai, Daiichi Sankyo, and Teijin Pharma. The remaining authors declare no competing financial interests.

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Author Contributions

M.A.-S. designed the study, performed the experiments, analyzed the results, and wrote the manuscript; A.Y. designed the study, performed the experiments, and revised the manuscript; Y.K. performed the experiments; T.S. revised the manuscript; J.H and H.I. diagnosed collected samples; N.T. supervised the study; N.G., T.K., H.H., and H.M. contributed to data interpretation and discussion; and all authors reviewed and approved the final manuscript.

Data Availability Statement

The data that support the findings of this study are openly available in DDBJ Sequence Read Archive at http://humandbs.biosciencedbc.jp/, NBDC Research ID: hum0335.v1 [DRA014819].
Further inquiries can be directed to the corresponding author.

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Figure Legends

Fig. 1. Pattern of mutations detected by NGS

Patients with mutations are shown in gray panels; patients with multiple mutations in the same gene are shown in black panels.

Fig. 2. Comparison of mutant allele frequency of *MYD88* L265P with other mutations

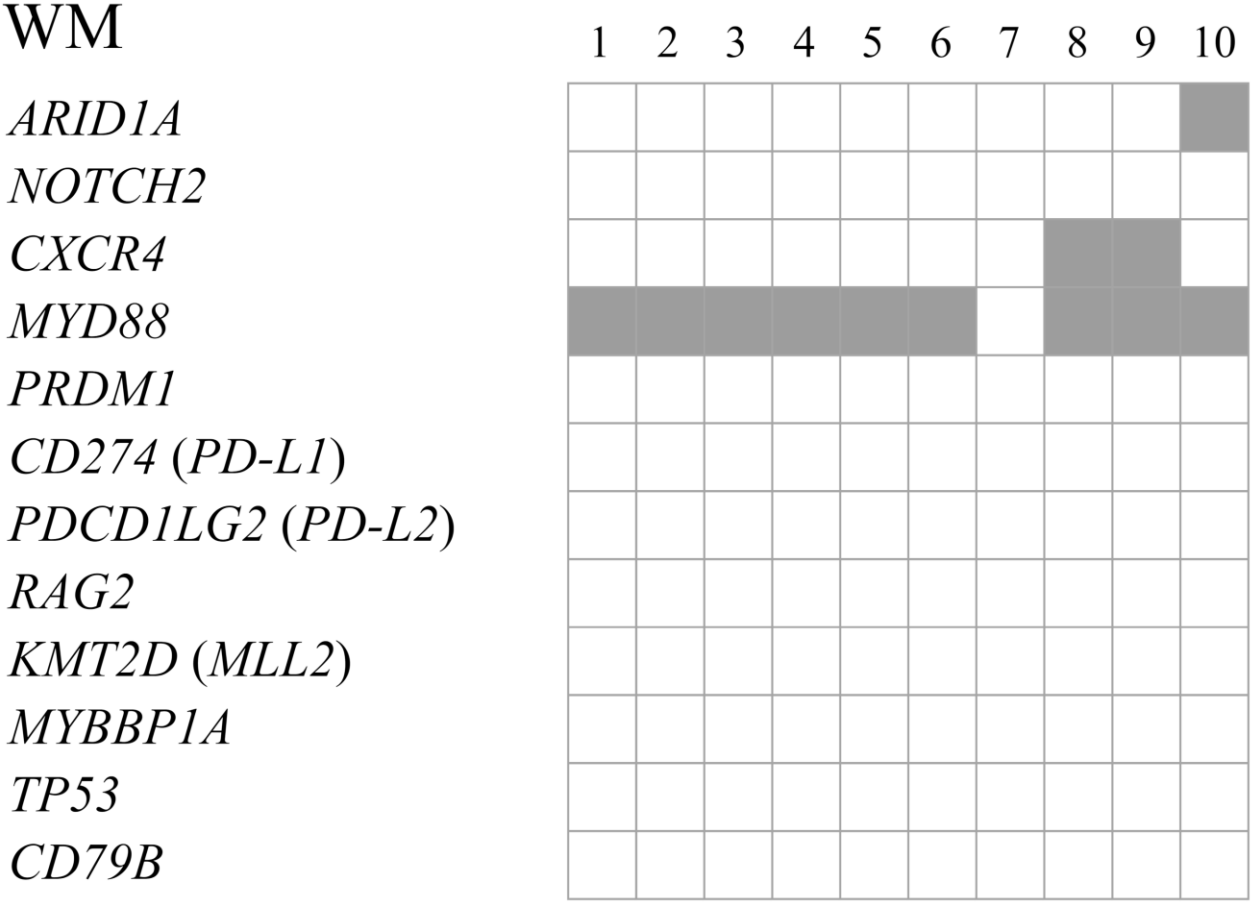
Mutant allele frequency of *MYD88* L265P in patients with WM and non-IgM-type LPL

A) Mutant allele frequency detected by NGS. B) The difference between patients with WM and non-IgM-type LPL is not significant (P = 1.0).

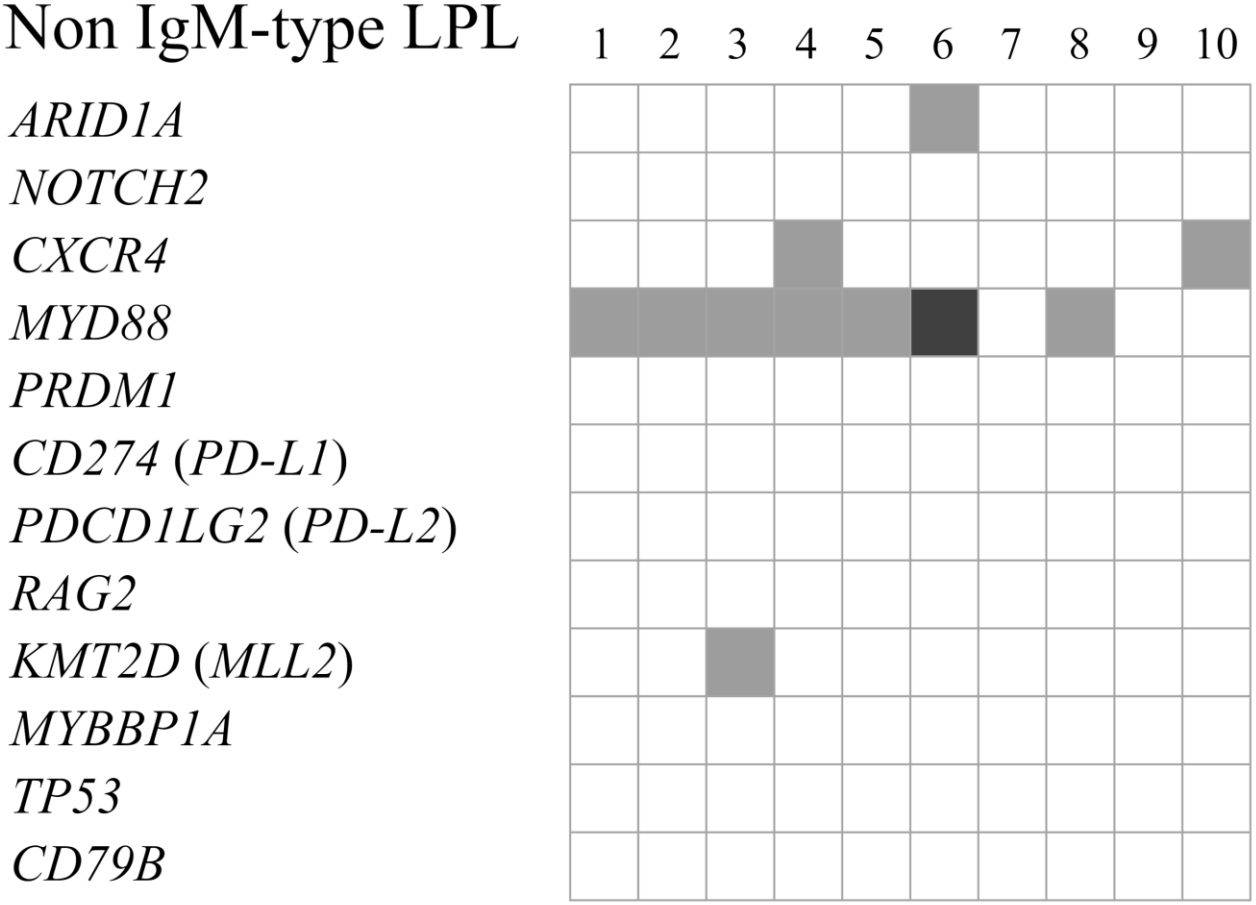
Mutant allele frequency in patients harboring multiple mutations detected by NGS

Most mutations are harbored at almost the same frequency or less than *MYD88* L265P. C) *MYD88* L265P and *CXCR4* mutations. D) *MYD88* L265P and other mutations.

WM



Non IgM-type LPL



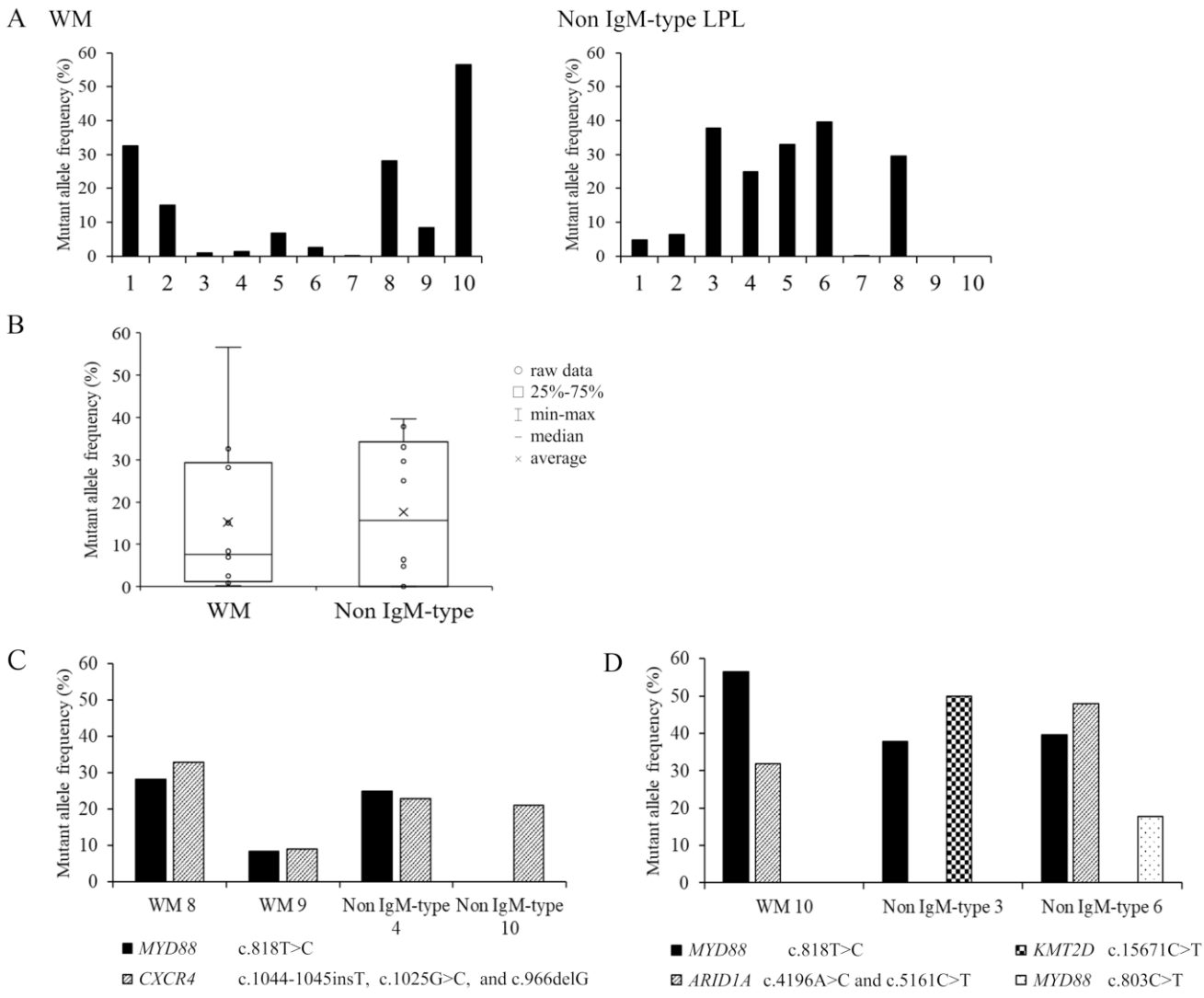


Table 1. Details of validated mutations detected by NGS

Gene	WM/non-IgM type	Chromosome	Position	Nucleotide substitution	Genotype	Amino acid	Note
ARID1A	0/1	1	27,100,914	c.4196A>C	het	Q→P missense	dbSNP: rs1064004
	1/0		27,105,550	c.5161C>T	het	R→X stop gained	COSMIC: COSM51441
CXCR4	1/0	2	136,872,465	c.1044-1045insT	het	-→X frameshift	**E345fs**
	0/1		136,872,485	c.1025G>C	het	S→X stop gained	**S338X** ClinVar: RCV000015067.26 dbSNP: rs104893626
	1/1		136,872,543	c.966delG	het	T→X frameshift	**T318fs**
MYD88	0/1	3	38,182,626	c.803C>T	het	A→V missense	dbSNP: rs781778676
	9/7		38,182,641	c.818T>C	het	L→P missense	**L265P** COSMIC: COSM5416224:COSM85940 ClinVar: RCV000030709.6 dbSNP: rs387907272
KMT2D	0/1	12	49,420,078	c.15671C>T	het	R→H missense	ClinVar: RCV000121442.1:RCV000146178.1 dbSNP: rs3782356

Amino acid substitutions previously reported in WM patients are listed in the note section in bold.