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Original Paper

Analysis of the MYD88 L265P mutation in IgM monoclonal gammopathy by semi-nested polymerase chain reaction-based restriction fragment length polymorphism method

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Abstract

MYD88 L265P mutation causes constitutive activation of NF-κB and possible driver mutation in B-cell lymphoid malignancies. It is frequently detected in Waldenstrom's macroglobulinemia (WM) (50%-100%), and its detection is important in diagnostic and therapeutic targets of this syndrome. Standard detection method of MYD88 L265P mutation in clinical practice has yet to be established. We developed semi-nested PCR-based restriction fragment length polymorphism (snPCR-RFLP) to detect the mutation. The snPCR-RFLP method is a modification of the PCR-RFLP method, which uses the restriction enzyme BsiEI that recognizes CGACT / CG, intending to increase detection sensitivity by amplification of mutated allele in the DNA sample using semi-nested PCR before enzyme digestion. The detection sensitivity of snPCR-RFLP was estimated as 0.1%, by detecting mutated allele in wild-type allele in the cloned plasmid DNA, which is comparable with allele-specific (AS) PCR method widely used as sensitive detection method. By analyzing 40 cases with IgM monoclonal gammopathy, snPCR-RFLP detected 29/40 (70%) of all cases, 22 / 31 (70.9%) of WM, and 6/9 (66.6%) of IgM-type monoclonal gammopathy with undetermined significance (IgMMGUS), including five cases (three cases of WM and two cases of IgMMGUS) in which the mutation was detected only by snPCR-RFLP but not by Sanger sequencing method. Regarding DNA sample status, particularly five cases, a case was extracted from formalin-fixed paraffin-embedded tissue and four cases were extracted from cells by Ficoll-Hypaque density gradient. In correlation with clinical features, the MYD88 mutation detected by snPCR-RFLP method was associated with the adverse prognostic index (WMIPSS) of WM using patient age, hemoglobin (Hb) level, platelet count, β2MG level, and serum IgM level (p=0.055). The snPCR-RFLP method is a clinically useful MYD88 mutation detection method that can be performed in general laboratories.

Key words: MYD88 L265P, IgM monoclonal gammopathy, snPCR-RFLP

Introduction

Since myeloid differentiation primary response 88 (MYD88) L265P mutation was reported as a commonly recurring mutation in Waldenstrom's macroglobulinemia (WM) in 2012[1], the importance of this particular mutation has been confirmed in several types of B-cell lymphoid tumors, including lymphoplasmacytic lymphoma (LPL) / WM and diffuse[2] large B-cell lymphoma with involvement of extranodal sites, such as the central nervous system

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and leg[1, 3]. Particularly, in WM, MYD88 L265P mutation has been found in >90% of cases and is an important prognostic and therapeutic biomarker in this clinical syndrome[4].

A number of methods for MYD88 L265P detection have been reported. These include Sanger sequencing[1], allele-specific PCR (AS-PCR) with or without real-time PCR[5-7], polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)[2], high-resolution melting analysis[8], and droplet digital PCR[9]. These methods have detection sensitivities of approximately 0.005%-5%. However, standardized methods for routine practice have yet to be established. Unresolved issues include 1) the requirement for specific equipment and 2) a tenfold difference in detection sensitivity among study groups in AS-PCR[5, 7].

Detection is also affected by DNA sample preparation. If DNA extracted from CD19-sorted B cells, PCR-based RFLP can detect 91.7% of cases of WM[10]. However, in practice, DNA can only be obtained from formalin-fixed paraffin-embedded (FFPE) tissues, such as bone marrow, because the diagnosis of WM is still based on the infiltration of lymphoplasmacytoid cells in addition to IgM monoclonal gammopathy[11].

In the present study, we report a snPCR-RFLP method, a modification of PCR-based RFLP, for the detection of MYD88 L265P mutation [8].

The present study aimed to establish a method for the detection of MYD88 L265P mutation, which is 1) sensitive, 2) applicable to any source of DNA in clinical settings, and 3) feasible for general laboratories in hospitals, without requiring special equipment.

Materials and methods

1. Patients

In 1984-2016, at Showa University Hospital, 40 patients subjected to serum monoclonal IgM detection were selected for the present study. According to the recommendations from the Second International

Diagnosed with WM and nine patients were diagnosed with IgM monoclonal gammopathy of undetermined significance (MGUS)[11]. The histopathological diagnosis of WM was based on evident infiltration of atypical lymphoid cells in the bone marrow.

This study was approved by Showa University, Human Genome, Gene Analysis Research Ethics Committee (No.189). Informed consent was obtained from all participants or substitutes for participants of the study.

2. Semi-nested PCR-based RFLP (snPCR-RFLP)

Genomic DNA was extracted using the QuickGene-AutoS DNA Tissue Kit (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) from unselected mononuclear cells isolated using a Ficoll-Hypaque density gradient of bone marrow cells in 33 cases. In seven cases, genomic DNA was obtained from FFPE bone marrow tissues using DNA Isolator PS-rapid Reagent (FUJIFILM Wako). Paraffin-embedded decalcified bone marrow trephine biopsy specimens were excluded.

To enable sufficient PCR amplification, we performed a snPCR before enzyme digestion. In the first PCR, 100 ng of genomic DNA was amplified in a total volume of 50 µl, including 0.5 µl of Taq DNA polymerase (5 µ/µl), 5 µl of 10×PCR-Buffer, and 1.0 µl of dNTPs obtained from the Taq DNA polymerase dNTPack (Roche Applied Science, Mannheim, Germany), with the primers 5′-TTAGATGGGGGATGGCTGTTG-3′ (F1) and 5′-GCGAGTCCAGAACCAAGATTTG-3′ (R1), yielding a 229-bp product (NG016964). The product obtained by the first PCR was purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). After purification, 2 µl of eluate was subjected to a second PCR using the primers F1 and 5′-ACCTCAGGATGCTGGGGAACTC-3′ (R2), which produced a 179-bp product. PCR conditions were initial denaturation at 94℃ for 5 min, 30 cycles of denaturation at 94℃ for 30 s, annealing at 57.5℃ for the first PCR and 57.2℃ for the second PCR for 1 min, and elongation at 72.0℃ for 2 min, followed by a final extension step at 72℃ for 7 min. After the second snPCR, 10 µl of purified semi-nested PCR product was digested using 20 U of BsiEI (New England Biolabs, Tokyo, Japan) in a total volume of 20 µl for 2 h, followed by separation by electrophoresis on a 5% acrylamide gel. After electrophoresis, the acrylamide gel was stained with SYBR Green I (Takara Bio Inc., Shiga, Japan) for 60 min and visualized using a 254-nm UV trans-illuminator. MYD88 L265P mutant alleles were digested into 125- and 48-bp fragments, which were visualized on acrylamide gel.

In snPCR-RFLP analysis, to exclude false positive results from repeated PCR, we performed first PCR to acrylamide gel analysis including plasmid DNA (10 ng) with or without mutation and genomic DNA obtained from patients.

In cases in which 100 ng of genomic DNA was unavailable for the first PCR, at least 5 ng of DNA was used for this step, following the same protocol described above.

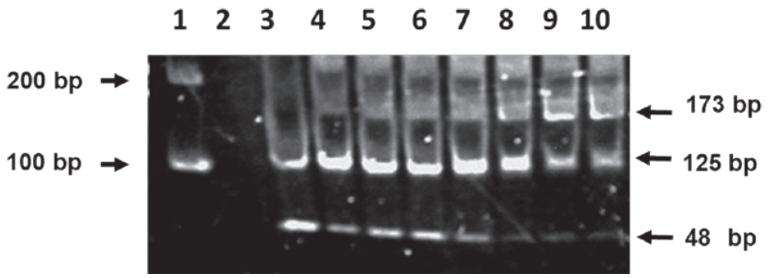

Fig. 1. Detection sensitivity analysis of snPCR-based RFLP for *MYD88* L265P mutation

Plasmid DNA with the *MYD88* L265P mutant allele was mixed with those carrying the wild-type *MYD88* allele at the indicated percentage. The mixed plasmid DNA was subjected to snPCR-based RFLP as described in the Materials and Methods section. In this experiment, we considered that as low as 0.1% of the *MYD88* L265P mutant allele was detectable in the examined DNA samples by performing this snPCR-based RFLP analysis.

Lane 1: 100 bp ladder; Lane 2: ddw; Lanes 3–10: 100%, 10%, 5%, 2.5%, 1%, 0.5%, 0.25%, and 0.1% mutant allele in wild-type allele, respectively.

173 bp band indicates wild-type allele of *MYD88* gene, while 125 bp and 48 bp band indicate mutant allele of *MYD88* gene in samples.

3. Determination of the detection sensitivity of snPCR-RFLP

A 418-bp DNA fragment that contained either c.794T or c.794C (NM 002468) was obtained by PCR (forward primer, 5′-GAAGATCTCTGCACACCTGA-3′; reverse primer, 5′-GATTCCTCCTACAACGAAAG-3′) using DNA extracted from bone marrow cells of a patient diagnosed with WM with the MYD88 L265P mutation (lane 6). Either the wild-type allele or mutant allele was cloned into the pGEM®-T Vector (Promega, Madison, WI, USA), and plasmids were used as MYD88 L265P-positive (mutant) and negative control (wild type).

The sensitivity of snPCR-RFLP was determined by serial dilution of the mutant plasmid with the wild-type plasmid (Fig. 1). In detail, 10 ng pGEM®-T vector with the cloned MYD88 L265P allele was serially diluted with those with the wild-type allele to 10%, 5%, 2.5%, 1%, 0.5%, 0.25%, and 0.1%. To determine the detection sensitivity of snPCR-RFLP analysis, approximately 10 ng of the plasmid DNA with various percentages of mutant allele was used as template DNA and analyzed by snPCR-RFLP protocol described in the Materials and Methods section (Fig. 1).

4. DNA sequencing

We performed Sanger method to determine the corresponding nucleotide substitution. The PCR product (229 bp) obtained by the primers for the first PCR (F1 / R1) was purified by 1.0% agarose gel electrophoresis. Both strands of the products were sequenced with the PCR primers using a BigDye Termination v1.1 Cycle Sequencing Kit (Life Technologies Japan, Ltd., Tokyo, Japan) and analyzed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

5. Correlations between clinical parameters and genotype and statistical analyses

Pretreatment data were extracted from the patient's medical records and hospital's computerized databases. Splenomegaly was evaluated by abdominal computed tomography findings at diagnosis.

The Mann-Whitney U test was performed to compare clinical values between cohorts with or without MYD88 L265P mutation. Fisher's exact test was used to evaluate differences in clinical features, such as karyotype, splenomegaly, and FFPE samples, between patients with or without MYD88 L265P mutation.

In the statistical analysis of the International Prognostic Scoring System of WM (IPSSWM)[12] and the revised IPSSWM (rIPSSWM)[13], the sum of scores for adverse prognostic markers was compared between patients with or without MYD88 L265P mutation.

All analyses were performed with two-sided tests at a significance level of 0.05 using JMP 15 (SAS Institute Inc., Cary, NC, USA).

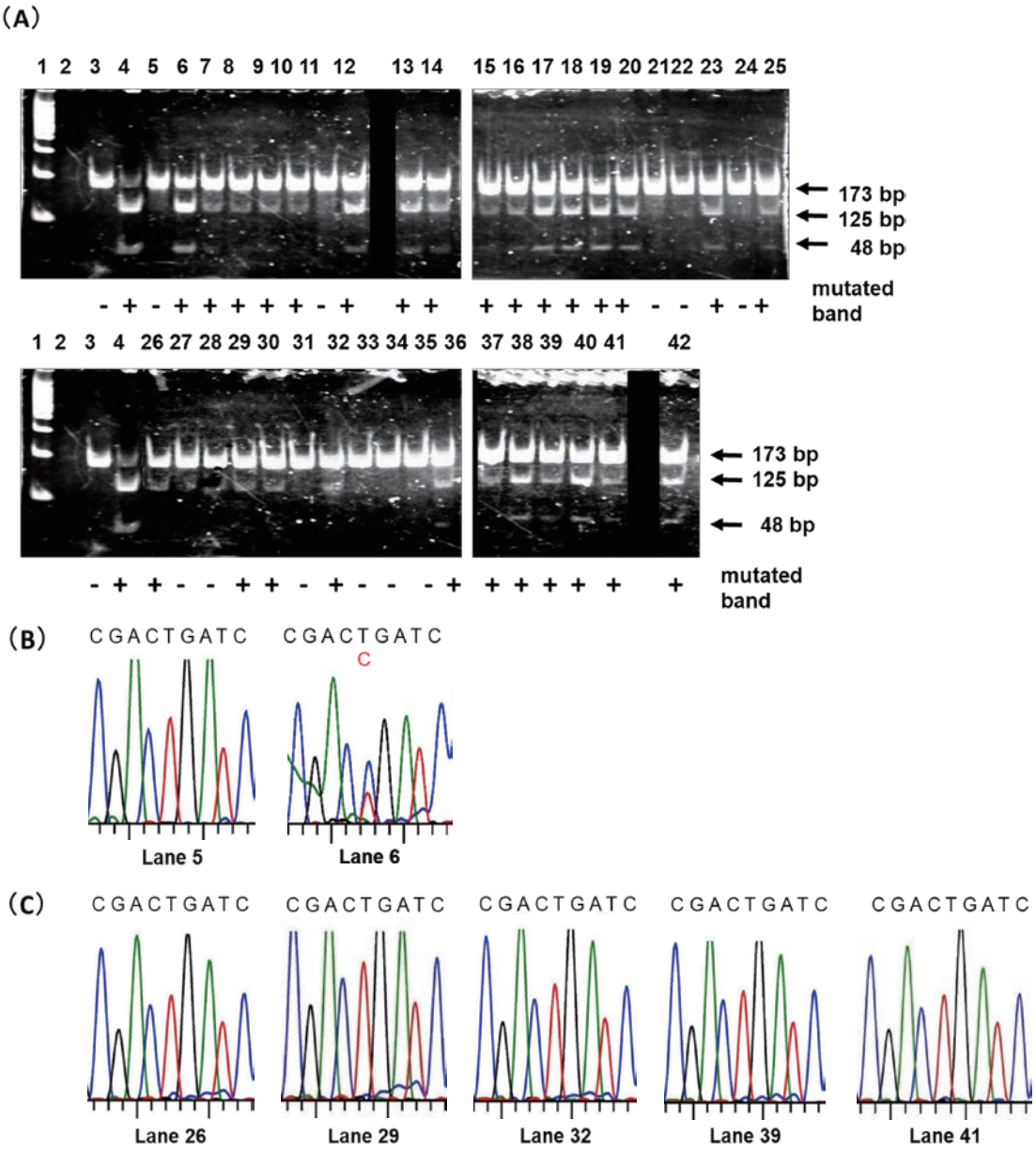

Fig. 2. Bone marrow DNA analysis of patients with IgM monoclonal gammopathy

Genomic bone marrow DNA was subjected to snPCR-based RFLP as described in the Materials and Methods section.

A : Lane 1: 100 bp ladder. Lane 2: ddw. Lane 3: Plasmid DNA with wild-type MYD88 allele. Lane 4: Plasmid DNA with mutant *MYD88* allele. Clinical diagnoses were as follows: lanes 5–31, 41, and 42: Waldenstrom macroglobulinemia; lanes 32–40: IgM MGUS.

Out of 40 cases analyzed by snPCR-RFLP, results of acrylamide gel analysis of 38 cases were shown.

By visualizing the SYBR Green-stained acrylamide gel, MYD88 L265P mutated allele was judged to be present in lanes 6–10, 12–20, 23, 25, 26, 29, 30, 32, 36–42, but not in lanes 5, 11, 21, 22, 24, 27, 28, 31, 33, 34, and 35.

Lanes 20, 21, 26, 31, and 40 were FFPE samples. Lanes 13, 14, 15, 22, and 30 were samples with DNA concentrations less than 100 ng for analysis.

Samples in lanes 26, 29, 32, 39, and 41 were judged to contain *MYD88* L265P mutant allele by snPCR-RFLP, but not by Sanger sequencing.

B : Sanger sequence results showing the *MYD88* gene with wild-type and L265P mutant alleles, corresponding to lanes 5 and 6, respectively.

C : Sanger sequence of five samples (lanes 26, 29, 32, 39, and 41) carrying *MYD88* wild-type allele, while the mutant allele was detected in these by snPCR-RFLP, as shown in Fig. 2(A).

Results

1. snPCR-RFLP and sensitivity

As shown in Fig. 2(A), plasmid DNA of MYD88 wild type (lanes 3) and plasmid DNA of MYD88 L265P (lanes 4) were analyzed by snPCR-RFLP. Samples with MYD88 wild type produced a 173-bp product by SNPCR, whereas those with MYD88 L265P produced 125- and 48-bp fragments.

As determined by the serial dilution of mutant plasmid DNA in wild-type plasmid, the sensitivity was at least 0.1% mutant allele (Fig. 1).

2. Analysis of the MYD88 L265P mutation

As shown in Fig. 2(A), in 38 examined cases, we were able to detect the mutant allele by observation of a 125-bp band with or without a 48-bp band in SYBR Green stained-acrylamide gel analysis.

We detected MYD88 L265P mutation using snPCR-RFLP method in 28 of 40 patients.

Table 1 shows the results of detection of MYD88 L265P mutation in the present study, with respect to the clinical diagnosis and analyzed DNA sample status. Compared with Sanger sequencing method, snPCR-RFLP showed higher detection rate of this mutation irrespective of clinical diagnosis, showing 70% (28/40) vs 57.5% (23/40) in IgM monoclonal gammopathy, 61.2% (19/31) vs 70.9% (22/31) in WM, and 44.4% (4/9) vs 66.6% (6/9) in IgMMGUS. Regarding DNA sample status, in both DNA extracted from Ficoll-Paque density gradient method and FFPE, snPCR-RFLP showed higher detection rate of this mutation compared with the Sanger method, showing 57.5% in the Sanger method and 69.6% in snPCR-RFLP and 57.1% in Sanger method and 71.4% in snPCR-RFLP, respectively.

In Figure 2(B), typical Sanger sequencing results for the absence and presence of c.794T>C are shown in lanes 5 and 6, respectively. In five cases, Sanger sequencing failed to detect mutant nucleotides, while snPCR-RFLP showed a 125-bp band, suggesting the presence of a mutant allele (Fig. 2(A)(C)).

In snPCR-RFLP, sample preparation, such as the preparation of DNA from FFPE sections, did not influence the detection of MYD88 L265P mutation (Table 2).

3. MYD88 L265P mutation and clinical parameters

The relationships between MYD88 L265P mutation and clinical parameters were analyzed in patients with

Table 1. Detection of MYD88L265P mutation in IgM monoclonal gammopathy by Sanger sequencing method and snPCR-RFLP

Clinical diagnosis / sample status / % of mutation detected	Sanger method result	snPCR-RFLP method result	
		L265P-detcted	L265P-undetected
IgM monoclonal gammmopathy (40)	L265P-detected (23)	23	0
	L266P-undetected (17)	5	12
	57.5%	70%	
Waldenstrom's macroglubulinema (31)	L265P-detected (19)	19	0
	L266P-undetected (12)	3	9
	61.2%	70.9%	
IgM MGUS (9)	L265P-detected (4)	4	0
	L266P-undetected (5)	2	3
	44.4%	66.6%	
icoll-Paque density gradient (33)	L265P-detected (19)	19	0
	L265P-undetected (14)	4	10
	57.5%	69.6%	
FFPE samples (7)	L265P-detected (4)	4	0
	L266P-undetected (3)	1	2
	57.1%	71.4%	
100 ng/μl DNA not available samples (5)	L265P-detected (4)	4	0
	L266P-undetected (1)	0	1
	80%	80%	

Table 2. Clinical characteristics of IgM monoclonal gammopathy stratified by MYD88L265P mutation status detected by snPCR-RFLP

	IgM MGUS			Waldenstrom's macroglobulinemia		
MYD88	Wild type (n=3)	L265P (n=6)	p-value	Wild type (n=9)	L265P (n=22)	p-value
Age	65 (48-77)	67 (36-87)	0.69	73 (34-88)	69 (40-86)	0.69
Sex (female / male)	2 / 1	2 / 4	0.86	5 / 4	5 / 17	0.1
IgM (mg/dl)	896 (287-1,527)	1,114 (552-1,744)	0.51	2,729 (1,123-5,862)	3,341 (402-6,438)	0.67
WBC (/mm^3)	8,700 (5,800-13,100)	8,280 (5,600-9,800)	0.89	4,000 (3,500-15,200)	5,350 (2,000-8,600)	1.00
Hb (g/dl)	13.5 (10.9-14.8)	10.8 (7.6-14.5)	0.15	10.5 (4.9-14.5)	9.4 (4.4-13.2)	0.32
PLT (/mm^3)	27.6 (18.9-41.8)	23.4 (0.3-41.7)	0.69	23.2 (5.7-39.1)	17.6 (3.2-41.7)	0.42
ALB (g/dl)	3.1 (1.1-4.1)	3.3 (1.2-4.6)	0.60	2.7 (1.3-4.3)	3.0 (1.2-4.2)	0.44
LDH (U/l)	195 (127-249)	259 (169-422)	0.51	228 (143-403)	171 (85-1,804)	0.06
β2MG (mg/l)	2.5 (2.5-2.5)	4.2 (1.7-8.9)	0.72	4.3 (0.1-19.9)	3.4 (2.4-6.0)	0.87
sIL2R (U/ml)	446 (446-446)	455 (288-581)	1.00	1,910 (434-7,612)	3,504 (697-16,750)	0.07
Abnormal karyotype	1	1	0.70	11	10	1.00
Splenomegaly	1	0	ND	5	10	0.70
IPSSWM				2 (1-3)	3 (1-4)	0.055
rIPSSWM				2 (1-5)	2 (1-4)	0.87

Statistical analysis of clinical data was performed using Mann-Whitney *U* test. Fisher's exact test was performed on the difference in clinical values in sex, karyotype, splenomegaly, or FFPE between with or without *MYD88*L265P mutation. In analysis of IPSSWM, age, Hb level, platelet count, and β2MG and serum IgM levels were considered adverse prognostic factors. In rIPSSWM, age, and β2MG, LDH, and serum albumin levels were considered adverse prognostic factors.

IgMMGUS (n=9) and patients with WM (n=31). In the IgMMGUS cohort, none of the clinical indices were significantly related to MYD88 L265P mutation. These indices included age, sex, serum IgM level, white blood cell count, Hb level, platelet count, serum albumin level, lactate dehydrogenase (LDH) level, β2-microglobulin (β2MG) level, soluble interleukin-2 receptor (sIL2R) level, abnormal karyotype, and splenomegaly. In the WM cohort, MYD88 L265P mutation was slightly related to an increase in LDH (p=0.06) or sILR (p=0.07). In both cohorts, abnormal karyotypes and splenomegaly were not significantly associated with MYD88 L265P mutation (Table 2).

To evaluate the prognostic relevance of MYD88 L265P mutation in WM, we studied the relationship between mutation and IPSSWM or rIPSSWM, which are established prognostic indices of WM. IPSSWM, but not rIPSSWM, tended to be associated with MYD88 L265P mutation in WM (Table 2).

Discussion

In the present study, we showed that snPCR-RFLP is sensitive and feasible method that does not require special equipment to detect MYD88 L265P mutation in clinical setting, such as IgM monoclonal gammopathy, in which MYD88 L265P mutation needs to be studied. snPCR-RFLP for MYD88 L265P mutation is widely applicable in various DNA samples in terms of difference in its extracted method and quality. It is the first study that describes the results, applying snPCR-RFLP method for MYD88 L265P detection in IgM monoclonal gammopathy.

In the detection of MYD88 L265P mutation, a number of methods of analysis has been reported with a wide range of difference in their detection sensitivity, showing 10% in Sanger sequencing method[2] to 0.0035% in ddPCR method[9]. The detection sensitivity of snPCR-RFLP (0.1%) estimated in the present study is comparable with 0.5%-0.1%

in PCR-RFLP[2] or 1.5%–0.1% in AS-PCR[5, 7], of which the latter is an analytical method widely used for detection of MYD88 L265P mutation. However, regarding detection sensitivity between analytical methods reported by different study groups, we need to be careful to compare their estimates, because these were derived from different methods, such as a group calculated them using genomic DNA obtained from patients with or without its mutation[2] and the other using DNA from cell line with its heterozygous mutation or wild type[7]. To more objectively estimate detection sensitivity, we employed plasmid DNA with wild-type and mutant allele.

We found a difference in detection rate between the Sanger method (57.5%) and snPCR-RFLP (70%). It is because PCR products obtained after snPCR contained a higher number of amplified mutated alleles compared with those after the first PCR by PCR primers (F1 and R1 in the Materials and Methods section), which was subjected to the Sanger method using cell cycle sequencing kit.

MYD88 L265P mutation has been reported in >90% of WM cases. Our estimate of 70.9% in WM was apparently lower than previous estimates. This is not likely due to an insufficient detection sensitivity (% of mutant allele) of snPCR-RFLP because the observed sensitivity of 0.1% is comparable to those of previously established, highly sensitive methods, such as AS-PCR, in which the MYD88 L265P mutation has been detected in 100% of WM [5]. The lower mutation frequency in WM in the present study compared with previous estimates can be explained by 1) a difference in methods for the collection of lymphoid cells for the mutation analysis and 2) a difference in the patient selection.

In CD19-sorted B cells, using PCR-RFLP, Argentou detected MYD88 L265P mutation in 91.7% of patients with WM[10]. In the present analysis, genomic DNA was obtained from unsorted bone marrow mononuclear cells, including both mononuclear cells isolated by Ficoll-Paque density gradient (33 patients) and cells in FFPE bone marrow tissues (7 patients). The purity of clonally mutated cells in samples may affect the present results.

Another explanation for the difference is our use of reported consensus criteria for the diagnosis of WM or IgM-MGUS, which is widely used in previous studies[5]. However, the particular diagnostic criteria for this disease are somewhat ambiguous, with disagreements. The criteria include the "unequivocal bone marrow infiltration of LPL with morphological evidence" for the diagnosis of WM. However, the Mayo Clinic group reported that >10% of LPL cells in the bone marrow are required in the diagnosis of WM[14]. In the present study, we diagnosed a patient with WM if the atypical lymphoid cells of any grade were detected histopathologically. As a result, the number of clonal B cells in the samples may be smaller than those in previous studies[2, 15]. This explanation is supported by our results for IgMMGUS; although the number of cases was small, the frequency of MYD88 L265P mutation (66.6%) was comparable to that in previous studies (47%–60%)[16, 17].

Calpaldi *et al.* also reported that MYD88 L265P mutation could be detected in samples obtained from decalcified BM biopsy containing <10% lymphoid cells with the differential diagnosis of low-grade B-cell lymphoma, including LPL / WM, essentially based on the recognition of lymphoma cells by the pathologist, irrespective of the percentage of cells in the bone marrow.

In the present study, we found a relationship between the mutant allele and increase in serum LDH and sIL2R levels, which are biological markers of lymphoid proliferation. Moreover, we found that these mutations tended to be associated with adverse prognostic scores in the IPSSWM. This is apparently contradictory to recent findings, suggesting that the presence of MYD88 L265P mutation is associated with a favorable prognosis in WM[4, 18].

A potential explanation for this difference in the prognostic significance of the mutation is that the mutational analysis in the present study includes two groups with IgM monoclonal protein, one accompanied by evident clonal B-cell infiltration with MYD88 L265P mutation and the other lacking a sufficient number of clones. We did not analyze the heterogeneity of WM based on the presence or absence of MYD88 L265P.

Another explanation for the difference is that the significance of this mutation in Japanese patients with WM may differ from that in populations in Europe and the United States because all patients with relapsed or refractory WM carried this mutation in a clinical study on Japanese patients[19]. To better understand the molecular basis of WM progression, additional analyses of gene mutations, such as mutations in CXCR4 and ARID1A, might be helpful[4].

Although MYD88 L265P mutation is defined as a driver mutation, present in >90% of WM cases, its frequency ranges from 57% to 100% among studies[3]. We believe that this relatively wide range of frequencies may be partially explained by unresolved

issues with respect to both diagnostic consensus and sample heterogeneity. In this context, the present results emphasize the usefulness and limitations of the molecular diagnosis of clinical syndromes with IgM paraproteinemia due to MYD88 L265P mutation in clinical practice.

The present results were obtained from retrospective and exploratory studies. The number of patients was small and limited in the single institution. DNA samples for mutation analysis were collected over a wide timespan (i.e., 1984–2016), and the medications administered to patients varied. Therefore, we did not analyze actual survival of patients with WM.

Nonetheless, we believe that snPCR-RFLP is a useful screening method for the differential diagnosis of MYD88 L265P mutation-associated lymphoid disease in clinical settings.

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