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Rapid and highly sensitive detection of *MYD88/CXCR4* mutations in Waldenström macroglobulinemia by MAS-qPCR: A pilot study

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ABSTRACT

Background: Waldenström macroglobulinemia (WM) is a subset of lymphoplasmacytic lymphoma characterized by bone marrow infiltration of tumor cells and IgM monoclonal gammopathy. *MYD88* mutations are found in more than 90% of WM patients and *CXCR4* mutations in approximately 30–40%. For this reason, *MYD88* mutation analysis is particularly important for the definitive diagnosis of WM. Furthermore, the *CXCR4* S338* mutation is clinically significant for treatment selection in WM patients, as Bruton's tyrosine kinase inhibitors have been reported to be less effective in patients harboring both *MYD88* and *CXCR4* mutations. However, there are currently no simple methods available for the simultaneous detection of *MYD88* and *CXCR4* mutations.
Objective: This study aimed to evaluate the multiplex allele-specific quantitative PCR (MAS-qPCR) assay we developed that is highly sensitive, inexpensive, and capable of simultaneously detecting these two WM-driver mutations using a single-tube reaction on a universal quantitative PCR instrument.
Method: We established optimal PCR conditions using plasmid DNA containing *MYD88* L265P(T > C) and *CXCR4* S338*(C > G) or S338*(C > A) mutations. Using the established method, 12 patient specimens were used for comparison with existing methods.
Results: The results demonstrated optimal PCR conditions at an annealing temperature of 64°C for 40 cycles. The detection sensitivity of *MYD88* L265P(T > C), *CXCR4* S338*(C > G), and *CXCR4* S338*(C > A) mutations were 0.1% for all variants. The MAS-qPCR method successfully detected mutations in WM patient samples with high accuracy.
Conclusion: MAS-qPCR may be a highly sensitive and cost-effective screening method for detecting *MYD88* L265P and *CXCR4* S338* mutations in WM.

1. Introduction

Waldenström macroglobulinemia (WM) is a subset of lymphoplasmacytic lymphoma characterized by bone marrow infiltration of tumor cells and IgM monoclonal gammopathy [1]. Myeloid differentiation factor 88 gene (*MYD88*) mutations are detected in more than 90% of patients and C-X-C chemokine receptor type 4 gene (*CXCR4*) mutations are detected in approximately 30–40% of patients [1–3]. In the *MYD88* gene, L265P (also called L252P, chr3: g.38141150 T > C) is the most frequently detected mutation and is known to activate the NF-kb signaling pathway [4]. As this gene mutation is highly specific to WM, it is important for differential diagnosis [1–3]. Among all *CXCR4* mutations, the S338* nonsense mutation is the most frequent subtype, accounting for approximately 50% of all *CXCR4* mutations, followed by S338 frameshift mutations in approximately 21% of cases [5]. Within S338* nonsense mutations, the base substitutions c.1013C > G (chr2:

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g.136114915G > C, S338*[C > G]) and c.1013C > A (chr2: g.136114915G > T, S338*[C > A]) account for 54% and 25% of cases, respectively [5]. These *CXCR4* mutations promotes enhanced AKT and subsequent MAPK1/2 signaling, leading to sustained survival signals for cancer cells [5]. Consequently, Bruton's tyrosine kinase inhibitors are effective in WM with the *MYD88* L265P mutation, but the response rate to these inhibitors is low in WM with both *MYD88* L265P and *CXCR4* S338* mutations. Therefore, the presence or absence of gene mutations may be important for predicting prognosis and selecting treatment [2,5]. In Japan, *MYD88* L265P is most commonly detected in commercial laboratories by Sanger sequencing, a technique that is straightforward but lacks analytical sensitivity [2]. Currently, no clinically established multiplex assay capable of detecting CXCR4 mutations with sufficient sensitivity and practicality has been widely implemented in routine clinical settings [1]. Although Xu et al. reported a method for detecting *MYD88* L265P, *CXCR4* S338*(C > A), and *CXCR4* S338*(C > G) using allele-specific PCR (AS-PCR) [6,7], this method uses an intercalating dye in the quantitative PCR (qPCR) detection mechanism, and thus, individual genes need to be detected separately, limiting efficiency and clinical utility [6,7]. Other methods include next-generation sequencing (NGS) and a method using digital PCR (dPCR) [8,9]. Compared with AS-PCR, NGS shows lower sensitivity for detecting MYD88 and CXCR4 mutations, particularly in samples with low tumor burden or subclonal disease, and may therefore yield false-negative results in a substantial proportion of patients [8,10]. As dPCR is highly sensitive but requires specific instruments, it has not been so commonly used in clinical settings [9]. To date, no study has achieved a 0.1% variant allele frequency (VAF) threshold for both *MYD88* L265P and *CXCR4* S338* within a single-tube reaction on the qPCR platform. This study aimed to develop a simple and highly sensitive diagnostic method for clinical application, enabling simultaneous detection in a single reaction tube and potentially overcoming limitations of commonly used methods such as Sanger sequencing, including high cost and limited sensitivity. We designed a multiplex allele-specific qPCR (MAS-qPCR) that couples AS-PCR primers with dual-labelled TaqMan probes, enabling simultaneous detection of *MYD88* L265P, *CXCR4* S338*(C > G), and *CXCR4* S338*(C > A) at dPCR-level sensitivity on a standard qPCR instrument. We describe in detail the analytical validation of this assay and its pilot evaluation in clinical WM specimens.

2. Material and method

2.1. Subjects

Wild-type and mutant-type plasmids of *MYD88* and *CXCR4* were prepared for basic examination. For *MYD88*, two plasmids were prepared with 388 bases inserted, including wild-type or L265P (g.38141150 T > C). For *CXCR4*, three plasmids were prepared with 388 bases inserted, including wild-type, S338*(C > G), or S338*(C > A) (all from Fasmac Co., Ltd., Kanagawa, Japan). Moreover, *MYD88* L265P and *CXCR4* S338*(C > G) and *MYD88* L265P and *CXCR4* S338*(C > A) were mixed at the same copy number, respectively. These mutant plasmids were diluted with the wild-type plasmid to prepare mixed dilution series with VAFs of 100, 50, 20, 10, 5, 1, 0.5, and 0.1%. These concentrations were adjusted to approximately 1,500 copies/μL, equivalent to 5 ng/μL of genomic DNA. Furthermore, a confirmation test was performed using 12 specimens (11 bone marrow specimens and 1 lymph node formalin-fixed paraffin-embedded [FFPE] specimen) of patients who were diagnosed as having WM at the Division of Surgical Pathology, Yamaguchi University Hospital, between 2016 and 2024. These specimens had already been analyzed using dPCR and Sanger sequencing. Table 1 shows the information on patient specimens and the results of genetic testing. Only one specimen was positive for both *MYD88* L265P and *CXCR4* S338*(C > G) mutations, and no specimen was positive for mutation *CXCR4* S338*(C > A). One specimen was negative for both mutations. To evaluate the presence of false-positive reactions, MAS-

Table 1
Clinical sample details with results of dPCR, MAS-qPCR, and Sanger sequencing.

Patient number	Specimen type	*MYD88* L265P				*CXCR4* S338*				BM involvement (%)	Treatment status[d]
		dPCR	MAS-qPCR		Sanger sequencing	dPCR	CXCR4 S338*(C > G) MAS-qPCR		CXCR4 S338*(C > A) MAS-qPCR		
		VAF (%)	Determination	Ct	Quantity (VAF,%)	(+/-)	VAF (%)	Determination	Ct	VAF (%)	Determination	Ct	Sanger sequencing (+/-)		
1	BM fluid	0.00	Neg.	UND	N/A	–	0.00	Neg.	UND	0.00	Neg.	UND	–	43.8[a]	Treated
2	BM fluid	5.06	Pos.	32.45	4.01	–	0.00	Neg.	UND	0.00	Neg.	UND	–	10.0[a]	Naive
3	BM fluid	0.54	Pos.	34.62	0.82	–	0.00	Neg.	UND	0.00	Neg.	UND	–	11.5[a]	Treated
4	Lymph node FFPE	7.47	Pos.	31.47	8.22	–	0.00	Neg.	UND	0.00	Neg.	UND	–	77.3[b]	Naive
5	BM smear	4.41	Pos.	33.44	1.94	–	0.00	Neg.	UND	0.00	Neg.	UND	–	4.8[c]	Naive
6	BM fluid	44.36	Pos.	28.50	72.86	+	0.00	Neg.	UND	0.00	Neg.	UND	–	79.4[c]	Treated
7	BM fluid	1.71	Pos.	35.19	0.54	–	0.00	Neg.	UND	0.00	Neg.	UND	–	3.0[c]	Naive
8	BM fluid	15.16	Pos.	32.10	5.18	+	0.00	Neg.	UND	0.00	Neg.	UND	–	22.9[a]	Naive
9	BM smear	19.11	Pos.	30.57	15.93	+	0.00	Neg.	UND	0.00	Neg.	UND	–	27.2[c]	Naive
10	BM smear	18.07	Pos.	30.19	21.13	+	0.00	Neg.	UND	0.00	Neg.	UND	–	33.6[a]	Naive
11	BM smear	22.89	Pos.	30.27	19.82	+	23.17	Pos.	29.15	0.00	Neg.	UND	+	41.2[c]	Naive
12	BM smear	19.79	Pos.	30.58	15.84	+	0.00	Neg.	UND	0.00	Neg.	UND	–	11.9[c]	Naive

dPCR, digital polymerase chain reaction; MAS-qPCR, multiplex allele-specific quantitive polymerase chain reaction; VAF, variant allele frequency; Ct, cycle threshold; BM, bone marrow; Neg., negative; UND, undetermined; N/A, not applicable; Pos., positive; FFPE, formalin-fixed paraffin-embedded.

[c] The proportions of CD19-positive and CD20-positive cells determined by flow cytometry are presented.

[a] BM involvement was estimated by morphological assessment of tumor cell proportion on BM smear specimens.

[b] For PN4 (lymph node FFPE), the value represents the proportion of tumor cells in the lymph node biopsy specimen.

[d] Treatment status at the time of specimen collection. "Treated" indicates patients who had received prior WM-directed therapy; "Naive" indicates patients who had not received any prior therapy.

qPCR was additionally performed using 10 bone marrow smear samples confirmed to be wild-type for both MYD88 and CXCR4 that were obtained from patients with 5 myelodysplastic syndromes, 2 acute myeloid leukemias, and 3 myeloproliferative leukemias. This study was conducted in compliance with the ethical principles of the Declaration of Helsinki and was approved by the institutional review board/research ethics committee of Yamaguchi University Hospital (approval numbers: 2023–195 and H29-109). This cohort was designed as a pilot validation series to benchmark MAS-qPCR performance against dPCR and Sanger sequencing prior to launching a multi-center study that will enroll more than 100 cases of B-cell lymphoma.

2.2. Methods

2.2.1. Design and validation of MAS-qPCR

Design of the allele-specific primer and detection system: Allele-specific forward primers were designed for each MYD88 and CXCR4 mutation ensuring a perfect match between the 3′ terminal nucleotide and the mutant base. Additionally, the intentional mismatch was introduced two bases upstream of the 3′ end to improve the distinction between mutant and wild-type alleles. The length of each primer was adjusted to achieve comparable melting temperatures (Tm). For simultaneous detection, TaqMan MGB (minor groove binder) probes (Thermo Fisher Scientific, Waltham, MA, USA) labeled with Victoria Dye (VIC) for MYD88 L265P and Fluorescein Amidite (FAM) for CXCR4 S338* were designed downstream of the forward primers. The primer and probe sequences and their Tm values are shown in Supplementary Table 1.

Genetic analysis: MAS-qPCR was performed as a single-tube assay in which all five primers and the two probes (Supplementary Table 1) were simultaneously added to the same reaction tube. Each primer was used at the final concentration of 0.2 μM, and each probe at the final concentration of 0.5 μM. The reaction mixture contained 1X TaqPath ProAmp Master Mix (Thermo Fisher Scientific), with a final volume of 20 μL. The instrument used was the QuantStudio5 (Thermo Fisher Scientific). Conditions that do not produce nonspecific reactions with the wild type were investigated, and as a result, 40 cycles of 95°C for 5 min followed by 95°C for 15 s and 64°C for 1 min were determined.

Validation: Validation was performed in accordance with The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) Guidelines for quantitative PCR [11]. As a template for PCR, dilution series of *MYD88* L265P + *CXCR4* S338*(C > G) and *MYD88* L265P + *CXCR4* S338*(C > A) were added at each concentration of 3,000 copies. The standard deviation (SD) of the threshold cycle (Ct) values and coefficient of variation (CV, %) for triplicate measurements were calculated to evaluate the sensitivity of detection and reproducibility. At the same time, calibration curves were prepared using the average value to calculate the coefficients of correlation, determination, and PCR efficiency.

2.2.2. Confirmation testing using patient specimens

DNA extraction from the specimens of WM patients was performed using a QIAamp® DNA Blood Mini Kit (QIAGEN, Hilden, Germany) for bone marrow specimens and a Maxwell® RSC FFPE Plus DNA Kit (Promega, Madison, WI, USA) for the FFPE specimen. For bone marrow aspirate samples in bone marrow specimens, mononuclear cells were separated using Lymphoprep (Cosmo Bio Co., Ltd., Tokyo, Japan) according to the manufacturer's protocol, and then DNA was extracted from these mononuclear cells. DNA concentrations were measured with a NanoDrop micro-spectrophotometer (Thermo Fisher Scientific). MAS-qPCR was performed according to the method described in Section 2.2.1, using 10 ng of template DNA. The concordance rate between the MAS-qPCR results and dPCR results and between the MAS-qPCR results and Sanger sequencing results were examined. In addition, the correlations between the VAFs of dPCR of the *MYD88* mutation-positive specimens and the VAFs calculated from the calibration curves using MAS-qPCR were examined. As supplementary information, for dPCR, the reagents and instrument used were Absolute Q™ DNA Digital PCR Master Mix (5X) and Taq Man probe and a QuantStudio Absolute Q (all, Thermo Fisher Scientific), respectively The reaction conditions were as follows: 96°C for 10 min, 96°C for 5 s, and 60°C for 30 s (40 cycles). The VAFs for dPCR were calculated as the percentage of the mutant copy number divided by the total copy number of the mutant and wild-type. For Sanger sequencing, the reagent and instrument used were a BigDye™ Terminator v3.1 Cycle Sequencing Kit and a 3500 Genetic Analyzer (both, Thermo Fisher Scientific), respectively. Supplementary Table 2 shows the details of the primers and probes used for sequencing.

2.3. Statistical analysis

Pearson's correlation coefficient (r) and coefficient of determination (r^2) were determined. Agreement between methods was further assessed by Bland-Altman analysis; mean bias and 95% limits of agreement (LoA = bias ± 1.96 SD) were derived and visualized. GraphPad Prism ver. 9 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis.

3. Results

3.1. Examination of sensitivity of mutation detection in MAS-qPCR

Using plasmid dilution series of *MYD88* L265P and *CXCR4* S338*(C > G) or (C > A), the sensitivity of MAS-qPCR to detect mutations in *MYD88* L265P, *CXCR4* S338*(C > G), and *CXCR4* S338*(C > A) were examined. The VAF was set as the standard at 0%, and the threshold line was determined for quantitative PCR (Fig. 1). The mean Ct values determined by triplicate measurements of VAFs of 100, 50, 20, 10, 5, 1, 0.5, and 0.1% were 28.02, 28.83, 30.68, 31.64, 31.82, 34.72, 36.13, and 36.94 for MYD88 (Fig. 2A); 28.92, 29.71, 30.99, 31.61, 32.71, 35.08, 35.64, and 37.18 for *CXCR4* S338*(C > G) (Fig. 2B); and 28.45, 28.79, 30.18, 31.21, 32.27, 35.73, 35.56, and 37.46 for *CXCR4* S338*(C > A) (Fig. 2C), respectively. Each mutation could be detected in all VAFs (from 100 to 0.1%) by the triplicate measurements (Fig. 2 and Supplementary Table 3).

3.2. Validation of each VAF

For each dilution series, the SD and CV of the Ct values from the triplicate measurements of each gene mutation by MAS-qPCR were calculated (Fig. 2 and Supplementary Table 3). The coefficient of determination (r^2) was calculated using the relationship between the Ct values and Log_VAF%, and PCR efficiency was calculated using the slope. The r^2 values and PCR efficiencies were 0.980 and 108.19% for MYD88 L265P (Fig. 2A), 0.994 and 123.91% for CXCR4 S338*(C > G) (Fig. 2B), and 0.980 and 102.66% for CXCR4 S338*(C > A) (Fig. 2C), respectively. In addition, to evaluate assay precision, the SD and CV of the Ct values were calculated at each VAF level (100%, 50%, 20%, 10%, 5%, 1%, 0.5%, and 0.1%), as summarized in Supplementary Table 3. In Fig. 2A–C, the mean Ct values showed a high correlation with the logarithmic values of the VAFs. As shown in Supplementary Table 3, all CV values across the dilution series were less than 5%, indicating good assay precision.

3.3. Confirmation testing using patient specimens

Table 1 shows the results of MAS-qPCR for the 12 patient specimens. The positive and negative results of all specimens corresponded to the results of dPCR. Even the specimens with a VAF of 0.54% could be detected by dPCR. The positive concordance rate with Sanger sequencing method was 54.5% (6/11), and the five specimens determined to be negative were those with a VAF of less than 10% by dPCR (Table 1).

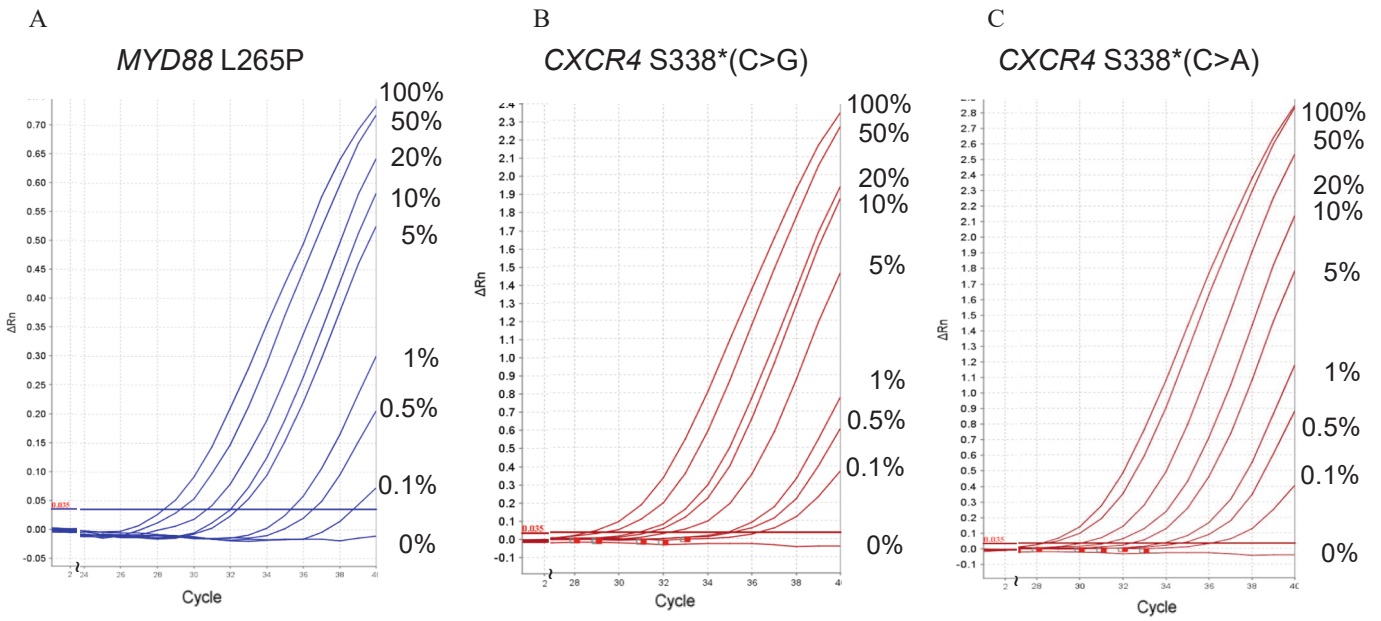

Fig. 1. Amplification curves of multiplex allele-specific quantitative PCR (MAS-qPCR) for *MYD88* L265P and *CXCR4* S338* mutations using plasmid DNA with assigned variant allele frequency (VAF) values. (A) Amplification curves for the *MYD88* L265P mutation using plasmid DNA samples with assigned VAF values ranging from 100% to 0%. Wild-type DNA (0% VAF) showed no amplification, confirming the specificity of the MAS-qPCR assay. Threshold cycle (Ct) values decreased as the VAF increased, demonstrating a clear concentration-dependent amplification pattern. (B) Amplification curves for the *CXCR4* S338* (C > G) mutation under similar conditions. No amplification was observed for wild-type DNA, and a VAF-dependent reduction in Ct values was evident. (C) Amplification curves for the *CXCR4* S338* (C > A) mutation showed consistent detection of target DNA with no amplification for wild-type DNA.

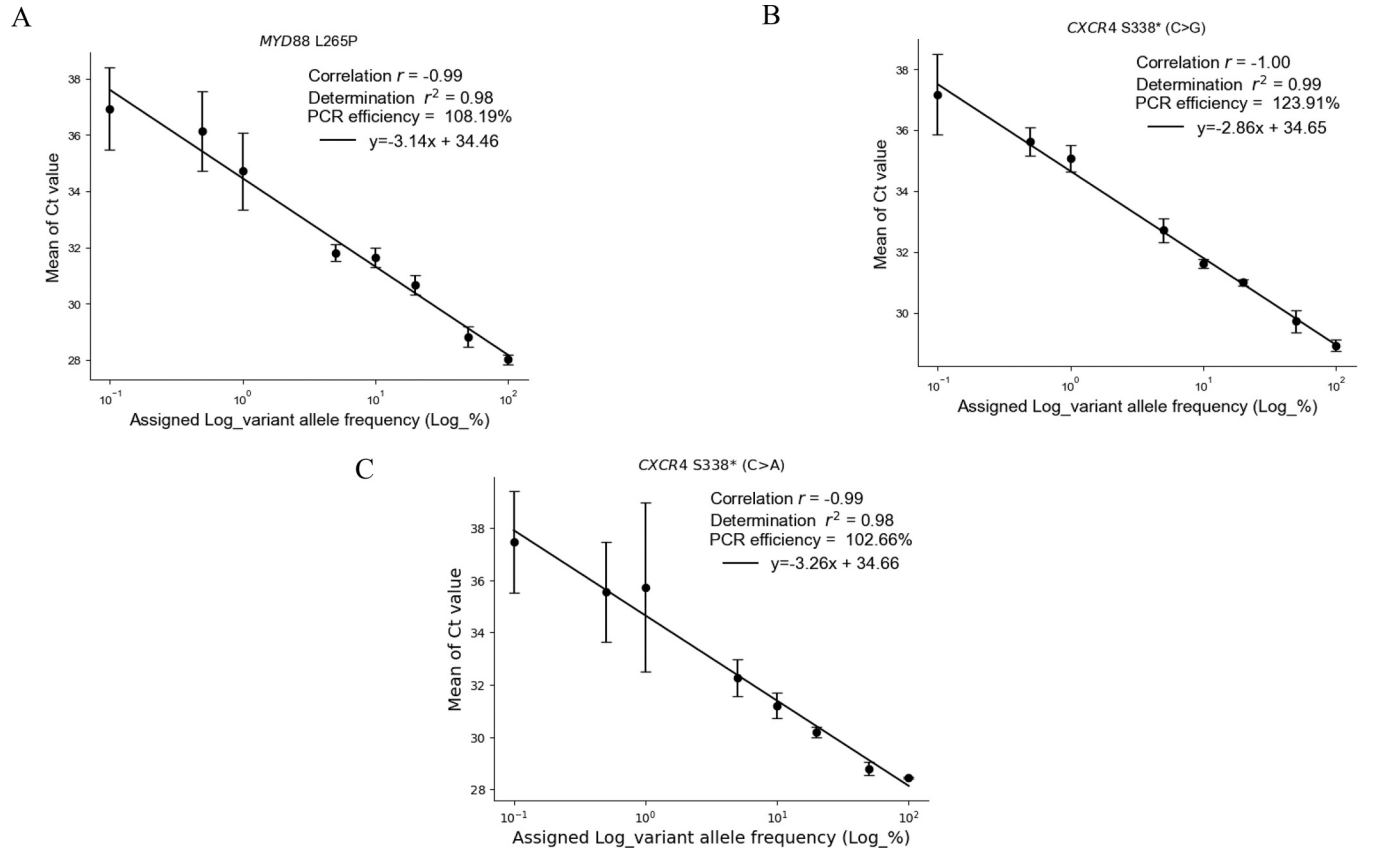

Fig. 2. Repeatability, sensitivity, and efficiency of multiplex allele-specific quantitative PCR (MAS-qPCR) for detecting *MYD88* L265P and *CXCR4* S338 mutations. Standard curve for the detection of the *MYD88* L265P mutation (A), *CXCR4* S338* (C > G) mutation (B), and *CXCR4* S338* (C > A) mutation (C). Mean threshold cycle (Ct) values were plotted against the assigned logarithmic values of variant allele frequency (Log_%). The correlation coefficient (r), coefficient of determination (r^2), PCR efficiency, and regression equation are indicated. Error bars represent ± 2 standard deviation (SD) of three independent measurements. High PCR efficiency and robust linear correlations (r ≥ 0.99) confirm the accuracy of the allele-specific PCR assays for detection of these mutations.

Fig. 3 shows the amplification curves of patient number (PN) 3, 4, and 11 in Table 1, each of which is a different specimen type, i.e., bone marrow aspirate, FFPE specimen, and bone marrow smear, respectively. The amplification curve for PN3 (bone marrow aspirate) shows positivity exclusively for the *MYD88* L265P mutation (Fig. 3A). That for PN4 (lymph node FFPE sample) reveals positivity only for the *MYD88* L265P mutation (Fig. 3B), and that for PN11 (bone marrow smear) indicates positivity for both the *MYD88* L265P and *CXCR4* S338* mutations (Fig. 3C). PN11 was the specimen with both *MYD88* L265P and *CXCR4* S338* mutations, which could be simultaneously detected by using MAS-qPCR (Fig. 3C). Regarding quantification, a strong correlation ($r = 0.936$) was observed between the VAFs of dPCR in *MYD88* L265P and the VAFs quantified from the calibration curve using MAS-qPCR (Fig. 4A). Bland-Altman analysis revealed a mean bias of + 0.70% with an SD of 9.80%, yielding 95% LoA of − 18.50% to + 19.91% (Fig. 4B). Eleven of 12 paired measurements (91.7%) fell within these LoA, indicating good agreement and no evident proportional bias across the tested VAF range. These data confirm the quantitative reliability of MAS-qPCR on a routine qPCR platform relative to dPCR.

4. Discussion

This is the first report to show that MAS-qPCR achieves dPCR-level sensitivity for detecting the two most prevalent WM mutations when using qPCR instruments. Other methods do not allow simultaneous detection of multiple genes due to the use of an intercalating dye [6,7]. In contrast, our method makes it possible to simultaneously detect multiple gene mutations using dual-color specific fluorescent probes. Furthermore, no nonspecific reactions were observed in any of the concentrations due to the use of specific fluorescent probes in our method, whereas other methods showed nonspecific reactions under a VAF of 0.4% [6,7]. In our results, a strong VAF-dependent decrease in Ct values was observed across the tested allele frequencies (Fig. 1). These results highlight the high specificity of MAS-qPCR, with no cross-reactivity to wild-type DNA, and demonstrate its sensitivity for detecting target mutations in a VAF-dependent manner, with reliable quantification down to a VAF of 0.1%. Because the length of the AS-PCR primer was adjusted to make the Tm value uniform so that each gene could be detected simultaneously, and the primer for detecting wild types was not used so as to focus on detection of mutations, our method reduced the

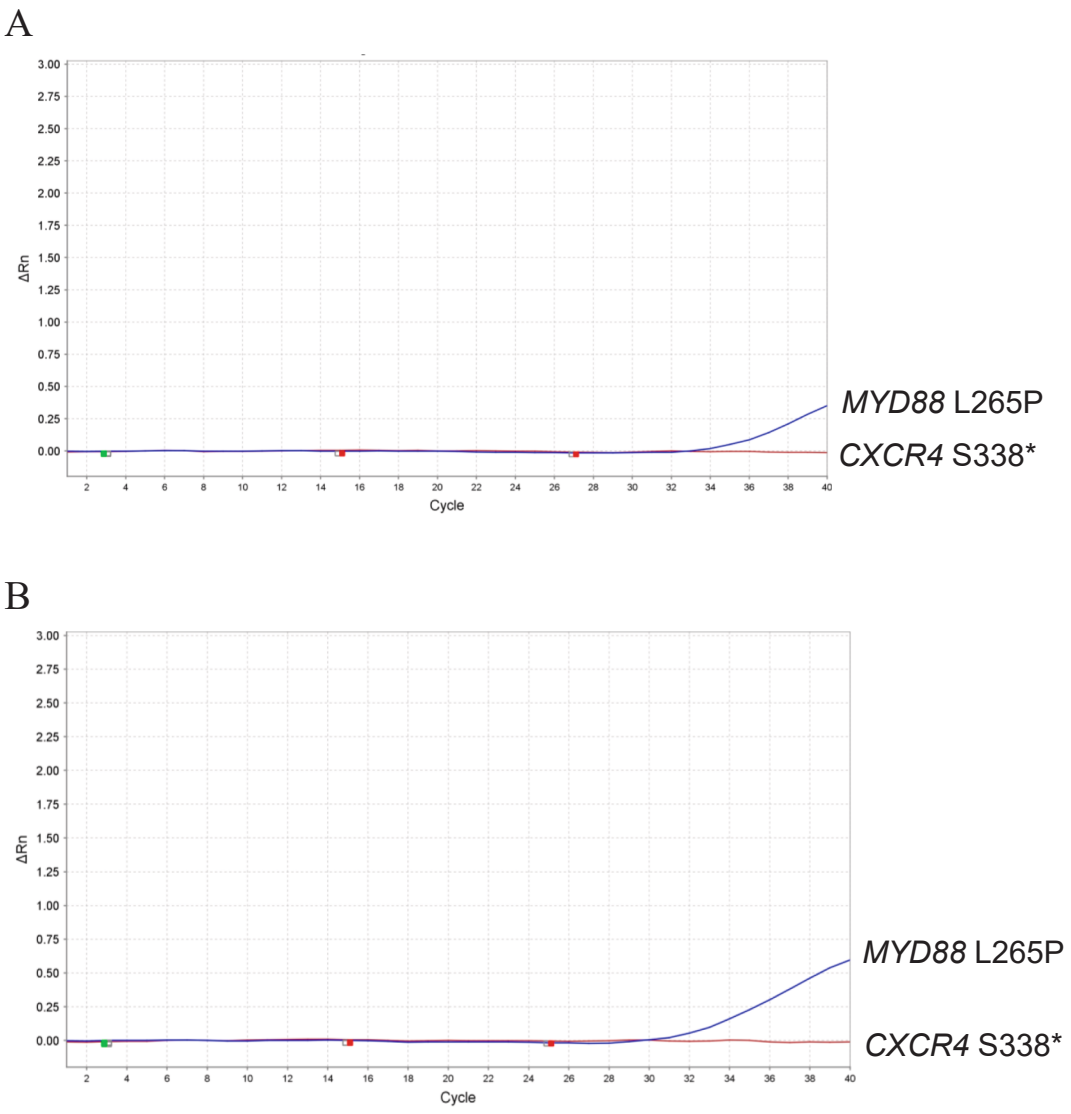

Fig. 3. Amplification curves of multiplex allele-specific quantitative PCR (MAS-qPCR). Amplification curves of MAS-qPCR are shown for PN3 with bone marrow aspirate specimen (A), PN4 with lymph node formalin-fixed paraffin-embedded sample (B), and PN11 with bone marrow smear (C). (D) Amplification curves obtained from 10 bone marrow smear samples confirmed to be wild-type for both MYD88 and CXCR4, together with three mutant plasmid controls (3,000 copies each), demonstrate the absence of nonspecific amplification in wild-type samples.

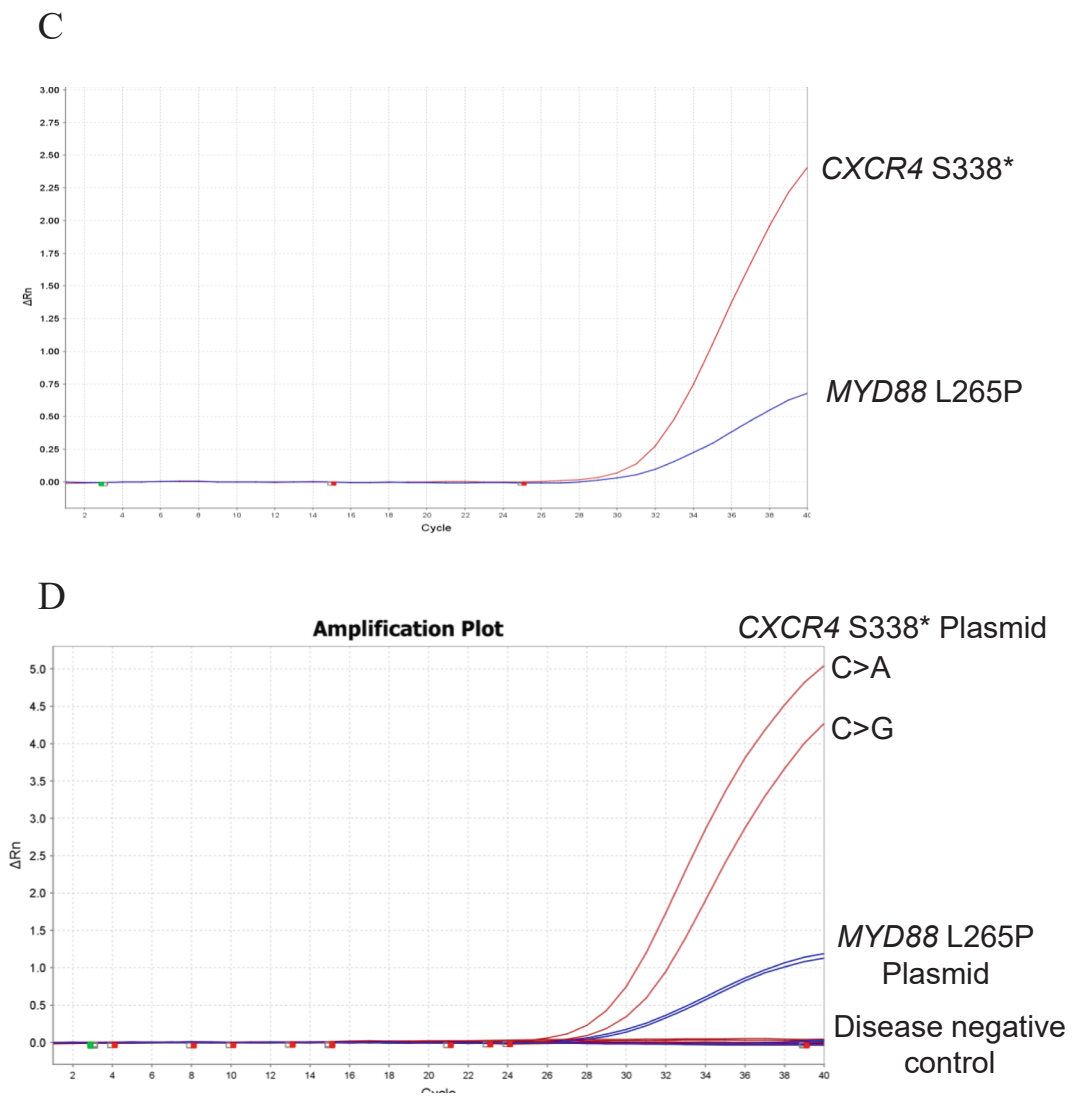

Fig. 3. (*continued*).

complexity of the detection system and achieved simpler and easier to understand results. Simultaneous detection led to reductions in cost, amount of DNA in the specimens used, and time required for workflow by approximately one-third compared to methods that separately detect the three mutations.

A previous study demonstrated that MYD88 L265P and CXCR4 S338X mutations can be detected in plasma cell-free DNA (cfDNA) using allele-specific PCR with high concordance rates to bone marrow genotyping and a cfDNA detection limit of 0.4% VAF [12]. In contrast, our MAS-qPCR combines allele-specific primers with dual-color TaqMan probes to simultaneously detect MYD88 L265P and both CXCR4 S338 variants in a single-tube reaction. This approach achieved a lower detection limit (0.1%) for all targets and showed excellent quantitative agreement with dPCR ($r = 0.936$). Thus, MAS-qPCR provides dPCR-level sensitivity and multiplex capability on standard qPCR instruments, offering a simple and cost-effective alternative for routine WM testing.

As our method can be used with two different types of specimens, i.e., bone marrow and FFPE specimens, it has high clinical applicability. Bone marrow specimens are generally used for testing WM; however, lymph node specimens are required in cases with few tumor cells in bone marrow or for patients with predominantly enlarged lymph nodes. In this case, lymph nodes are often stored as FFPE specimens, making our method highly useful. Actually, PN4 did not show bone marrow infiltration and was morphologically suspected to have lymphoplasmacytic lymphoma. However, these results did not match those of immunostaining. For this reason, the FFPE specimen preserved at the time of lymph node biopsy was examined by genetic testing to reveal that *MYD88* L265P was detected. As a result, this case was diagnosed definitively as WM with *MYD88* L265P detected (Table 1 and Fig. 3B). Consequently, MAS-qPCR is expected to lead to simplification of workflow, easy determination, and lowered costs of specimen use in the clinical examination of WM.

MAS-qPCR showed high correlation and agreement with dPCR (Fig. 4). This suggests that MAS-qPCR does not require specialized equipment such as dPCR and that MAS-qPCR can perform highly sensitive quantification with general-purpose instruments such as quantitative PCR devices. In the clinical setting, our method can assist in the diagnosis of specimens with low tumor burden. Furthermore, the determination of efficacy during and after treatment, and the determination of treatment strategy after recurrence can be diagnosed [13].

Three limitations of this study include the following: 1) our method mainly identifies hot spot mutations of three genes, *MYD88* L265P, *CXCR4* S338*(C > G), and *CXCR4* S338*(C > A), and cannot detect other minor gene mutations or frameshift CXCR4 variants; 2) it cannot distinguish between *CXCR4* S338*(C > G) and *CXCR4* S338*(C > A); and 3) the number of clinical specimens measured (12 specimens) is small—this work therefore represents a pilot validation study—and CXCR4 S338*(C > A) has not yet been validated in clinical material.

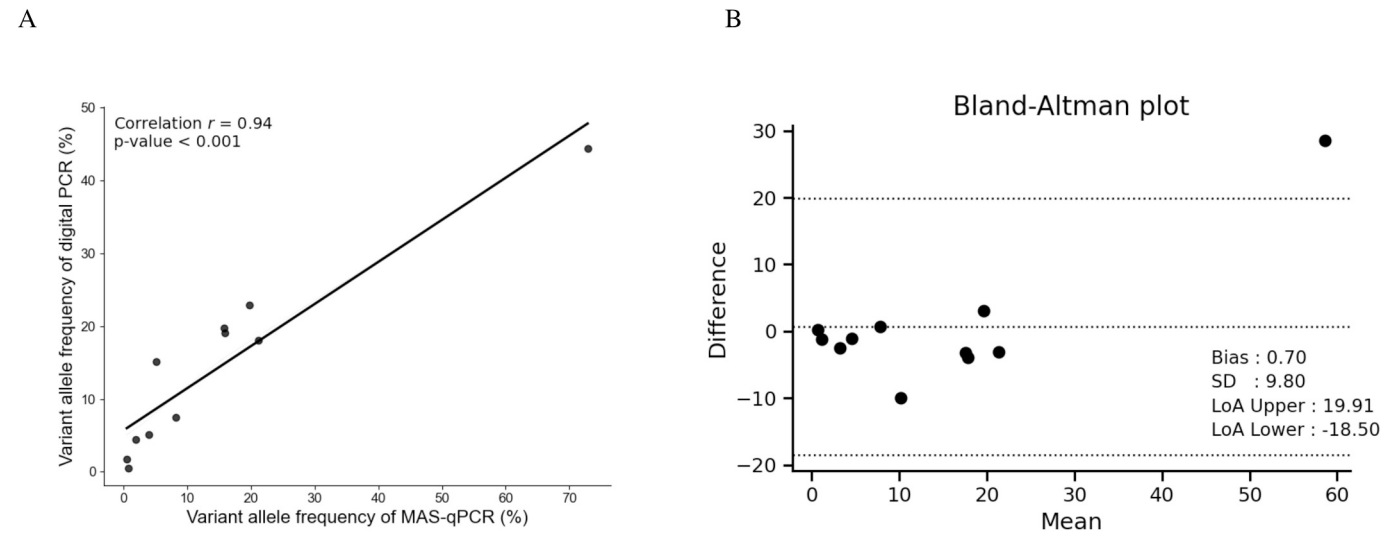

Fig. 4. Comparison of variant allele frequency (VAF) values between digital PCR (dPCR) and multiplex allele-specific quantitative PCR (MAS-qPCR) for the MYD88 L265P mutation. (A) Correlation between VAF values determined by dPCR and those obtained using MAS-qPCR. A strong positive correlation was observed (r = 0.936), highlighting the quantitative capability of MAS-qPCR. (B) Bland-Altman analysis revealed a mean bias of + 0.70% with SD of 9.80%, yielding 95% limits of agreement (LoA) from − 18.50% to + 19.91%. Eleven of 12 paired measurements (91.7%) fell within these LoA, indicating good agreement and no evident proportional bias across the tested VAF range.

Regarding limitations 1) and 2), as the gene mutations targeted in this study account for approximately 90% of WM, this method is more cost-effective than comprehensively examining individual genes using NGS as front-line testing [1]. Frameshift CXCR4 mutations, which are scattered throughout exon 2, will be addressed in a planned two-step workflow: MAS-qPCR will serve as an initial screen for S338; samples that are MYD88 L265P positive/CXCR4 S338* negative but clinically suspicious will reflex to dPCR or targeted NGS for rare frameshift variants. To distinguish between *CXCR4* S338*(C > G) and *CXCR4* S338*(C > A), further identification by sequencing is necessary. However, as shown in the results, six of 11 positive specimens could not be detected by Sanger sequencing due to its low sensitivity, and combined use of Sanger sequencing would likely be of little value. Rather than distinguishing between the two, it is clinically important to detect the presence or absence of the CXCR4 S338* mutation [14]. Regarding limitation 3), only one CXCR4 S338*-positive case was identified among the MYD88-mutated specimens in this pilot cohort. This reflects the limited availability of CXCR4-mutated cases encountered during the study period rather than the true prevalence of CXCR4 mutations in WM. Clinical specimens in this study were intentionally collected to include multiple specimen types (bone marrow smear, bone marrow aspirate, and FFPE specimen), primarily from MYD88-positive cases, to evaluate assay performance across heterogeneous clinical materials rather than mutation frequency. The observed CXCR4 S338* mutation prevalence in our cohort (1/11 MYD88-mutated patients, 9.1%) is lower than the 30–40% reported in predominantly Western WM populations [1–3], which may partly reflect ethnic or geographic differences, as published data in Asian WM populations remain scarce. The absence of an independent external validation cohort with confirmed CXCR4 S338* mutation status is another limitation; validation using externally collected samples will be essential prior to widespread clinical implementation of MAS-qPCR. A multi-center prospective trial involving more than 100B-cell lymphoma cases is currently being planned to confirm analytical robustness, evaluate inter-operator reproducibility, and clarify the true frequency of CXCR4 mutations in Japanese WM patients, along with the potential use of peripheral blood and plasma cfDNA samples. Although MAS-qPCR was successfully applied to an FFPE specimen, validation in FFPE material was limited to a single case, and further studies are required to confirm broader applicability. As this method uses allele-specific primers that do not amplify wild-type alleles, an internal control system must be incorporated for routine clinical application to confirm successful PCR amplification and adequate DNA quality. We plan to develop a simple, cost-effective internal control assay in future studies to distinguish true wild-type samples from technical failure.

Future studies should expand clinical validation to include larger cohorts and additional disease controls in which MYD88 and CXCR4 mutations are rarely observed. Evaluation of FFPE specimens from other B-cell lymphomas, such as diffuse large B-cell lymphoma, may further clarify assay robustness and specificity in archival tissue. Moreover, application of MAS-qPCR to peripheral blood samples from WM patients and to IgM monoclonal gammopathy of undetermined significance, a precursor condition of WM, may enable sensitive monitoring of low-level mutations and longitudinal assessment of tumor burden during disease progression.

In conclusion, we developed a screening method for detecting *MYD88* L265P and *CXCR4* S338* mutations in WM that uses MAS-qPCR, a simple and highly sensitive assay capable of simultaneous measurements.

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CRediT authorship contribution statement

Yuki Kunimune: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mitsuaki Nishioka:** Writing – review & editing, Writing – original draft, Conceptualization. **Haruka Namba:** Writing – original draft, Investigation, Formal analysis, Data curation. **Kimika Todaka:** Writing – original draft, Investigation, Formal analysis, Data curation. **Tomohiro Fujii:** Writing – original draft, Investigation, Formal analysis, Data curation. **Nayu Kiso:** Investigation, Formal analysis, Data curation. **Yukiko Nakahara:** Methodology, Formal analysis. **Kanae Shinkawa:** Methodology, Formal analysis. **Yumiko Kumano:** Methodology, Formal analysis. **Masaki Kodama:** Methodology, Formal analysis. **Tomoya Ebisui:** Validation, Methodology, Formal analysis. **Ryoga Nishimoto:** Validation, Methodology, Formal analysis. **Naoko Okayama:** Methodology, Formal analysis. **Yutaka Suehiro:**

Writing – review & editing, Supervision, Resources. **Takahiro Yamasaki:** Writing – review & editing, Supervision, Resources. **Toshiaki Yujiri:** Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clinbiochem.2026.111141.

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