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RESEARCH ARTICLE

Complete detection of FR1 to FR3 primer-based PCR patterns of immunoglobulin heavy chain rearrangement in the BIOMED-2 protocol is associated with poor prognosis in patients with diffuse large B-cell lymphoma

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Abstract

Somatic hypermutations (SHMs) in the variable region (V_H) of the immunoglobulin heavy chain (IgH) gene are common in diffuse large B-cell lymphoma (DLBCL). Recently, IgH V_H SHMs have become known as immunogenic neoantigens, but few studies have evaluated the prognostic impact of the frequency of V_H SHMs in DLBCL. The BIOMED-2 protocol is the gold standard polymerase chain reaction (PCR) for clonality analysis in lymphoid malignancies, but can produce false negatives due to the presence of IgH V_H SHMs. To overcome this problem, three primer sets were designed for the three framework regions (FR1, FR2, and FR3). We evaluated the predictive value of this PCR pattern in patients with DLBCL. To evaluate the prognostic impact of complete detection of the clonal amplifications (V_HFR1–J_H, V_HFR2–J_H, and V_HFR3–J_H) in the BIOMED-2 protocol, we retrospectively analyzed 301 DLBCL patients who were initially treated with anthracycline-based immunochemotherapy. Complete detection of the FR1 to FR3 primer-based IgH V_H PCR patterns in the BIOMED-2 protocol was associated with low frequency of V_H SHMs ($p < 0.001$). Patients who were positive for all these three PCRs ($n = 79$) were significantly associated with shorter 5-year overall survival (OS; 54.2% vs. 73.2%; $p = 0.002$) and progression-free survival (PFS; 34.3% vs. 59.3%; $p < 0.001$) compared to patients with other PCR patterns ($n = 202$). Specifically, the successful FR3-J_H detection was associated with significantly worse OS ($p < 0.001$) and PFS ($p < 0.001$). PCR patterns of complete IgH rearrangement using the BIOMED-2 protocol are clinically meaningful indicators for prognostic stratification of DLBCL patients.

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1 | INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common form of B-cell malignancy, accounting for 30%–40% of all malignancies but has a broad spectrum of biological, pathological, and clinical features [1]. DLBCL usually contains a number of somatic hypermutations (SHM) of the variable region (V_H) of the immunoglobulin heavy chain (IgH) gene with highly variable frequencies [2, 3]. In several other B-cell malignancies, including B-cell chronic lymphocytic leukemia (B-CLL) and mantle cell lymphoma (MCL), the SHM status of the V_H region (immunoglobulin heavy variable [IGHV]) significantly correlates with clinical outcome [4, 5]. Recently, IGHV SHMs have been recognized as a type of neoantigen specific to B-cell malignancies because it generates a novel tumor-specific sequence [6, 7], and its importance has gained increasing attention. The prognostic significance of IGHV SHM frequency has been reported using next-generation sequencing (NGS) in DLBCL patients [8], but its validity and clinical applicability are not well studied.

Clonality analysis using polymerase chain reaction (PCR) methods plays a critical role in the diagnosis of B-cell malignancies. In 2003, the European BIOMED-2 network (now referred to as the EuroClonality consortium) established a standardized multiplex PCR protocol [9] that has been validated by multiple studies from different institutions [10–20]. The IgH rearrangement starts with a D to J (incomplete) rearrangement, followed by a V to D–J (complete) rearrangement [21]. The BIOMED-2 protocol is designed to detect both incomplete and complete IgH rearrangements; however, PCRs for complete IgH rearrangement often fail to detect clonally rearranged genes, because annealing of the primers is sometimes hampered by SHMs in V_H regions. To decrease the false-negative rates in the BIOMED-2 protocol, three sets of primers were designed for the three framework regions (FRs), FR1, FR2, and FR3 [9]. In addition, no previous studies have evaluated the correlation between BIOMED-2 PCR patterns and the clinical prognosis of DLBCL patients, although many studies reported the clinical utility of the BIOMED-2 protocol for DLBCL patients [22–26]. In this study, we first evaluated whether this PCR assay could be useful for estimating the frequency of V_H SHMs in DLBCL. We then investigated whether the complete detection of the FR1 to FR3 primer-based PCR patterns of IgH rearrangement in the BIOMED-2 protocol is associated with poor prognosis in patients with DLBCL.

2 | METHODS

2.1 | Clonal IgH-rearrangement detection using the BIOMED-2 protocol

Genomic DNA was extracted from fresh samples from DLBCL patients using the QIAamp DNA mini kit (Qiagen) according to manufacturer protocol [9]. PCR amplifications of IgH rearrangement were performed using the standard BIOMED-2 protocol (Figure 1A). To discriminate among monoclonal, oligoclonal, or polyclonal PCR products, dominant amplification was obtained by heteroduplex analysis. The PCR products were analyzed by electrophoresis on 8.0% polyacrylamide gels and visualized by ethidium bromide staining and ultraviolet light (Figure 1B).

2.2 | V_H sequencing analysis

PCR amplifications of IgH rearrangement using the standard BIOMED-2 protocol were performed using 45 cryopreserved DNA samples. After purification of the PCR products using the ExoSAP-IT system (Affymetrix), direct sequencing was performed using forward and reverse primers. FR1 primers were used as the first option of forward primers; however, when they did not yield PCR amplifications, FR2 primers were used as alternative forward primers. The sequencing conditions included a final volume of 20 μL containing 1 μL of purified PCR product, 0.5 μM of both primers, and reagents from the BigDye Terminator version 1.1 cycle sequencing kit (Applied Biosystems). Each sequenced sample was assigned a percentage of V_H identity to the closest germline gene using the IMGT/V-QUEST database (http://www.imgt.org/). The percentage of V_H identity was calculated based on the ratio of the number of mutations to the length in the most homologous germline V_H gene: 100 − (mutations × 100/most homologous V_H length).

2.3 | Survival analysis

We conducted a historical cohort study of consecutive patients that were newly diagnosed with DLBCL at the Kobe City Medical Center General Hospital, between January 2005 and June 2015. DLBCL

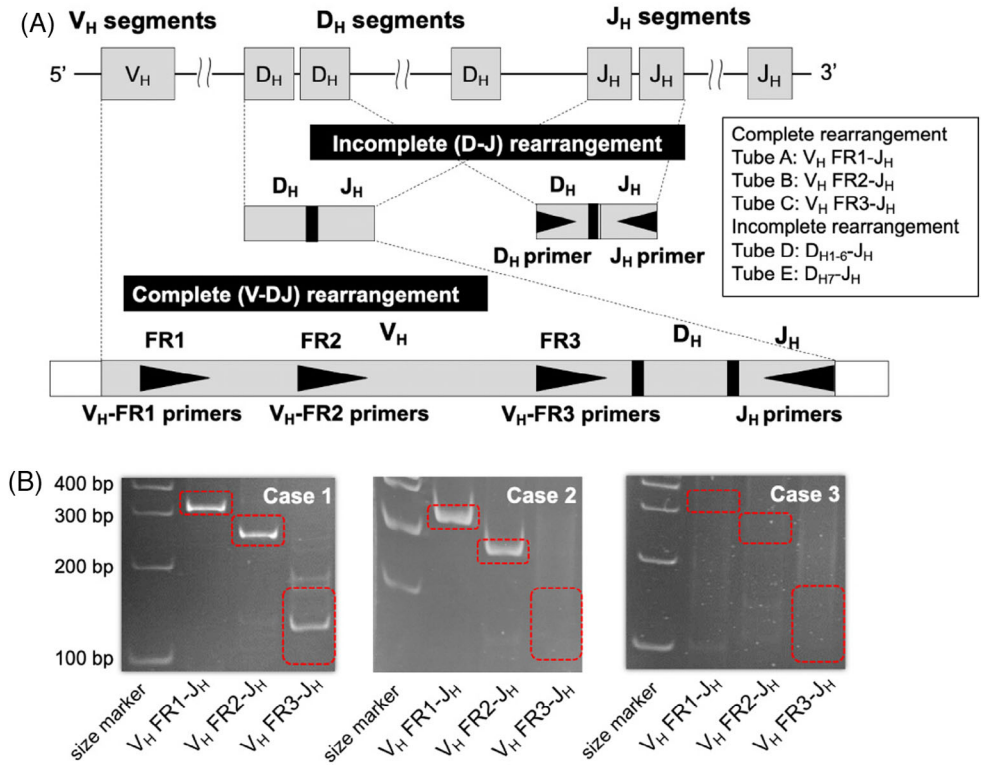

FIGURE 1 Complete immunoglobulin heavy chain (IgH) rearrangement detected using the BIOMED-2 protocol. (A) Schematic description of the BIOMED-2 protocol. Three sets of reactions were designed to detect complete IgH rearrangement (tube A: $V_HFR1–J_H$; tube B: $V_HFR2–J_H$; and tube C: $V_HFR3–J_H$), and two sets of reactions were designed to detect incomplete IgH rearrangement (tube D: $D_{H1-6}–J_H$ and tube E: $D_{H7}–J_H$). (B) Examples of reaction patterns in complete IgH rearrangement. The size of clonal polymerase chain reaction (PCR) products was 310–360 base pairs (bp) in $V_HFR1–J_H$ products, 250–295 bp in $V_HFR2–J_H$, and 100–170 bp in $V_HFR3–J_H$. Case 1 shows all positive reactions in complete IgH rearrangement (all-positive), Case 2 shows two positive reactions and a pseudo-negative one in FR3-J_H, and Case 3 shows all negative reactions (all-negative).

diagnosis was confirmed by the tissue biopsy according to the fourth edition of the World Health Organization classification published in 2008 [27]. Patients were included in this analysis if they were curatively treated with ≥1 cycle of anthracycline-based immunochemotherapy. Patients with primary mediastinal large B-cell lymphoma, intravascular large B-cell lymphoma, primary effusion lymphoma, and primary central nervous system lymphoma were excluded, as were patients with a prior history of indolent B-cell lymphoma and infection with human immunodeficiency virus.

In principle, the treatment regimen comprised conventional thrice-weekly R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone). Fourteen patients presenting with limited stage received three cycles of R-CHOP, followed by field irradiation, instead of six cycles of R-CHOP [28, 29]. Five patients with cardiac dysfunction received pirarubicin on Day 1 instead of doxorubicin (R-THPCOP regimen) [30, 31]. The study protocol complied with the Declaration of Helsinki and current ethical guidelines and was approved by the institutional review board of Kobe City Medical Center General Hospital. Informed consent was obtained from participants through posters and/or website using the opt-out method.

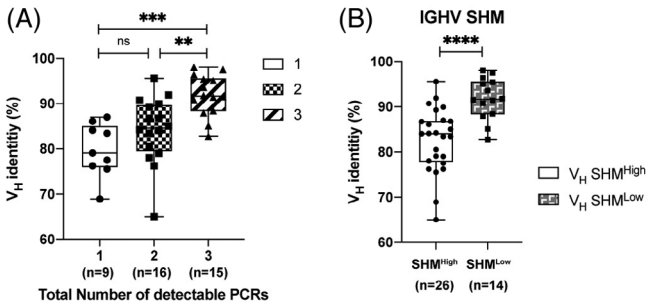

FIGURE 2 Correlation between V_H identity and the total number of successfully detectable polymerase chain reaction (PCRs). (A) Differences in V_H identity were analyzed based on the numbers of detectable clonal amplifications for 38 evaluable samples by one-way analysis of variance (ANOVA). $^{**}p < 0.01$, $^{***}p < 0.001$. (B) After categorization into the V_H^{Low} group and the V_H^{High} group, differences in V_H identity were assessed by the Mann–Whitney *U* test. $^{****}p < 0.0001$.

2.4 | Definition of BLNER

A total of 70 major histocompatibility complex (MHC)-II immunoglobulin neoantigens were identified from nine B-cell malignancies (DLBCL, follicular lymphoma, and chronic lymphocytic leukemia) using antigen presentation profiling by integrated tumor genomic profiling and

TABLE 1 Baseline clinical characteristics of the patients of our survival analysis according to the SHM status of IGHV in complete IgH rearrangement.

		SHM status of IGHV in DLBCL patients		
Patient characteristics	**Total ($N = 301$)**	**IGHV SHM^{High} ($n = 222$)**	**IGHV SHM^{Low} ($n = 79$)**	***p* value**
Median age (range), year	69 (32–93)	69 (32–93)	69 (37–90)	0.43
Male sex	165 (54.8%)	121 (54.5%)	44 (55.7%)	0.59
ECOG performance status				
≧2	95 (31.6%)	65 (32.1%)	30 (37.9%)	0.16
Serum LDH				
Elevated	167 (55.4%)	123 (58.5%)	44 (55.7%)	0.99
Ann Arbor stage				
Stage III or IV	183 (60.8%)	138 (62.1%)	45 (57.0%)	0.42
Extranodal sites				
≧2 sites	87 (28.9%)	68 (30.6%)	19 (24.1%)	0.31
IPI (International Prognostic Index)				
Low	89 (29.6%)	68 (34.1%)	21 (26.6%)	
Low—intermediate	65 (21.6%)	38 (18.8%)	27 (34.1%)	
High–intermediate	62 (20.6%)	54 (26.7%)	8 (10.1%)	
High	85 (28.2%)	62 (31.1%)	23 (29.1 %)	0.10
Bulky lesion				
Present	37 (12.3%)	24 (11.8%)	13 (16.5%)	0.23
Bone-marrow involvement				
Present	46 (15.3%)	36 (17.8%)	10 (12.7%)	0.59

Note: Data are presented as numbers with percentages unless otherwise indicated.
Abbreviations: DLBCL, diffuse large B-cell lymphoma; ECOG, Eastern Cooperative Oncology Group; IgH, immunoglobulin heavy chain; IGHV, immunoglobulin heavy variable; LDH, lactate dehydrogenase; SHM, somatic hypermutations.

proteomic characterization of tumor-derived MHC-II ligands by immunoprecipitation and liquid chromatography–tandem mass spectrometry [6, 7]. Based on those reports, we defined a region of amino acids shared at a high frequency (more than 25 peptides) among those 70 neoantigen peptides as BLNER (B-cell lymphoma neoantigen enriched region).

2.5 | Statistical methods

A one-way analysis of variance (ANOVA) was used to test an association between the total detectable numbers of V_H clonal amplifications and the frequency of V_H SHMs. A Mann–Whitney test was used to test the statistical significance of V_H identity between the V_H SHM^{Low} group and the V_H SHM^{High} group. Patient baseline characteristics were compared through the use of the exact chi-square test if they were categorical and the Mann–Whitney *U* test if they involved continuous variables. The primary endpoint was 5-year overall survival (OS), and the secondary endpoint was 5-year progression-free survival (PFS). OS was calculated from the time of initial diagnosis to the date of death, irrespective of the cause. PFS was measured from the time of diagnosis to disease progression or death from any cause. The observation time was censored at the date of the last follow-up. OS and PFS curves were constructed using the Kaplan–Meier method and compared using the log-rank test. The Cox proportional-hazard model was used to estimate the hazard ratio (HR) and its 95% confidence interval (CI) for each variable for 5-year OS. Potential confounder variables for adjustment were as follows: age, sex, performance status, lactate dehydrogenase (LDH) levels, Ann Arbor stage, number of extranodal sites, bulky lesions, and bone-marrow involvement. The clinical parameters of International Prognostic Index (IPI) risk stratification (patient age, LDH level, Eastern Cooperative Oncology Group [ECOG] performance status, Ann Arbor stage, and numbers of extranodal sites) were included in this multivariable analysis. All *p* values were two-tailed and considered significant at <0.05. All statistical analyses were performed using EZR software (v3.1.1; R Development Core Team; https://www.r-project.org/) [32].

3 | RESULTS

3.1 | Full detection of FR1 to FR3 primer-based IGHV PCR patterns in BIOMED-2 protocol is a useful surrogate for low SHM frequency

We first evaluated whether the FR1 to FR3 primer-based PCR patterns in the BIOMED-2 protocol (Figure 1) could be a surrogate for

IGHV SHM frequency. We analyzed the SHM frequency in the V_H segment of the IgH gene in 44 cryopreserved DLBCL samples. These samples were amplified by PCR according to the BIOMED-2 protocol (Figure 1) and directly sequenced, yielding results from 40 samples. The other four were unsuccessfully sequenced due to insufficient fluorescence intensity for accurate sequencing ($n = 2$) and high polyclonal background ($n = 2$). Of the 40 successful sequences, 38 (95.0%) were productive rearrangements and two (5.0%) were unproductive. Thirty-eight (95.0%) rearrangements were sequenced from the FR1 region. All analyzed V_H sequences contained SHMs with a median percentage of 13.3%. As shown in Figure 2A, the median value of V_H germline identity was 91.8% (range: 82.8%–98.0%) in patients positive for all FR1 to FR3 primer-based PCR amplification using the BIOMED-2 protocol (triple-positive: $n = 15$), 84.6% (range: 65.0%–95.6%) in patients positive for two primers (double-positive: $n = 16$), and 79.1% (range: 68.9%–87.0%) in patients positive for one primer (single-positive: $n = 9$). Patients in the double-positive group had higher V_H homology as compared with the single-positive group, although the difference was not statistically significant ($p = 0.095$). Patients in the triple-positive group displayed significantly higher homology to the germline sequence than either the double-positive or single-positive patients ($p = 0.004$ and $p < 0.001$, respectively). These results showed that complete detection of FR1 to FR3 primer-based PCR patterns in the BIOMED-2 protocol is a convenient surrogate marker reflecting the significantly lower frequency of V_H SHMs. Based on this, we classified patients with successful triple-positive PCR amplifications using all three sets of V_H primers as the V_H SHM^{Low} group, and the other cases with at least one false-negative PCR result as the V_H SHM^{High} group (Figure 2B).

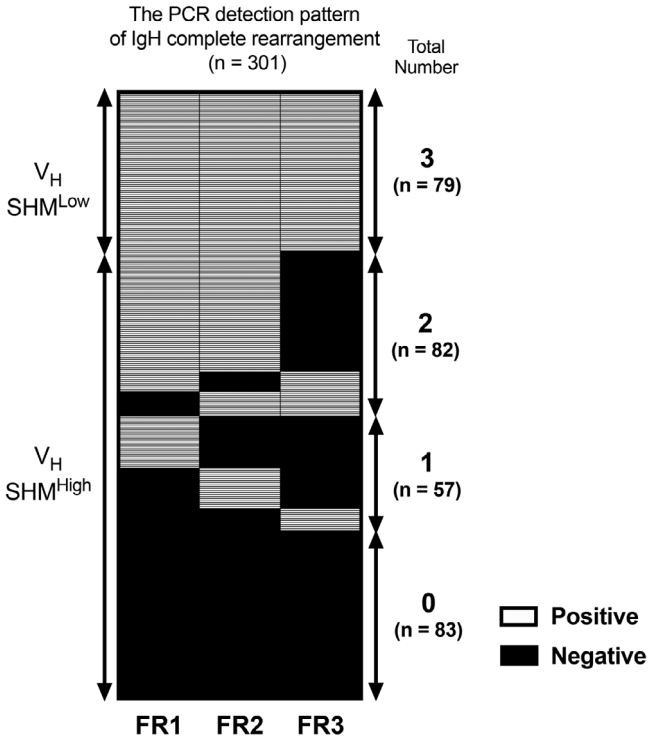

FIGURE 3 Multiplex polymerase chain reaction (PCR) results of immunoglobulin heavy chain (IgH) complete rearrangement based on BIOMED-2 protocol for 301 patients used for survival analysis. White squares indicate successful detection of clonal amplifications and black squares indicate false-negative results.

3.2 | The V_H SHM^{LOW} group has a significantly poor prognosis in DLBCL

To investigate the prognostic impact of IGHV SHM frequency in DLBCL, a total of 301 patients with de novo DLBCL were included. The baseline characteristics of the patients are summarized in Table 1. The study population comprised 165 males and 136 females, with a median age of 69 years (range: 22–93 years), with 183 patients (60.7%) presenting Ann Arbor Stage III or IV disease, and 147 (48.8%) having a high–intermediate or high IPI score. Regarding IGHV PCR results, a total of 254 patients (84.3%) were found to have an IgH clonal rearrangement. Incomplete IgH D–J rearrangement was detected in 98 patients (32.6%), and complete IgH V–D–J rearrangement was detected in 218 patients (72.5%). FR1-J_H clonal amplifications were detected in 175 patients (58.1%), FR2-J_H clonal amplifications in 171 patients (56.8%), and FR3-J_H clonal amplifications in 112 patients (37.2%; Figure 3). Such detection rates are comparable to those reported in the original BIOMED-2 protocol [20] and other subsequent studies of IgH clonality in DLBCL [23, 25, 26], assuring that the BIOMED2 protocol is implemented with fidelity at our institution. Based on the PCR results obtained, the enrolled patients were divided into the V_H SHM^{Low} group ($n = 79$) and the V_H SHM^{High} group ($n = 222$). There was no statistical difference in the distribution of clinical parameters between the patient groups (Table 1). We compared the 5-year OS and PFS rates between the V_H SHM^{Low} group and the V_H SHM^{High} group. The 5-year OS and PFS rates were significantly worse in the V_H SHM^{Low} group relative to the V_H SHM^{High} group (OS: 54.2% vs. 73.2%; $p = 0.002$; PFS: 34.3% vs. 59.3%; $p < 0.001$; Figure 4A,B). A multivariable Cox proportional-hazard model identified the lower frequency of V_H SHMs as an independent predictor for worse 5-year OS (HR: 2.34; 95% CI: 1.50–3.67; $p < 0.001$), even after adjustment for the five clinical parameters of IPI risk stratification. Furthermore, patients for whom immunohistologic specimens were available were also analyzed for survival based on cell of origin and complete IgH-rearrangement pattern. Of the 124 evaluable DLBCL patient samples, 65 were germinal center B-cell (GCB) type and 59 were non-GCB (activated B-cell like)-type (5-year OS: GCB type 79.0% vs. non-GCB type 70.1%, $p = 0.79$; 5-year PFS: GCB type 63.7% vs. non-GCB type 39.6%, $p = 0.046$). In GCB-DLBCL, the 5-year OS and PFS rates were significantly worse in the V_H SHM^{Low} group than the V_H SHM^{High} group (OS: 47.3% vs. 88.8%; $p = 0.012$; PFS: 37.5% vs. 72.9%; $p = 0.011$; Figure 4C,D). In non-GCB DLBCL, the 5-year OS were not significantly different in the V_H SHM^{Low} group and the V_H SHM^{High} group (65.7% vs. 72.2%; $p = 0.93$), but the 5-year PFS rates were significantly worse in the V_H SHM^{Low} group than the V_H SHM^{High} group (19.2% vs. 47.9%; $p = 0.015$; Figure 4E,F). These data suggest that full detection of the FR1 to FR3 primer-based IgH VH PCR patterns is an indicator of poor

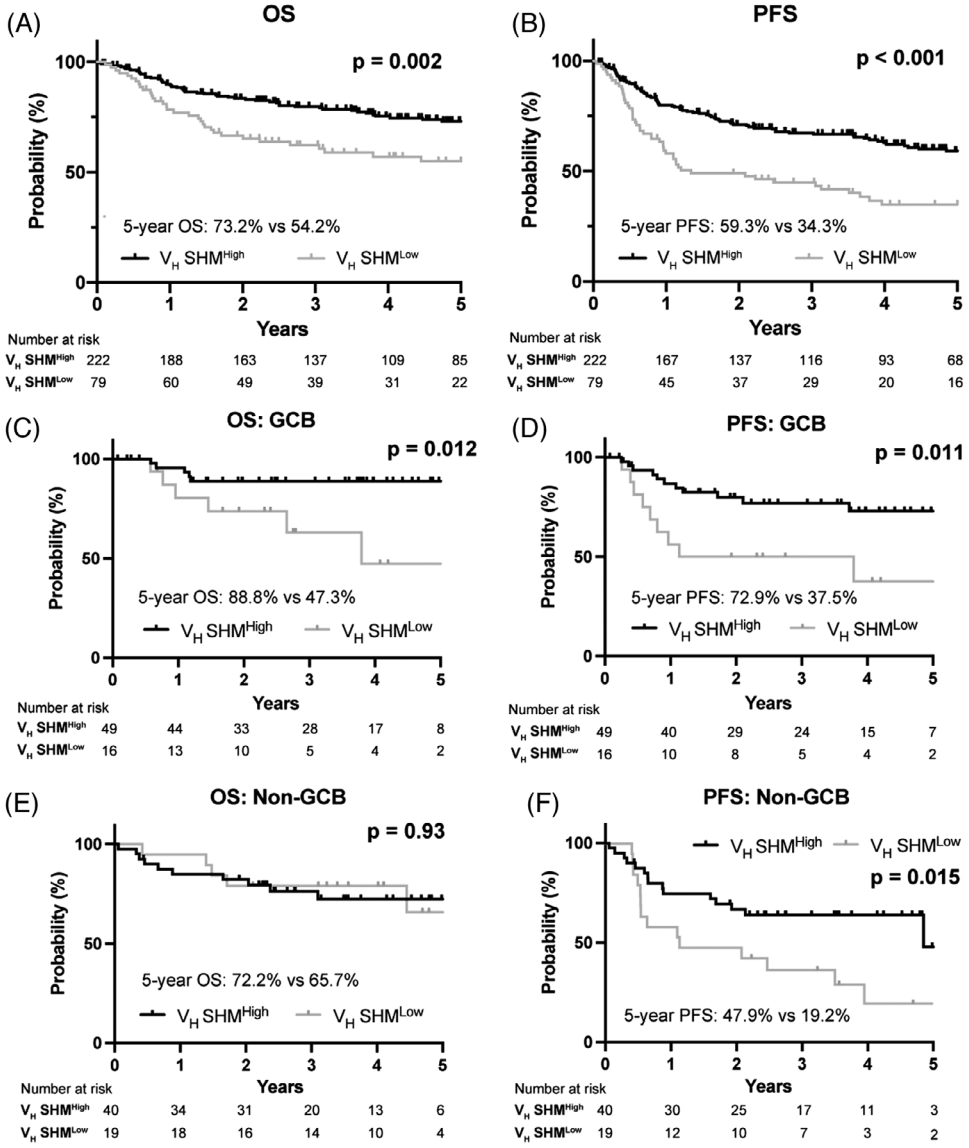

FIGURE 4 Kaplan–Meier curves for overall survival (OS; A) and progression-free survival (PFS; B) between the V_H SHM^{Low} group and the V_H SHM^{High} group. (C and D) Prognostic analysis between the V_H SHM^{Low} group and the V_H SHM^{High} group in the GCB subtype of diffuse large B-cell lymphoma (DLBCL; $n = 65$). (E and F) Prognostic analysis between the V_H SHM^{Low} group and the V_H SHM^{High} group in the non-GCB subtype of DLBCL ($n = 59$). The number of patients at risk per year is shown below each figure.

prognosis of DLBCL independent of other known prognostic factors, including cell of origin.

3.3 | Successful detection of FR3-J_H clonal amplification in the BIOMED-2 protocol is significantly associated with poor prognosis in DLBCL

Finally, to evaluate the impact of the SHMs on specific individual regions (FR1–FR3), we evaluated the prognostic impact of the presence of each clonal amplification (FR1-J_H, FR2-J_H, and FR3-J_H). There was no significant difference in 5-year OS between the presence and absence of FR1-J_H (66.2% vs. 71.0%; $p = 0.37$, Figure 5A) or FR2-J_H (64.1% vs. 75.5%; $p = 0.07$, Figure 5B). On the other hand, the 5-year OS and PFS were significantly worse in the FR3-J_H detectable group than in the FR3-J_H undetectable group (OS: 54.6% vs. 76.2%, $p < 0.001$, Figure 5C; PFS: 37.0% vs. 62.1%, $p < 0.001$, Figure 5D). In the V_H SHM^{high} subgroup, we also found that both 5-year OS and PFS were significantly worse in the FR3-J_H detectable group than in the FR3-J_H undetectable group (OS: 53.8% vs. 76.2%, $p = 0.049$, PFS: 40.7% vs. 62.1%, $p = 0.031$; Figure 5E,F). These results suggest that the high frequency of SHMs of FR3 regions of the V_H segment may contribute to improve prognosis in patients with DLBCL. FR3 has recently been focused on as a region responsible for B-cell lymphoma neoantigen. We defined BLNER as the most concentrated region of B-cell lymphoma neoantigen in the FR3 region based on previous studies about B-cell lymphoma neoantigen [6, 7]. We found that FR3 primers, which consisted of seven oligonucleotides capable of annealing to the

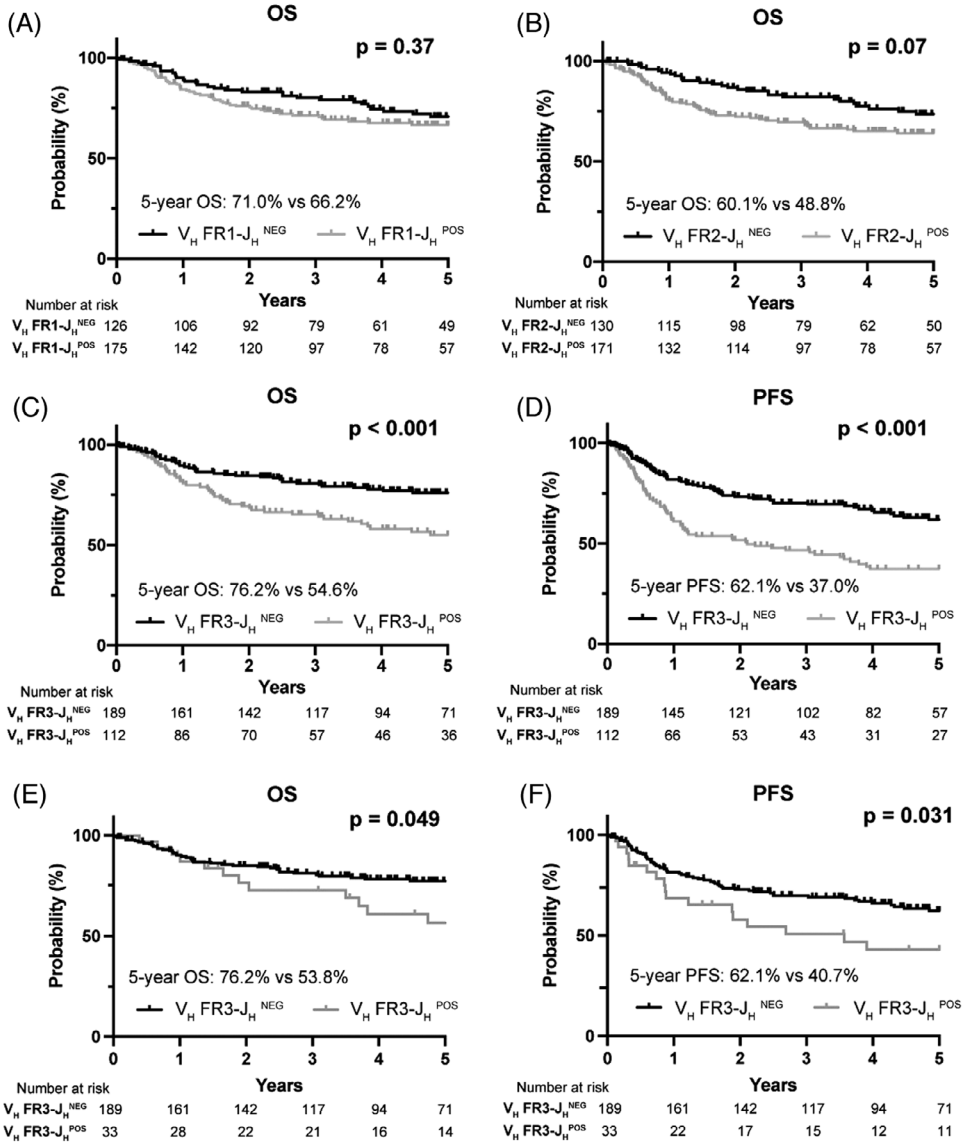

FIGURE 5 (A and B) Kaplan–Meier curves for overall survival (OS) of patients with diffuse large B-cell lymphoma (DLBCL; $n = 301$) based on the positivity of V_HFR1–J_H (A) and V_HFR2–J_H (B). (C and D) Kaplan–Meier curves for OS (C) and progression-free survival (PFS; D) based on the positivity of V_HFR3–J_H. (E and F) Kaplan–Meier curves for OS (E) and PFS (F) based on the positivity of V_HFR3–J_H in the V_H SHM^{High} subgroup ($n = 222$). The number of patients at risk per year is shown below each figure.

corresponding major V_H segments (VH1–VH7), completely annealed the BLNER without any mismatches (Figure 6). This means that the detection of FR3-JH clonal amplification is highly dependent on the SHM frequency of the BLNER sequences. The poor clinical impact of successful detection of FR3-J_H clonal amplification in this study may be due to the loss of neoantigen conferred by BLNER SHMs.

4 | DISCUSSION

IGHV SHMs have revealed the ontogeny of several B-cell lymphomas, including DLBCL [4, 5, 33–36], and also act as antigens against T cells and exhibit immunogenicity as a neoantigen [6, 7, 37–39]. Thus, a new biomarker related to IGHV SHMs may allow the identification of DLBCL patients who would potentially benefit from more specific and/or aggressive treatment strategies. In this study, we focused on the PCR pattern of IgH complete (V–D–J) rearrangement according to the BIOMED-2 protocol as a potential surrogate for SHM analysis of the V_H gene. We demonstrated that DLBCL patients who were positive for all three PCRs for IgH complete rearrangement had significantly lower V_H SHMs and worse prognosis than those with other PCR patterns.

Over the past two decades, several PCR assays have been developed for IgH clonality testing [12, 40–44]. The BIOMED-2 protocol has been the gold standard for clonality testing in lymphoid malignancies; however, it has several limitations and pitfalls, including pseudo-negative results [9]. In PCR assays for B-cell malignancies, false-negative results are usually caused by unsuccessful PCR amplification of clonal IgH

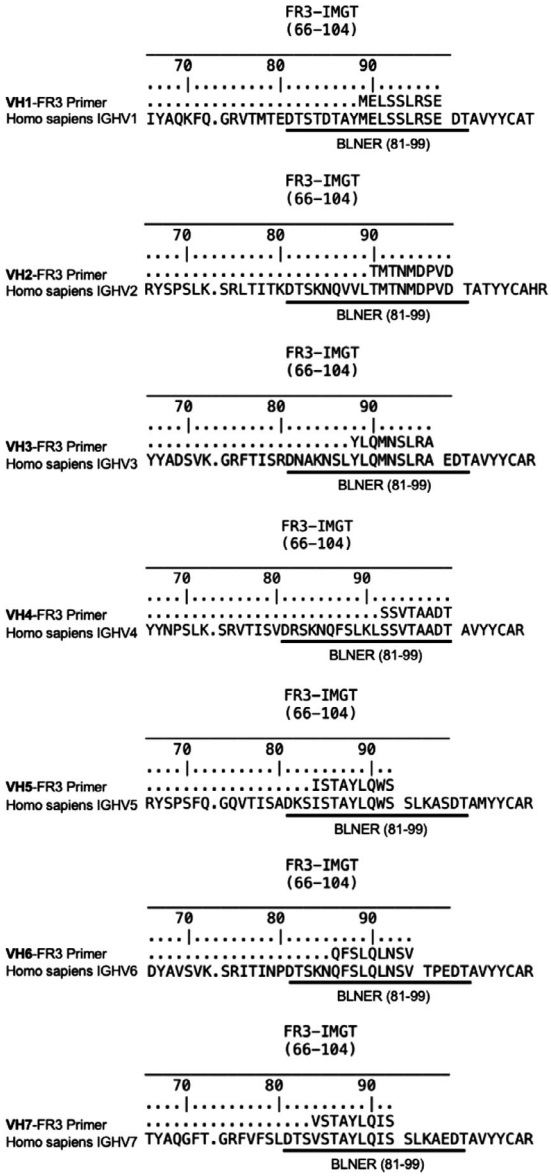

FIGURE 6 Framework region 3 (FR3) sequences of the major V_H segments (V_H1–V_H7) and FR3 primers for the corresponding V_H segments (V_H1–V_H7). All primers completely annealed to the corresponding V_H segments and ranged within BLNER (B-cell lymphoma neoantigen enriched region).

rearrangement, which is mainly associated with two different mechanisms: insufficient primer coverage for all Ig gene regions and poor primer annealing due to SHMs in the VH gene introduced in the germinal center [20]. In this protocol, the first problem was almost solved by designing primers that were maximally homologous to all relevant Ig gene regions [18]. In the general testing phase of this protocol, it was concluded that false-negative results were mainly caused by SHMs of the primer binding sites in the VH gene region [9]. In the present study, using 40 available DLBCL samples, we confirmed that patients positive for all three PCRs had a significantly lower frequency of SHMs in the V_H gene (higher homology to the VH germ line sequence) compared to those with other PCR patterns, indicating that FR1 to FR3 primer-based IGHV PCRs in the BIOMED-2 protocol would be a convenient and clinically applicable surrogate method for SHM analysis of the V_H gene.

B-cell malignancies commonly retain various characteristics of normal cells in the corresponding stage of B-cell differentiation [45]. The ontogeny of neoplastic B cells has been elucidated partly by SHM analysis of the V_H gene [4, 5, 33–36]. In B-CLL and MCL patients, the presence of subpopulations with nonmutated V_H genes has been confirmed, and these subpopulations are associated with poor prognosis. A number of extensive studies have shown that in B-CLL, patients with nonmutated IgH (>98% homology to the V_H germline sequence) have a worse prognosis than those with mutated IgH (<98% homology) [4]. Similarly, ~20%–30% of MCL patients harbor SHMs of the V_H gene, with this type of MCL characterized by non-nodal disease presentation, stable karyotype, and indolent clinical course [5, 36, 46]. Gene expression profiling studies have demonstrated that DLBCL comprises two molecular categories, GCB and non-GCB (ABC) type, based on the cell of origin [47]. This molecular classification has become critical for prognostic stratification and determination of optimal treatment strategies especially in the current molecular era. Two groups have shown that the ABC subtype has a significantly lower frequency of SHM in the V_H gene than the GCB subtype [2, 48]; however, Xu-Monette et al. observed no significant differences in SHM status by cell of origin. Our study also did not show any significant association between the PCR pattern of the complete IgH rearrangement and cell of origin, so further research is needed to verify this controversial point.

In addition to tumor-intrinsic features, recent studies have shown that V_H SHMs are also important as neoantigens in eliciting immune responses against B-cell malignancies. Neoantigens are non–self-peptides derived from tumor-specific gene products that are recognized as foreign and presented MHC-restricted T cells to induce antitumor effects [38, 39]. In most cancers, somatic mutations can generate neoantigens. In B-cell malignancies, however, neoantigens have been reported to derive from Ig variable chain peptides with SHMs and they are processed by B cells and presented by MHC class I and class II to both CD4+ and CD8+ T cells [37]. Of all the MHC-binding peptides, somatic neoantigens were mainly of IGHV origin [6]. Recently, Xu-Monette et al. reported that high levels of IGHV SHM (SHM^{High}) were associated with prolonged OS in DLBCL patients especially those without BCL2 or MYC translocation, and that the SHM^{High} group harbored more potentially immune-stimulatory neoantigens with high binding affinity and rare T-cell exposed motifs by their prediction models. In the present study, we retrospectively investigated the prognostic impact of VH SHMs in DLBCL using FR1 to FR3 primer-based IGHV PCR patterns as a surrogate for VH SHMs, and also showed that the SHM^{high} group had better OS and PFS.

Most of these neoantigens found in B-cell malignancies are derived from FR 3 (FR3) and are generated by V–D–J recombination or SHMs [6, 7]. In this study, we also found that the prognostic significance of the complete detection of IGHV PCR products via the BIOMED-2 protocol in this study was mainly due to the successful PCR of the FR3

region. Therefore, the present results suggest that IGHV SHM-derived neoantigens (especially in the FR3 region) have immunogenicity that may account for a favorable prognosis in patients receiving standard DLBCL therapy and that they deserve further consideration as immunotherapy targets in the current era of immunotherapy.

Our study has several limitations in that it is a retrospective and single-center study with limited resources. In addition, this study could not assess the IGHV SHM frequency in all the patients from the survival cohort because of sample DNA availability. Recently, the IGHV SHM status has been reported to have association with BCL2/MYC translocation and PD-L1/L2 (9p24.1) amplification, but in our study such molecular relationships could not be fully clarified.

Nevertheless, considering its availability, cost, and diagnostic utility, our results suggest that the complete IgH-rearrangement pattern of the BIOMED-2 protocol is highly effective as a biomarker for prognostic stratification in DLBCL.

5 | CONCLUSION

Our results showed that complete detection of FR1 to FR3 primer-based PCR patterns of IgH rearrangement in the BIOMED-2 protocol was associated with poor prognosis in de novo DLBCL patients. Combined with the results of V_H sequencing analysis, this may suggest that a subset of DLBCL with lower levels of V_H SHMs, especially in the FR3 region, includes patients with unfavorable prognosis. Further large prospective studies are needed to validate these findings and the prognostic implications of SHM frequency in the V_H gene in DLBCL.

AUTHOR CONTRIBUTIONS

Tomohiro Yabushita and Hayato Maruoka designed the research, obtained the samples, and performed the study; Yoshimitsu Shimomura, Nobuhiro Hiramoto, Satoshi Yoshioka, and Noboru Yonetani collected clinical information; Daisuke Yamashita and Yukihiro Imai diagnosed the pathology of tumor samples; Tomohiro Yabushita, Hayato Maruoka, Yoshimitsu Shimomura, and Takeshi Morimoto contributed to data analysis and interpretation; Tomohiro Yabushita, Hayato Maruoka, Hironaga Satake, Momoko Nishikori, and Takayuki Ishikawa wrote the manuscript; and all authors revised the report and approved the final version.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author (Yoshimitsu Shimomura), upon reasonable request.

ETHICS STATEMENT

This study complied with the Declaration of Helsinki and was approved by the Ethical Committee of Kobe City Medical Center General Hospital (Approval No. zn171212).

PATIENT CONSENT STATEMENT

Based on the approval by the Ethical Committee in our institution, patient consent to participate was obtained using the opt-out method.

PERMISSION TO REPRODUCE MATERIAL FROM OTHER SOURCES

NA.

CLINICAL TRIAL REGISTRATION (INCLUDING TRIAL NUMBER)

The authors have confirmed clinical trial registration is not needed for this submission.

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