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Ig Gene Clonality Analysis Using Next-Generation Sequencing for Improved Minimal Residual Disease Detection with Significant Prognostic Value in Multiple Myeloma Patients

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Next-generation sequencing (NGS) of rearranged Ig genes is an effective technology for identifying pathologic clonal cells in multiple myeloma (MM) and tracking minimal residual disease. The clinical effect of implementing NGS in Ig gene clonality analysis was evaluated via a retrospective chart review. A total of 312 patients diagnosed with MM were enrolled in the study. Ig gene clonality was determined by fragment analysis using BIOMED-2 multiplex PCR assays and by NGS using the LymphoTrack *IGH* FR1 Assay and LymphoTrack *IGK* Assay. The clonality detection rates in diagnostic samples obtained using fragment analysis and NGS were 96.7% and 95.4%, respectively (statistically nonsignificant difference; $P = 0.772$). Among samples of patients in complete remission, the clonality detection rates obtained using fragment analysis and NGS were 33.3% and 60.3%, respectively (statistically significant difference; $P = 0.034$). Progression-free survival was significantly longer in negative than positive patients by NGS analysis ($P = 0.03$). Clonality detection by NGS-based methods using *IGH* FR1 and *IGK* assays in routine clinical practice is feasible, provides good clonality detection rates in diagnostic samples, and allows monitoring of samples in MM patients with significant prognostic value. *(J Mol Diagn 2022, 24: 48—56; https://doi.org/10.1016/j.jmoldx.2021.09.006)*

Multiple myeloma (MM) is characterized by clonal proliferation of neoplastic plasma cells in bone marrow.[1] Traditionally, several factors have been known to be associated with the prognosis of MM patients, such as cytogenetic abnormalities and serum levels of β2-microglobulin, lactate dehydrogenase, and albumin. Recent myeloma therapies have achieved high response rates; however, most patients eventually relapsed due to the persistently low levels of malignant plasma cells after treatment. Such minimal residual disease (MRD) assessment is crucial for evaluating treatment response and risk stratification in MM patients. The attainment of MRD negativity is associated with prolonged progression-free and overall survival in MM patients.[2,3]

Conventional methods of MRD assessment include allele-specific oligonucleotide PCR and multiparametric flow cytometry (MFC).[4,5] Although allele-specific oligonucleotide PCR provides high sensitivity for the detection of residual clonotype sequences, it is not widely used for routine clinical testing because it is a laborious and time-consuming process, owing to the design and validation of patient-specific primers and probes for quantitative PCR. The most commonly used method in clinical laboratories for detecting clonal Ig gene

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rearrangements is fragment analysis using multiplex PCR primers established by the EuroClonality/BIOMED-2 consortium.[6] However, the detection sensitivity of fragment analysis is approximately 5%, which makes it unsuitable for MRD assessment. MFC, another MRD assessment technique, is widely available in hematology laboratories, and has broad applicability in patients. However, obtaining consistent sensitivity between laboratories requires additional standardization efforts, such as those dedicated toward selecting antibody panels and gating strategies.[7]

Recently, the next-generation sequencing (NGS) technology has been applied to clonality detection and quantification of Ig gene rearrangements in MM.[3,8] NGS has the advantage of universal applicability, with the use of off-the-shelf consensus primers. In addition, high sensitivity and specificity can be expected because the initial clonotype sequence is tracked in massively parallel sequencing data from follow-up samples. The purpose of this study was to explore the clinical usefulness of NGS-based clonality tests in MM patients.

Materials and Methods

Patients and Clinical Data

A total of 312 patients diagnosed with MM from January 2013 to July 2019 were included in this study. The medical records of the patients were retrospectively reviewed. This study protocol was approved by the institutional review board of the Severance Hospital, Yonsei University College of Medicine, Seoul, Republic of Korea (institutional review board number 4-2019-0815). Clinical response and disease progression were assessed according to the International Myeloma Working Group criteria for MM.[9] Disease staging and results of ancillary test results, including morphologic assessment of bone marrow aspiration and biopsy, multicolor flow cytometry, serum protein electrophoresis, serum immunofixation electrophoresis, and serum free-light chain assay, were reviewed. The overall patient characteristics and disease types are summarized in Table 1.

Sample Preparation

Bone marrow aspirate samples at initial diagnosis and follow-up were obtained in K2 EDTA tubes. Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Venlo, the Netherlands) for clonality testing.

Clonality Testing Using Fragment Analysis

For fragment-based clonality testing, the IdentiClone *IGH* Gene Clonality Assay and the IdentiClone *IGK* Gene Clonality Assay kits (Invivoscribe Technologies, San Diego, CA) were used for PCR amplification. The assays use BIOMED-2 multiplex primer sets in five master mixes targeting the *IGH* (IGH_A: $V_HFR1\text{-}J_H$; IGH_B: $V_HFR2\text{-}J_H$; IGH_C: $V_HFR3\text{-}J_H$; IGH_D: $D_H1{-}6\text{-}J_H$; and IGH_E: $D_H7\text{-}J_H$) and two targeting the *IGK* locus (IGK_A: Vκ-Jκ; IGK_B: Vκ-kappa-deleting element (Kde); and JκCκ intron-Kde). The fluorescently labeled PCR products were analyzed by fragment analysis on ABI 3130 DNA Analyzer (Thermo Fisher Scientific, Life Technologies Corp., Carlsbad, CA) and GeneMapper software version 3.2 (Thermo Fisher Scientific). Clonality in samples was interpreted on the basis of the manufacturer's recommendations; the suspected peak in diagnostic samples within the valid size range should be at least three times higher than the height of the third largest peak in the background. In follow-up samples, peaks with identical sizes in diagnostic samples and with a height exceeding that of an adjacent peak were interpreted as positive.

Clonality Testing Using Next-Generation Sequencing

NGS analysis was performed with the LymphoTrack *IGH* FR1 Assay and LymphoTrack *IGK* Assay (Invivoscribe Technologies), according to the manufacturer's instructions. Genomic DNA was quantified using the Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA). Then, 200 to 400 ng of DNA was amplified using a single multiplex master mix for each target (*IGH* FR1 and *IGK*). After PCR amplification, the libraries were purified using the Agencourt AMPure XP system (Beckman Coulter, Brea, CA). Quantified libraries were sequenced on the MiSeq system using MiSeq Reagent Kit v2 (Illumina, San Diego, CA). For each batch, the positive and negative control materials contained in the kit were tested simultaneously. Bioinformatics analysis was done with commercially available LymphoTrack Dx MiSeq Data Analysis version 2.4.3 (Invivoscribe Technologies). The cutoff for clonality and clonotype sequence determination was followed by manufacturer's guidelines. The presence of MRD in follow-up samples was analyzed using the LymphoTrack MRD Data Analysis Tool (Invivoscribe Technologies). In the sequencing data from the follow-up sample, read frequencies of the previously characterized index clonotype sequences as an exact match and up to 2-bp mismatches (>99% sequence homology) were detected and calculated. The median (range) sequencing reads for monitoring samples were 249,895 (20,550 to 2,580,828). The manufacturer guarantees a sensitivity level of 10^{-4} with about 95% confidence when sequencing the 300 ng of DNA with 250,000 reads.

Multicolor Flow Cytometry

Multiparametric flow cytometric immunophenotyping was performed using monoclonal antibodies (Beckman Coulter, Miami, FL) against CD138, CD38, CD45, CD56, CD19, CD20, cytoplasmic (cyt)KAPPA, and cytLAMBDA following the procedure for intracytoplasmic determination. Backbone markers CD38, CD45, and CD138 were included in all tests. All measurements were performed on a flow cytometer, Navios (Beckman Coulter, Miami, FL). Gating procedure was based on CD38 versus CD138 antigen expression. In this analysis, 100,000 events are evaluated. Positive criteria of multicolor

Table 1 Patient and Disease Characteristics at the Time of Initial Diagnosis

Characteristics	Fragment analysis (n = 182)	NGS (n = 130)	P value
Sex			
Male	89 (48.9)	78 (60.0)	0.06834
Female	93 (51.1)	52 (40.0)	0.06834
Age, median (range), years	64.5 (37–92)	66 (31–91)	0.1355
Ig isotype*			
IgG	97 (53.3)	68 (52.3)	0.9541
IgA	35 (19.2)	23 (17.7)	0.844
IgD	8 (4.4)	5 (3.8)	1.000
IgM	0 (0.0)	1 (0.8)	0.8656
Light chain only	41 (22.5)	31 (23.8)	0.8916
Nonsecretory	1 (0.5)	2 (1.5)	0.7686
Creatinine, mg/dL, median (range)	0.9 (0.46–11.55)	1.0 (0.46–19.57)	0.05799
Hemoglobin, g/dL, median (range)	11.9 (4.2–18.0)	11.2 (6–17.6)	0.4347
Calcium, mg/dL, median (range)	9.4 (7.4–17.1)	9.2 (7.3–15.1)	0.9259
β2-Microglobulin, mg/L, median (range)	4.2 (1.05–36.92)	3.9 (1.24–54.03)	0.3553
Bone marrow plasma cell %, median (range)	35.5 (4.9–97.9)	37.5 (3.3–97.8)	0.8456
ISS			
I	33 (25.8)	26 (29.2)	0.6863
II	40 (31.2)	29 (32.6)	0.9526
III	55 (43.0)	34 (38.2)	0.5742
Missing	54	41	

Data are expressed as n (%) of patients, unless otherwise indicated.

*Ig heavy chain was not detected in light chain disease (n = 72; 24.0%) and nonsecretory type (n = 3; 1.0%).

ISS, International Staging System; NGS, next-generation sequencing.

flow cytometry for MRD assessment were defined as the presence of a population consisting of at least 20 cells with an abnormal immunophenotype.

Statistical Analysis

Shapiro-Wilk test was conducted to assess whether continuous variables follow normal distribution, followed by the Levene test for determining the homogeneity of variance. Hemoglobin was normally distributed, as were homogeneous variables, but age, creatinine, calcium, β2-microglobulin, and bone marrow plasma cells were not. The t-test was performed in normally distributed and homogeneous data among the two groups with different methods. Otherwise, Mann-Whitney-Wilcoxon test was applied where appropriate. Categorical variables were compared using χ^2 tests. Logistic regression analysis was used to investigate the relationships between plasma cell percentage and positivity. Linear regression was used to analyze relationships involving NGS and conventional six-color MFC assays. Time to progression was evaluated using Kaplan-Meier plots and log-rank tests. Differences with $P < 0.05$ were considered statistically significant. Statistical analysis was

Table 2 Positive Rates of Ig Gene Rearrangement, Serum Light Chain Ratio, and Multicolor Flow Cytometry Tests in Fragment and NGS Periods

Test	Evaluation time points	Period of fragment analysis (January 2013–March 2017)*	Period of NGS assay (April 2017–August 2019)†	P value
Positive rate of clonality of Ig gene rearrangement, %	Diagnosis	96.7 (n = 182)	95.4 (n = 130)	0.772
	Monitoring	44.7 (n = 76)	70.3 (n = 118)	<0.001
Positive rate of serum light chain ratio, %‡	Diagnosis	93.4 (n = 182)	96.2 (n = 130)	0.411
	Monitoring	55.3 (n = 76)	48.3 (n = 118)	0.421
Positive rate of multicolor flow cytometry, %§	Diagnosis	99.4 (n = 178)	100.0 (n = 63)	1.000
	Monitoring	37.8 (n = 74)	27.1 (n = 48)	0.305

*Fragment-based clonality testing was performed with the IdentiClone Gene Clonality Assay kits (Invivoscribe Technologies) that use BIOMED-2 multiplex primer sets in five master mixes targeting the *IGH* (*IGH*$_A$: V_HFR1-J_H; *IGH*$_B$: V_HFR2-J_H; *IGH*$_C$: V_HFR3-J_H; *IGH*$_D$: D_H1–6-J_H; and *IGH*$_E$: D_H7-J_H) and two targeting the *IGK* locus (*IGK*$_A$: Vκ-Jκ; *IGK*$_B$: Vκ-Kde; and JκCκ intron-Kde).

†NGS analysis was performed with the LymphoTrack *IGH* FR1 Assay and LymphoTrack *IGK* Assay (Invivoscribe Technologies).

‡Positive criterion of serum light chain ratio was defined as <0.26 or >1.65.

§Positive criterion of multicolor flow cytometry was defined as the presence of a population consisting of at least 20 cells with an abnormal immunophenotype.

NGS, next-generation sequencing.

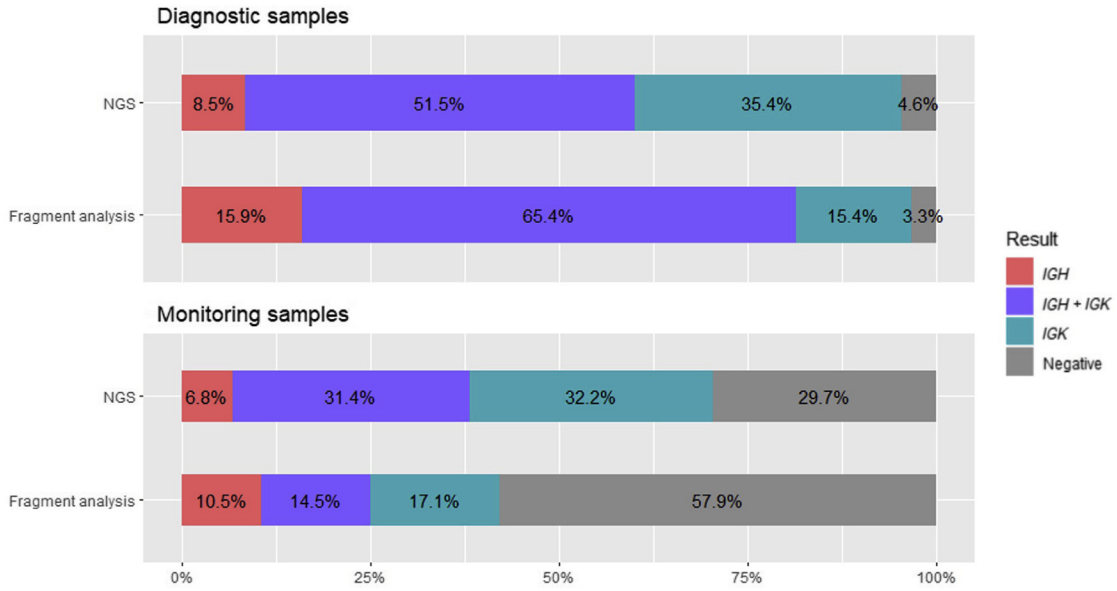

Figure 1 Overall clonal characterization. By fragment analysis using *IGH* (FR1, FR2, and FR3) and *IGK* primers and next-generation sequencing (NGS) with *IGH* (FR1) and *IGK* primers, positive rates in diagnostic samples were 96.7% (176/182) and 95.4% (124/130), respectively. Positive rates for *IGH* clonality assessment using fragment analysis and NGS assay in diagnostic samples were 81.3% (148/182) and 60.0% (78/130), respectively. Positive rates for *IGK* clonality assessment using fragment analysis and NGS assay in diagnostic samples were 80.8% (147/182) and 86.9% (113/130), respectively. In monitoring samples, the clonality detection rates obtained using fragment analysis and NGS were 44.7% (34/76) and 70.3% (83/118), respectively, with a statistically significant difference ($P < 0.001$). Positive rates for *IGH* clonality assessment using fragment analysis and NGS assay were 33.9% (19/56) and 63.4% (45/71), respectively. Positive rates for *IGK* clonality assessment using fragment analysis and NGS assay in monitoring samples were 53.3% (24/45) and 81.5% (75/92), respectively.

performed using R software version 3.6.3 (R Core Team 2020, R Foundation for Statistical Computing, Vienna, Austria; *https://www.R-project.org*, last accessed September 30, 2021).

Results

Overall Clonality Detection

The clonality detection rates in diagnostic samples obtained using fragment analysis and NGS were 96.7% (176/182) and 95.4% (124/130), respectively, with no statistically significant difference ($P = 0.772$) (Table 2). Among the monitoring samples, the clonality detection rates obtained using fragment analysis and NGS were 44.7% (34/76) and 70.3% (83/118), respectively, with a statistically significant difference ($P < 0.001$). Among the samples of patients in complete remission, the clonality detection rates obtained using fragment analysis and NGS were 33.3% (6/18) and 60.3% (35/58), respectively, with a statistically significant difference ($P = 0.034$). Between the two periods during

Table 3 Positive Rates of Ig Gene Rearrangement According to Ig Type in Diagnostic Samples

	IGH				*IGK*			
Variable	Fragment analysis, % (*n*/total)†		NGS assay, % (*n*/total)‡		Fragment analysis, % (*n*/total)†		NGS assay, % (*n*/total)‡	
Light chain myeloma	65.9	(27/41)*	25.8	(8/31)**	70.7	(29/41)	90.3	(28/31)
κ	68.2	(15/22)	37.5	(6/16)	50.0	(11/22)**	81.3	(13/16)
λ	63.2	(12/19)	13.3	(2/15)**	94.7	(18/19)	100.0	(15/15)
Conventional myeloma§	85.7	(120/140)	72.2	(70/97)	83.6	(117/140)	85.6	(83/97)
κ	90.0	(63/70)	71.9	(41/57)	68.6	(48/70)	78.9	(45/57)
λ	81.4	(57/70)	72.5	(29/40)	98.6	(69/70)**	95.0	(38/40)
Nonsecretory myeloma	100.0	(1/1)	0.0	(0/2)	100.0	(1/1)	100.0	(2/2)
Total	81.3	(148/182)	60.0	(78/130)	80.8	(147/182)	86.9	(113/130)

*$P < 0.05$, **$P < 0.01$.

†Fragment-based clonality testing was performed with the IdentiClone Gene Clonality Assay kits (Invivoscribe Technologies) that use BIOMED-2 multiplex primer sets in five master mixes targeting the *IGH* (IGH_A: V_HFR1-J_H; IGH_B: V_HFR2-J_H; IGH_C: V_HFR3-J_H; IGH_D: D_H1—6-J_H; and IGH_E: D_H7-J_H) and two targeting the *IGK* locus (IGK_A: Vκ-Jκ; IGK_B: Vκ-Kde; and JκCκ intron-Kde).

‡NGS analysis was performed with the LymphoTrack *IGH* FR1 Assay and LymphoTrack *IGK* Assay (Invivoscribe Technologies).

§Conventional myelomas refer to myeloma with Ig with both heavy and light chain molecules.

NGS, next-generation sequencing.

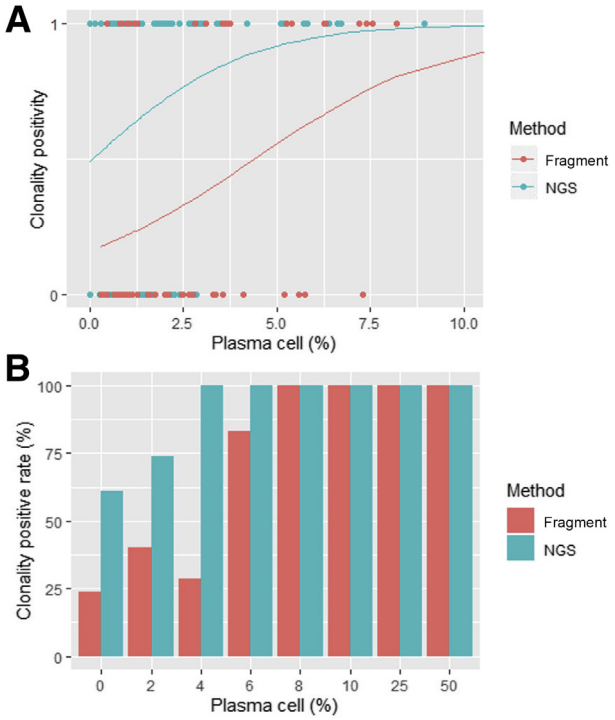

Figure 2 Positive rate of clonality and plasma cell percentage in the monitoring samples. **A:** Points plotted along the top border represent samples where clonality was detected, whereas no clonality could be found in the samples at the bottom. Regression lines show the probability of clonality detection as a function of bone marrow plasma cell percentage by aspirate smear. **B:** Clonality positive rate by next-generation sequencing (NGS) method was higher than fragment method at low plasma cell percentage.

which the different methods were performed (fragment analysis, from January 2013 to March 2017; NGS, from April 2017 to August 2019), there was no difference in the results obtained from other clonality-associated tests with regard to parameters, such as serum light chain ratio and multicolor flow cytometry−positive rates.

Positive rates for *IGH* clonality assessment using fragment analysis and NGS assays in the diagnostic samples were 81.3% (148/182) and 60.0% (78/130), respectively (Figure 1). Positive rates for *IGK* clonality assessment using fragment analysis and NGS assays in the diagnostic samples were 80.8% (147/182) and 86.9% (113/130), respectively. In the monitoring samples, positive rates for *IGH* clonality assessment using fragment analysis and NGS assay were 33.9% (19/56) and 63.4% (45/71), respectively. Positive rates for *IGK* clonality assessment using fragment analysis and NGS assay in the monitoring samples were 53.3% (24/45) and 81.5% (75/92), respectively.

When compared with Ig typing in diagnostic samples, positive rates of *IGH* clonality tests in light chain myeloma were lower than that in the case of conventional myeloma ($P = 0.008$ and $P < 0.001$ by fragment analysis and NGS assays, respectively) (Table 3). In particular, *IGH* clonality assessment, performed using NGS, showed the lowest positive rates of 25.8% in light chain myeloma, 37.5% in κ light chain disease, and 13.3% in λ light chain disease. *IGK* clonality assessment for λ-type myeloma, both light chain myeloma and conventional myeloma, exhibited significantly higher positive rates (ie, 97.8% and 96.4%) than those obtained for κ type myeloma (ie, 64.1% and 79.5%) ($P < 0.001$ and $P = 0.005$), using fragment analysis and NGS assays, respectively.

Positive Rate of Clonality and Plasma Cell Percentage

To examine the correlation between plasma cell percentage and clonality positivity, an analysis was conducted using the plasma cell percentage estimated from the aspiration smear, and the median (range) values were 17.1% (0.2% to 97.9%) and 9.7% (0.0% to 97.8%) for fragment analysis and NGS assays, respectively. In diagnostic samples, the average plasma cell percentages counted on aspirate smear of patients with negative clonality results were 21.5% (7.9% to 53.6%) and 26.7% (8.6% to 79.6%), which were significantly lower than those of the patients with positive clonality [ie, 43.3% (7.7% to 97.9%) and 44.1% (7.3% to 97.8%)], using fragment analysis and NGS assays, respectively ($P = 0.018$ and $P = 0.041$). In the monitoring samples, positive rates were associated with plasma cell percentage estimated using aspirate smears in the logistic regression analysis ($P = 0.001$) (Figure 2A). In bone marrow samples with plasma cell percentages <10%, the positive rates of clonality obtained using fragment analysis and NGS assays were 34.3% (23/67) and 67.6% (73/108), respectively (Figure 2B).

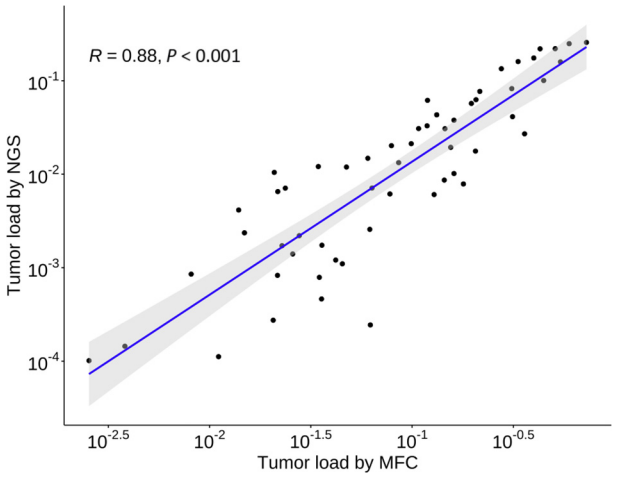

Figure 3 Comparison of tumor load determined by the next-generation sequencing (NGS) and multiparametric flow cytometry (MFC) assays. Correlation analysis between NGS- and MFC-based measurement of tumor load showed excellent correlation between the NGS and MFC assays for tumor burden levels ($R = 0.88$). Tumor load by NGS (%) = (the percentage of total reads from the target sequence shown by NGS) × (the percentage of B lymphocytes defined as CD19 positive and plasma cells defined as CD138 and CD38 positive determined by MFC) × 100. Shown is the correlation line (blue line) with 95% CI (gray shading).

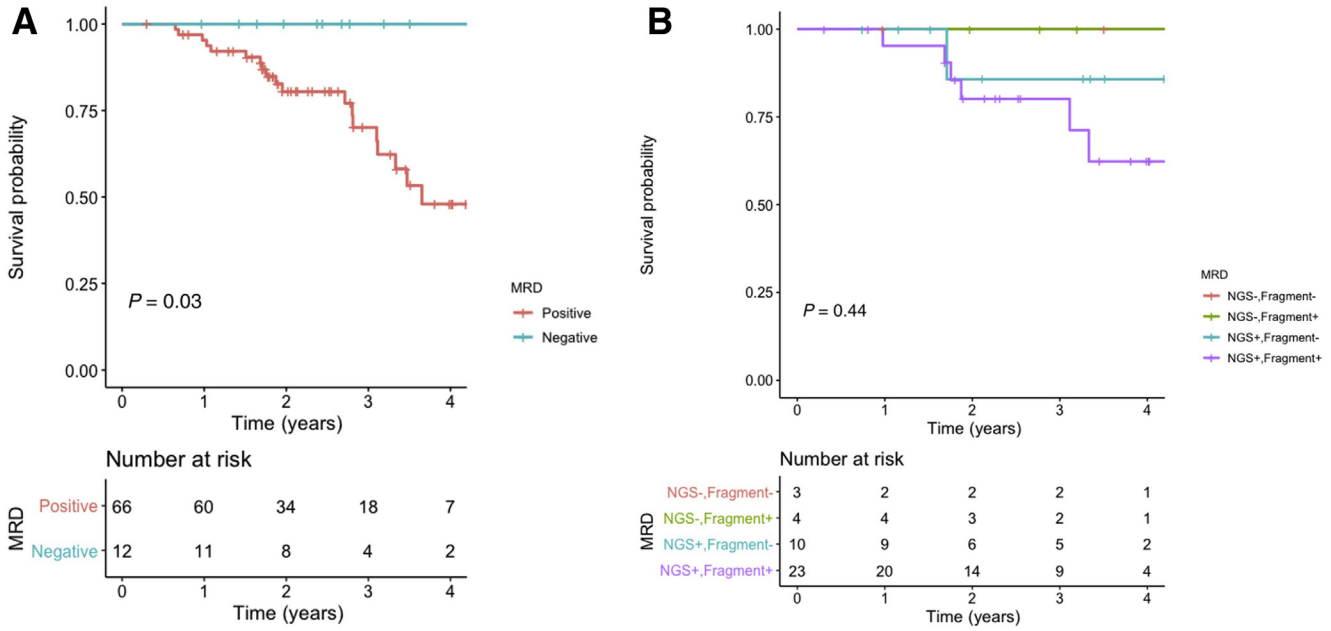

Figure 4 Kaplan-Meier plots for progression-free survival according to results of next-generation sequencing (NGS) assay and fragment analysis. **A:** Results of NGS analysis. **B:** Results of fragment analysis on the available samples out of samples tested with NGS analysis. Shown is the cumulative incidence of progression, according to the presence of positive (+) or negative (−) results for the detection of clonality of *IGH* or *IGK* gene rearrangement on NGS and fragment analysis (Fragment). MRD, minimal residual disease.

Comparison of Tumor Load Determined by the NGS and MFC Assays

To evaluate the quantitative results presented as clonal percentage from NGS assays, comparative studies involving NGS and conventional six-color MFC assays were performed. Samples with clonal plasma cells detected by MFC and for which data regarding the B-cell fraction were available were included. A total 56 diagnostic samples were selected. Tumor load was calculated on the basis of B-cell population size determined by flow cytometry: MRD% = (the percentage of total reads from the target sequence shown by NGS) × (the percentage of B lymphocytes defined as CD19 positive, and plasma cells defined as CD138 and CD38 positive determined by MFC) × 100. There was an excellent correlation between the NGS and MFC assays for tumor burden levels ($R = 0.88$) (Figure 3). Conversely, percentages of clonal plasma cells among the entire B-cell and plasma cell populations were calculated on the basis of B-cell numbers, as determined by flow cytometry and compared with the clonal percentage obtained using NGS. Likewise, there was an excellent correlation between the NGS and MFC assays (Supplemental Figure S1).

Clinical Significance of Clonality Assessment by NGS Assay

The median (range) follow-up period was 29.3 (3.7 to 54.9) months. Survival analysis was restricted to the 78 multiple myeloma patients who underwent gene rearrangement testing using monitoring sample within 1 year from the diagnosis. The first follow-up gene rearrangement test was performed within a median of 4.4 months (range, 3.1 to 11.8 months) after the diagnosis and second follow-up within 8.2 months (range, 4.9 to 11.7 months). Patients who obtained negative results of gene rearrangement test within 1 year were categorized into the negative group, and those who did not have negative results were assigned to the positive group. Figure 4 displays the Kaplan-Meier curve for progression-free survival (PFS) from the time of diagnosis. PFS was significantly longer in MRD-negative than MRD-positive patients ($P = 0.03$). Although the median PFS was 37.8 months in MRD-positive group, median PFS was not reached in MRD-negative group. The estimated 2-year PFS from MRD assessment in MRD-negative patients was 100% versus 80.5% in MRD-positive patients.

Head-to-Head Comparison between Fragment Analysis and NGS Assay

Because this was a retrospective study analyzing positive rates and PFS of two different time periods, fragment analysis was performed on 103 samples with available samples of patients who were already characterized by NGS assay to overcome the limitation of this study. In diagnostic samples, *IGH* and *IGK* clonality assessment using fragment analysis showed a higher positive rate compared with NGS assay (Table 4). In monitoring samples, *IGH* and *IGK* clonality assessment using NGS assay showed higher positive rates

Table 4 Head-to-Head Comparison between Fragment Analysis and NGS Assay

Variable	Type of sample	NGS assay*								
		IGH					*IGK*			
Fragment analysis†			Positive, *n*	Negative, *n*	Total, *n*	Positive rate, %		Positive, *n*	Negative, *n*	Total, *n*	Positive rate, %
	Diagnostic samples	Positive, *n*	20	5	25	72.7	Positive, *n*	25	4	29	83.9
		Negative, *n*	4	4	8		Negative, *n*	1	1	2	
		Total, *n*	24	9	33		Total, *n*	26	5	31	
		Positive rate, %	75.8				Positive rate, %	93.5			
	Monitoring samples	Positive, *n*	8	2	10	77.4	Positive, *n*	24	3	27	89.2
		Negative, *n*	16	5	21		Negative, *n*	9	1	10	
		Total, *n*	24	7	31		Total, *n*	33	4	37	
		Positive rate, %	32.3				Positive rate, %	73.0			

*NGS analysis was performed with the LymphoTrack *IGH* FR1 Assay and LymphoTrack *IGK* Assay (Invivoscribe Technologies).

†Fragment-based clonality testing was performed with the IdentiClone Gene Clonality Assay kits (Invivoscribe Technologies) that use BIOMED-2 multiplex primer sets in five master mixes targeting the *IGH* (IGH_A: $V_HFR1\text{-}J_H$; IGH_B: $V_HFR2\text{-}J_H$; IGH_C: $V_HFR3\text{-}J_H$; IGH_D: $D_H1\text{—}6\text{-}J_H$; and IGH_E: $D_H7\text{-}J_H$) and two targeting the *IGK* locus (IGK_A: Vκ-Jκ; IGK_B: Vκ-Kde; and JκCκ intron-Kde).

NGS, next-generation sequencing.

compared with fragment analysis (*IGH*, 77.4% versus 32.3%; *IGK*, 89.2% versus 73.0%). In particular, 16 of 24 (66.6%) and 9 of 33 (27.3%) NGS-positive samples were negative in fragment analysis for *IGH* and *IGK* clonality assessment, respectively. PFS was better in the order of two NGS (−) groups, NGS (+)-fragment (−) group, NGS (+)-fragment analysis (+) group, but the difference was not statistically significant, probably because of the small number of samples ($P = 0.44$) (Figure 4).

Discussion

During lymphocyte development, Ig genes undergo a complex rearrangement process to generate antibody diversity.[10] Clonal expansions of malignant cells result in identical copies of unique rearrangements of genes. Therefore, analysis of *IGH* and *IGK* rearrangement is widely used as markers for residual disease assessment of MM patients.[11] One of the widely used methods for measuring rearrangements is fragment assay, which identifies clonality based on PCR product size, separated by gel or capillary electrophoresis.[12] Because fragment assays cannot differentiate between clonal populations that have the same PCR product size but different sequences, MRD detection is limited. However, sequence-based method using NGS could track a specific rearrangement sequence at low level, enabling accurate MRD detection.[13] Several studies have been conducted to evaluate NGS-based methods for clonality assessment of MM patients. Clonal detection rates of diagnostic samples were ≥95% when using *IGH* FR1-3, Leader, and *IGK* assays in MM.[3,14] In this present study, we described the clonality detection rates of diagnostic and monitoring samples of MM patients in routine clinical practice using an NGS-based method in comparison to the widely used fragment analysis assay.

IGH gene rearrangements, identified using NGS analysis, were analyzed by FR1 assays without FR2, FR3, and the Leader assays in our study. The *IGH* FR1-positive rate was 60.0% in diagnostic samples, which was much lower than that obtained using the fragment assay performed with FR1-3 primers (81.3%). Because of somatic hypermutations in the B-cell development process for diversification, the PCR primer cannot anneal to the intended target, necessitating the use of multiple primers. In MM, low clonal detection rates for the *IGH* gene were reported (ie, 72%) compared with 96% for B-lymphoblastic leukemia when using the FR1 assay alone.[14] However, in this study, the combination of the *IGH* FR1 and *IGK* assays exhibited a high clonality detection rate of 95.4%, which was not significantly lower than that of the fragment assay (96.7%). The *IGK* assay could raise the clonality detection rate by 35.4%, compared with the *IGH* FR1 assay alone. Therefore, combining the *IGH* FR1 and *IGK* assays could be an alternative approach to reducing time and costs, and increasing the positive detection rate in MM.

In this study, we found that the positive rate of *IGH* gene rearrangements tested by NGS analysis was particularly low in light chain disease (κ type, 37.5%; λ type, 13.3%). This could be explained by the fact that somatic mutations occur more frequently in nonproductively rearranged VH genes than in productively rearranged VH genes.[15] In addition, clonal *IGK* rearrangements were identified in 96.4% of λ-restricted MM, which was significantly higher than that for κ-restricted MM (79.5%) obtained using the NGS assays ($P = 0.005$). The fact that several unique rearrangements,

such as inactivating rearrangements involving the κ-deleted element (Kde), are more common and that somatic hypermutation is minimal in λ-type MM could lead to a high positive rate of clonality.[3,16,17]

Clonal detection rate was reported to be dependent on the use of good quality aspirates and subsequent tumor cell enrichment.[3] In this study, there was a significant difference in the average plasma cell percentage in patients with positive and negative results for clonality assays in the diagnostic samples. Furthermore, plasma cell percentage was significantly correlated with clonal positivity in the monitoring samples. However, even in bone marrow samples with low plasma cell percentages, the positive rate of clonality obtained using NGS analysis was much higher than that for fragment analysis (67.6% versus 34.3% in samples with plasma cell percentage <10% for NGS and fragment analysis, respectively). NGS-based MRD assessment is required in samples with low tumor burden to distinguish genuinely negative results from MRD from low levels of MRD in MM patients.

The percentage of unique reads of total sequence reads provided by NGS is derived only from lymphoid cells because gene rearrangements only occur in such cells. To generate a percentage of the total number of nucleated cells, reagents with PCR primers directed at a housekeeping gene or an external spike-in control are required. As an alternative, the percentage of total cells can be estimated by multiplying the percentage obtained using NGS assays by the percentage of B lymphocytes and plasma cells. In this study, the estimated tumor load using B-cell percentages and clonal plasma cell percentages detected by MFC showed good correlations. Such a calculation method using B-cell percentages obtained using flow cytometry analyses could be used to estimate tumor burden in routine clinical settings.

MRD status determined by NGS assay could identify patient subpopulations with highly different prognoses, and achieving MRD negativity was the strongest prognostic indicator to overcome the known adverse factors (ie, disease stage or cytogenetic risk profile).[2,8] We conducted survival analysis of patients who underwent gene rearrangement testing for MRD assessment in 1 year from diagnosis. Consistent with the studies showing that MRD negativity, as assessed by NGS analysis, was a significant prognostic factor in multiple myeloma, PFS was significantly longer in MRD-negative patients than in MRD-positive patients by NGS analysis. NGS-based MRD assessment could improve the risk stratification of multiple myeloma patients in clinical practice.

One limitation of this study may be its retrospective design, and other inherent shortcomings. Although it was evaluated using other biomarkers that there were no significant differences in treatment responses during the two periods when fragment analysis and NGS were performed, it should be taken into consideration that such analyses were performed over two different time periods, which could have influenced the outcomes. Since flow cytometry was performed using the two-tube six-color method and 100,000 events were analyzed, this method was much less sensitive than the next-generation flow cytometry, which analyzed 10 million cells. Therefore, NGS and next-generation flow cytometry could not be compared. Flow cytometry was only used to analyze the linearity of the estimated tumor load.[4] In addition, the tumor load was estimated using the theoretical background that the read percentage from NGS assay is a percentage among cells undergoing gene rearrangement. However, this tumor load calculation method is rather a rough estimation, because plasma cells are underestimated by flow cytometry. Therefore, it is difficult to accurately detect all cells undergoing gene rearrangement. In addition, as only a small number of patients were included in the survival analysis and the median follow-up time was relatively short, further studies considering these aspects should be undertaken to validate our findings.

Taken together, clonality detection by NGS-based method using *IGH* FR1 and *IGK* assays in routine clinical practice is feasible, provides good clonality detection rates in diagnostic samples, and enables improved MRD detection in MM patients with significant prognostic value.

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.jmoldx.2021.09.006*.

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