Detection of Immunoglobulin Heavy Chain Gene Clonality by Next-Generation Sequencing for Minimal Residual Disease Monitoring in B-Lymphoblastic Leukemia

Saeam Shin, M.D.[1,2,*], In Sik Hwang, M.S.[3,*], Jieun Kim, M.D.[1], Kyung-A Lee, M.D.[1], Seung-Tae Lee, M.D.[1], and Jong Rak Choi, M.D.[1]

Department of Laboratory Medicine[1], Yonsei University College of Medicine, Seoul; Department of Laboratory Medicine[2], Hallym University College of Medicine, Kangnam Sacred Heart Hospital, Seoul; Brain Korea 21 PLUS Project for Medical Science[3], Yonsei University, Seoul, Korea

Minimal residual disease (MRD) following B-lymphoblastic leukemia (B-ALL) treatment has gained prognostic importance. Clonal immunoglobulin heavy chain (*IGH*) gene rearrangement is a useful follow-up marker in B-ALL owing to its high positivity rate. We evaluated the performance and clinical applicability of a next-generation sequencing (NGS) assay for *IGH* rearrangement in B-ALL MRD monitoring. *IGH* rearrangement was tested by using fluorescence PCR-fragment analysis and the NGS assay in eight B-ALL patients. The NGS assay was run on two platforms: the Ion Torrent PGM (Thermo Fisher Scientific, USA) (18 samples from 1st to 7th patients) and the MiSeq system (Illumina, USA) (four samples from 8th patient). All initial diagnostic samples and four follow-up samples were positive for clonal *IGH* rearrangement with fluorescence PCR-fragment analysis and the NGS assay, and six follow-up samples were positive only with NGS. In one case with *BCR-ABL1* translocation, *BCR-ABL1* quantitative PCR was negative but the NGS *IGH* assay was positive just prior to full-blown relapse, suggesting the high sensitivity and clinical utility of the NGS assay. The NGS assay is proposed for MRD monitoring in B-ALL Additional studies are needed to confirm the clinical implications of cases showing positive results only in NGS.

Key Words: Immunoglobulin heavy chain gene rearrangement, Minimal residual disease, B-lymphoblastic leukemia, Next-generation sequencing

The presence of minimal residual disease (MRD) after chemotherapy or bone marrow transplantation has been an important prognostic factor in B-lymphoblastic leukemia (B-ALL) [1, 2]. Conventionally, MRD can be detected by using multi-color flow cytometry or real-time PCR with high sensitivity. However, the detection of MRD can be limited in a subset of patients that lack leukemia-specific cell markers or harbor genetic abnormalities [1, 3].

In the early stage of B-cell differentiation, the immunoglobulin gene cluster regions undergo a complex rearrangement process

to produce diverse antibody-coding sequences [4]. When the immunoglobulin gene cluster regions are amplified by using specific primers, normal polyclonal cells show amplicons with variable sizes reflecting the broad antibody repertoire, whereas tumor cells derived from a single clone show amplicons with one or a few fixed sizes [5]. Therefore, clonal immunoglobulin gene rearrangement can be a useful diagnostic and monitoring marker in a broad range of B-cell lymphoproliferative neoplasms, including B-ALL, plasma cell myeloma, and mature B-cell lymphoma [6-8]. However, conventional fluorescent PCR and fragment analysis have drawbacks, including difficulty in interpretation of peak patterns in some cases and relatively low sensitivity compared with other highly sensitive assays [9].

Rapid progression in next-generation sequencing (NGS) technologies has enabled highly sensitive cancer genomic testing in clinical laboratories [10]. Although some NGS-based clonal immunoglobulin heavy chain gene (*IGH*) rearrangement assays have already been tried for MRD monitoring of B-ALLs [6, 11], a considerable need still remains for a standardized *IGH* clonality testing with acceptable performance and streamlined workflow suitable to clinical laboratory. LymphoTrack *IGH* Assay (InVivoScribe Technologies, San Diego, CA, USA) includes a primer set targeting *IGH* loci for preparing multiplex PCR library preparation and can be used on two NGS platforms: Ion Torrent Personal Genome Machine (PGM; Thermo Fisher Scientific, Waltham, MA, USA) and the MiSeq system (Illumina, San Diego, CA, USA). We further validated the clinical utility of NGS for *IGH* rearrangement in a clinical setting, using amplicon-based library preparation kit and two NGS platforms: Ion Torrent PGM (for 1st-7th patients) and the MiSeq system (for 8th patient).

Written informed consent was obtained from the patients' parents, according to the ethical guidance of the institutional review board of the Severance Hospital, Yonsei University College of Medicine, Seoul, Korea. Genomic DNA was extracted from bone marrow samples by using the QIAamp DNA Blood Mini Kit (Qiagen, Venlo, The Netherlands).

PCR amplification for fragment analysis was performed by using the IdentiClone *IGH* Gene Clonality Assay kit (InVivoScribe Technologies). Multiplex primer sets in five master mixes target the variable, joining, and diversity region of the *IGH* locus. Fragment detection and analysis was performed by using 3130 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) and Gene-Mapper 3.2 software (Applied Biosystems). Interpretation of clonal peaks in diagnostic samples was based on two criteria. The suspected peak should fall within the valid size range supplied by the manufacturer and 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.

For NGS library preparation, DNA concentration was checked by using a Qubit 2.0 fluorometer (Thermo Fisher Scientific) and the Qubit dsDNA HS assay kit (Thermo Fisher Scientific), and adjusted to 10 ng/μL. The library was generated from 50 ng of DNA per sample using the LymphoTrack *IGH* assay kit. The kit uses single multiplex master mixes targeting the conserved site in the variable region and the joining region of *IGH*. For each run, positive and negative controls included in the kit were assayed simultaneously. After PCR amplification, the libraries were purified by using the Agencourt AMPure XP system (Beckman Coulter, Brea, CA, USA). Eighteen samples from patient 1–7 were tested by using the Ion Torrent PGM. Emulsion PCR was conducted by using the Ion OneTouch 400 template kit, and the enriched libraries were sequenced on the 318 chip with the Ion PGM Sequencing 400 kit (all from Thermo Fisher Scientific). Four samples from patient 8 were sequenced on the MiSeq System using the MiSeq Reagent Nano Kit v2 (Illumina) and the MiSeq Reagent Kit v2 (Illumina). Raw sequence data in FASTQ format were analyzed by using the Lymphotrack software package (InVivoScribe Technologies). The frequency of identical sequences in NGS was calculated by dividing the number of sequence reads by the total number of sequence reads in the sample. For interpretation, we adopted previously reported criteria [12] and determined a 5% frequency as the cut-off for a leukemic clone in initial diagnostic samples. The presence of MRD in follow-up samples was determined by the presence of identical sequences of leukemic clones in the initial samples, even if the frequency of clone was less than 5%.

Patient demographics and the results of *IGH* clonality tests are summarized in Table 1. Total read counts ranged from 7,422 to 518,728. Interpretable results were obtained with higher sensitivity compared with fragment analysis, even in samples with relatively fewer reads (patients 3 and 7). Seven patients (patients 1–7) had no molecular markers other than the clonal *IGH* rearrangement, whereas patient 8 was positive for t(9;22)(q34;q11.2); *BCR-ABL1*. All initial diagnostic samples were positive for *IGH* clonality by both fragment analysis and NGS.

Two patients (patients 7 and 8) had a single monoclonal clone according to the sequences identified by NGS, while five patients (patients 2–6) had two different clones and one patient (patient 1) had three clones. The proportion of leukemic clones in the *IGH* repertoire varied among samples (median 51.4%; range

Table 1. Patient demographics and results of bone marrow analysis and clonality tests

ID	Sex	Age (yr)	Sample ID	Time‡	Blasts in BM (%)	Leukemic cells in flow cytometry (%)	Cytogenetics	Abnormal cells by FISH§ (%)	Molecular analysis	Fragment analysis	Clone 1 (%, type)	Clone 2 (%, type)	Clone 3 (%, type)	% of total clones	Total N of reads
1	M	4	1-1*	Diagnosis	92.5	56.9	Not interpretable	53.6	Negative‖	Positive	44,001 (27.3, VH1-J4)	9,877 (6.0, VH1-J4)	9,765 (6.0, VH3-J4)	39.3	160,971
			1-2*	10 days	4.0	Negative	ND	4.0	ND	Positive	27,547 (11.6)	9,019 (3.8)	4,846 (2.1)	17.5	236,250
			1-3*	30 days	1.5	Negative	46,XY [20]	Negative	ND	Negative	0	0	0	0	78,806
2	M	3	2-1*	Diagnosis	87.0	88.2	Not interpretable	70.1	Negative‖	Positive	119,153 (23.0, VH3-J4)	114,278 (22.0, VH2-J4)	-	45.0	518,728
			2-2*	2 weeks	NA	Negative	ND	ND	ND	Negative	3,212 (0.7)	12,524 (2.6)	-	3.2	489,937
			2-3*	30 days	0.7	Negative	46,XY [20]	Negative	ND	Negative	0	0	-	0	298,570
3	F	2	3-1*	Diagnosis	91.4	77.0	Not interpretable	62.8	Negative‖	Positive	8,350 (23.7, VH6-J5)	3,741 (10.6, VH6-J5)	-	34.3	35,182
			3-2*	2 weeks	6.8	1.0	ND	11.3	ND	Positive	7,585 (10.5)	3,569 (4.9)	-	15.4	72,494
			3-3*	30 days	1.0	Negative	46,XX [20]	Negative	ND	Negative	515 (0.5)	247 (0.2)	-	0.7	100,618
4	F	4	4-1*	Diagnosis	87.8	75.3	Not interpretable	75.3	Negative‖	‖Positive	232,951 (54.8, VH1-J5)	41,075 (9.7, VH1-J5)	-	64.5	425,184
			4-2*	10 days	NA	Negative	ND	Negative	ND	Positive	51,167 (11.8)	8,317 (1.9)	-	13.7	434,643
			4-3*	30 days	0.9	Negative	46,XX [20]	Negative	ND	Negative	0	0	-	0	214,809
5	M	8	5-1*	Diagnosis	96.8	53.5	Not interpretable	65.9	Negative‖	Positive	207,993 (53.8, VH3-J6)	39,502 (10.2, VH3-J6)	-	64.0	386,903
			5-2*	2 weeks	0	Negative	46,XY[20]	Negative	ND	Negative	468 (0.1)	0	-	0.1	339,695
6	M	3	6-1*	Diagnosis	87.0	95.6	Not interpretable	76.8	Negative‖	Positive	172,072 (54.3, VH3-J4)	23,264 (7.3, VH3-J4)	-	61.6	316,654
			6-2*	2 weeks	0	Negative	ND	Negative	ND	Negative	9,290 (2.0)	1,208 (0.3)	-	2.3	462,911
7	M	7	7-1*	Diagnosis	92.0	90.8	45,XY,der(7;9)(q10;q10)[20]	96.3	Negative‖	Positive	2,429 (25.5, VH4-J6)	-	-	25.5	9,513
			7-2*	30 days	1.0	Negative	ND	Negative	ND	Negative	0	-	-	0	7,422
8	M	15	8-1†	Diagnosis	93.0	88.2	52,XY,+8,t(9;22)(q34;q11.2),+der(22)t(9;22),+mar1,+mar2,+mar3,+mar4[11]/46,XY[17]	80.8	3.25¶	Positive	8,555 (57.7, VH3-J4)	-	-	57.7	14,825
			8-2†	30 days	0	Negative	ND	ND	3.94×10^{-4}¶	Negative	3,501 (14.3)	-	-	14.3	24,427
			8-3†	10 months	0.2	Negative	ND	Negative	Negative¶	Negative	400 (2.8)	-	-	2.8	14,099
			8-4†	Relapse**	87.0	71.8	52,XY,+8,t(9;22)(q34;q11.2),+der(22)t(9;22),+mar1,+mar2,+mar3,+mar4[25]/46,XY[1]	83.1	4.36¶	Positive	35,456 (65.8)	-	-	65.8	53,881

*Eighteen samples from Patients 1–7 were tested by using the Ion Torrent PGM (Thermo Fisher Scientific); †Four samples from Patient 8 were tested by using MiSeq (Illumina); ‡Days, weeks, or months after induction chemotherapy; §Five probe sets were tested by using the *IGH/MYC/CEP8* tri-color dual fusion translocation probe, *BCR/ABL* dual color dual fusion probe, *CDKN2A* spectrum orange/*CEP9* spectrum green probe, *TEL/AML1* dual color extra signal probe, and *MLL* break-apart arrangement probe (Vysis, Downers Grove, IL, USA) at initial diagnosis. Follow-up tests were done by using the probe with abnormal results at initial diagnosis; ‖Molecular analysis was done by using HemaVision kit (DNA technology, Aarhus, Denmark); ¶*BCR-ABL1* transcript frequency (relative ratio of *BCR-ABL1* to *G6PDH*) was quantified by using the LightCycler t(9;22) Quantification (Roche Molecular Systems, Branchburg, NJ, USA) kit; **Patient 8 relapsed at one year after diagnosis.
Abbreviations: NGS, next-generation sequencing; BM, bone marrow; ND, not done; NA, not available due to dry-tap.

25.5–64.5%). Among 14 follow-up samples, excluding one in relapse, three were positive in both fragment analysis and NGS (median 15.4%; range 13.7–17.5%). In contrast, six follow-up samples were negative by fragment analysis but positive by NGS (median 2.6%; range 0.1–14.3%). The proportions decreased as chemotherapy progressed, and converted to negative in four patients at 30 days after initial diagnosis.

Patient 8 was under long-term monitoring with real-time PCR for the *BCR-ABL1* fusion transcript. He received an allogenic bone marrow transplantation after eight months of chemotherapy. However, the disease relapsed one year after initial diagnosis and the patient died six months thereafter from pneumonia and systemic shock. At 10 months after initial diagnosis, which was just before full-blown relapse, *BCR/ABL1* was negative but *IGH* NGS showed the presence of initial clones (Fig. 1).

Clonal *IGH* rearrangement in B-ALL has an advantage as an MRD marker owing to its broad applicability and high positivity rate (84–95%) in all B-ALL cases [12, 13]. Because the conventional method , fluorescence PCR-fragment analysis using a capillary electrophoresis sequencer, has a limitation of low sensitivity (1–10%), other sensitive methods such as real-time PCR with patient-specific primers have been tested for *IGH* clonality detection in MRD monitoring [9]. However, the labor- and time-consuming procedures of designing and validating patient-specific primers have been major hurdles of its widespread application in clinical laboratories [12]. Moreover, there is a possibility of false-negative results because primers specifically designed for initial diagnostic clones can miss newly emerging clones with different genetic properties [12].

The sensitivity of NGS for detecting *IGH* clonality has been reported at 0.001–0.00001% in recent studies using different platforms [6, 12, 14]. In addition to fragment analysis, we detected six more positives by NGS in follow-up samples with a low proportion of clonal leukemic cells. Four patients (patients 2, 3, 5, and 6) with discrepant results were followed up with a complete remission state until 30–59 months after diagnosis. However, in one relapsed patient with the *BCR-ABL1* fusion, the MRD level detected by NGS was comparable to or even more sensitive than *BCR-ABL1* detection with real-time PCR. An additional experiment with six follow-up samples from six other B-ALL patients (data not shown) revealed a similar pattern: five samples showed concordant results (two positives and three negatives), and one sample showed a positive result in NGS and a negative result in fragment analysis. The clinical implication of these findings should be further validated in a study with a larger patient cohort.

Another advantage of NGS is that it can identify the number
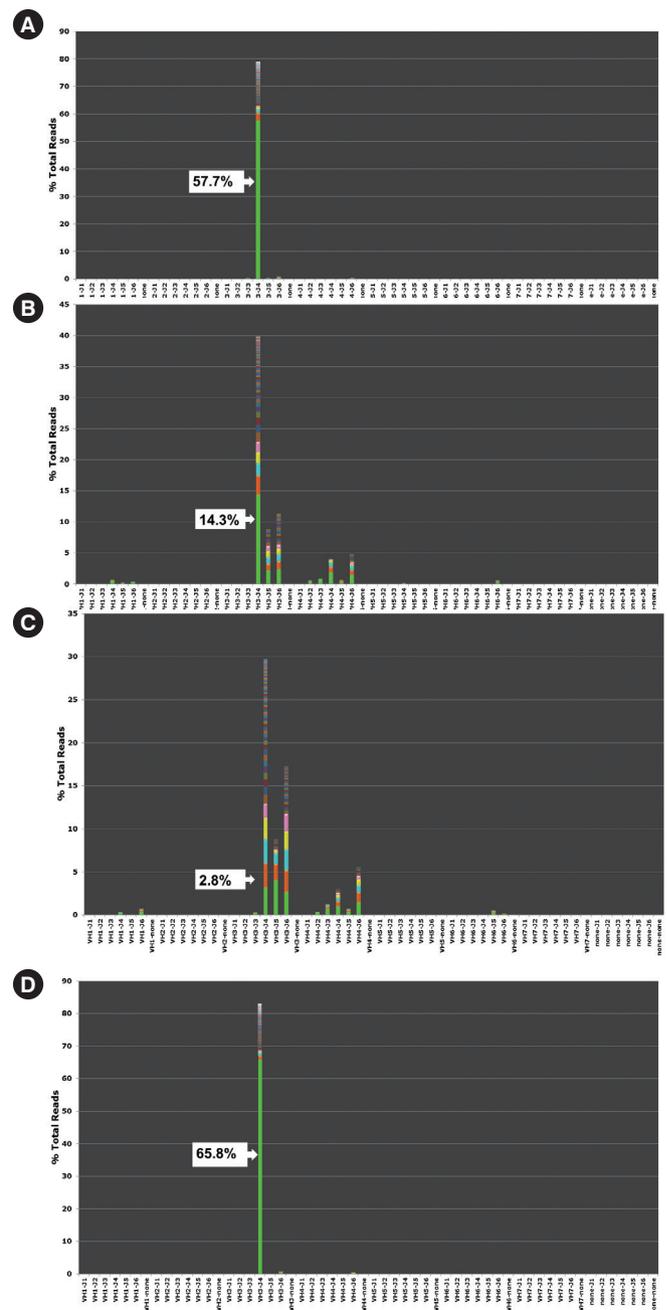
Fig. 1. Next-generation sequencing (NGS) results using MiSeq (Illumina) for *IGH* clonality detection at diagnosis (A), follow-up (B, C), and full-blown relapse (D) in Patient 8. Each colored box represents the frequency of reads with an identical sequence. NGS showed a leukemic clone frequency of 57.7% (A) at initial diagnosis and of 14.3% (B) at 30 days after diagnosis (white arrows). (C) A residual leukemic clone frequency of 2.8% was found by NGS at two months before full-blown relapse, although PCR fragment analysis for *IGH* clonality detection and real-time PCR for *BCR-ABL1* transcript quantification showed negative results.

and DNA sequence of tumor clones within a sample. In this study, the numbers of clones at initial diagnosis ranged from one to

three among patients. Previous studies reported the possibility of multiple leukemic clones in each B-ALL sample because of ongoing clonal evolution [13]. Because of the small number of cases in this study, the clinical significance of the number and type of leukemic clones could not be analyzed. This should be explored in future studies involving a large and diverse patient series.

In summary, we evaluated an NGS-based *IGH* clonality assay using initial and follow-up samples of B-ALL patients. Using predesigned primer set in library preparation kit and the provided software optimized for bioinformatics analysis, the experimental and bioinformatics analysis processes were streamlined. Furthermore, compared with conventional fluorescence PCR-fragment analysis, the NGS method was more sensitive in detecting positive follow-up samples. The prognostic impact of these lowlevel MRD detected by NGS should be validated with more data and further investigation. Nevertheless, we suggest that this NGS assay can be applicable for MRD monitoring in B-ALL in clinical laboratories.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

Acknowledgments

REFERENCES

1. Borowitz MJ, Devidas M, Hunger SP, Bowman WP, Carroll AJ, Carroll WL, et al. Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia and its relationship to other prognostic factors: a Children's Oncology Group study. Blood 2008;111:5477-85.

2. Logan AC, Vashi N, Faham M, Carlton V, Kong K, Buño I, et al. Immunoglobulin and T cell receptor gene high-throughput sequencing quantifies minimal residual disease in acute lymphoblastic leukemia and predicts post-transplantation relapse and survival. Biol Blood Marrow Transplant 2014;20:1307-13.

3. Bassan R, Spinelli O, Oldani E, Intermesoli T, Tosi M, Peruta B, et al. Improved risk classification for risk-specific therapy based on the molecular study of minimal residual disease (MRD) in adult acute lymphoblastic leukemia (ALL). Blood 2009;113:4153-62.

4. Schwartz RS. Shattuck lecture: Diversity of the immune repertoire and immunoregulation. N Engl J Med 2003;348:1017-26.

5. Grove CS and Vassiliou GS. Acute myeloid leukaemia: a paradigm for the clonal evolution of cancer? Dis Model Mech 2014;7:941-51.

6. Wu D, Emerson RO, Sherwood A, Loh ML, Angiolillo A, Howie B, et al. Detection of minimal residual disease in B lymphoblastic leukemia by high-throughput sequencing of IGH. Clin Cancer Res 2014;20:4540-8.

7. Vij R, Mazumder A, Klinger M, O'Dea D, Paasch J, Martin T, et al. Deep sequencing reveals myeloma cells in peripheral blood in majority of multiple myeloma patients. Clin Lymphoma Myeloma Leuk 2014;14:131-9. e1.

8. He J, Wu J, Jiao Y, Wagner-Johnston N, Ambinder RF, Diaz LA Jr, et al. IgH gene rearrangements as plasma biomarkers in Non-Hodgkin's lymphoma patients. Oncotarget 2011;2:178-85.

9. Langerak AW, Groenen PJ, Jm van Krieken JH, van Dongen JJ. Immunoglobulin/T-cell receptor clonality diagnostics. Expert Opin Med Diagn 2007;1:451-61.

10. Singh RR, Patel KP, Routbort MJ, Reddy NG, Barkoh BA, Handal B, et al. Clinical validation of a next-generation sequencing screen for mutational hotspots in 46 cancer-related genes. J Mol Diagn 2013;15:607-22.

11. Pulsipher MA, Carlson C, Langholz B, Wall DA, Schultz KR, Bunin N, et al. IgH-V(D)J NGS-MRD measurement pre- and early post-allotransplant defines very low- and very high-risk ALL patients. Blood 2015;125:3501-8.

12. Faham M, Zheng J, Moorhead M, Carlton VE, Stow P, Coustan-Smith E, et al. Deep-sequencing approach for minimal residual disease detection in acute lymphoblastic leukemia. Blood 2012;120:5173-80.

13. Gawad C, Pepin F, Carlton VE, Klinger M, Logan AC, Miklos DB, et al. Massive evolution of the immunoglobulin heavy chain locus in children with B precursor acute lymphoblastic leukemia. Blood 2012;120:4407-17.

14. Langerak AW, Groenen PJ, Jm van Krieken JH, van Dongen JJ. Immunoglobulin/T-cell receptor clonality diagnostics. Expert Opin Med Diagn 2007;1:451-61.

15. Ladetto M, Brüggemann M, Monitillo L, Ferrero S, Pepin F, Drandi D, et al. Next-generation sequencing and real-time quantitative PCR for minimal residual disease detection in B-cell disorders. Leukemia 2014;28:1299-307.