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Considerations for monitoring minimal residual disease using immunoglobulin clonality in patients with precursor B-cell lymphoblastic leukemia

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ABSTRACT

Background: Minimal residual disease (MRD) monitoring is a powerful tool to predict the risk of relapse. Herein, we present an MRD monitoring strategy for B-cell lymphoblastic leukemia (B-ALL) using high-throughput sequencing (HTS) of immunoglobulin (Ig) clonality before implementation into routine practice.

Methods: We selected 74 bone marrow (BM) specimens from 47 patients who were diagnosed with B-ALL. Ig clonality was analyzed using both fragment analysis and HTS. The performance of Ig clonality was evaluated through comparison of the results from real-time quantitative polymerase chain reaction (qPCR) of leukemia-specific fusion transcripts and flow cytometry.

Results: *IGH* clonality was observed in all patients, and the sum of clonal burden varied (9.47%–96.77%). *IGK* clonality was identified in 70% of patients and availed in cases with low *IGH* clonal burden. The total *IGH* clonal burden was significantly correlated with the proportion of leukemic blasts, leukemia-specific fusion transcripts, and flow cytometry. We recognized the different responses of each clone and emerging clones originating from the trace of Ig rearrangement presented in the initial specimen. *IGH* clonal burden after chemotherapy represented patient outcomes well. *IGH* assay also provided information of repertoire diversity of *IGH* rearrangement.

Conclusion: The Ig clonality assay via HTS will be a promising tool for MRD monitoring of B-ALL through an adequate strategy to identify and monitor individual clones and determine repertoire diversity.

1. Introduction

Minimal residual disease (MRD) is one of the most powerful tools to predict the risk of relapse [1,2]. MRD monitoring based on immunoglobulin (Ig) gene rearrangement is well established and has become routine practice in patients with B-cell lymphoblastic leukemia (B-ALL) [3]. Recent studies focusing on MRD monitoring via high-throughput sequencing (HTS) have shown its higher sensitivity and precision in bone marrow (BM) and peripheral blood samples compared with multi-parameter flow cytometry or quantitative allele-specific oligonucleotide-polymerase chain reaction (PCR) [4,5]. This suggests that HTS might replace the quantitative PCR because of its higher analytical performance and the additional impact of predicting relapses [6,7]. Moreover, HTS is now affordable for clinical laboratories, and HTS-based quantification of Ig rearrangements is the best application for many routine MRD monitoring practices.

The analytical performance of Ig clonality via HTS has been continuously assessed along with the introduction of new primers, and it remains in development for MRD monitoring under various conditions, including at the time of initial diagnosis and after treatment. In this

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Table 1
Overview of patient characteristics and outcome data.

Evaluable		Total	*ETV6-RUNX1*	*BCR-ABL1*	Polysomy 14	Hematogone
		N = 47	N = 14	N = 18	N = 13	N = 2
Age	Median	8	5.5	51.5	5	5.5
	< 19 yrs	33	14	4	13	2
	≥19 yrs	14	0	14	0	0
Gender	Male	26	9	8	8	1
	Female	21	5	10	5	1
Subtype	pro B	1	1	0	0	0
	c/pre B	46	13	18	13	2
BM blast	Median (%)	93	91	95	94	62.5
	< 70%	3	0	2	0	1
	≥70%	44	14	16	13	1
Response to induction	CR	45	14	16	13	2
	CMR	14	11	3	0	0
	MMR	8	3	5	0	0
Long-term outcome	Continuous CR (≥5 yrs)	28	13	2	11	2
	HSCT	11	1	8	2	0
	Relapse	8	0	8	0	0

BM: bone marrow; CR: complete remission; CMR: complete molecular response; MMR: major molecular response; HSCT: hematopoietic stem cell transplantation.

study, we propose a feasible MRD monitoring strategy for B-ALL by evaluating real specimens considering the clinical conditions that affect HTS-based Ig gene rearrangement.

2. Materials and Methods

2.1. Patients and study design

We selected 74 BM specimens from 47 patients who were diagnosed with B-ALL and followed after treatment at the Catholic Blood and Marrow Transplantation Center, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea. Diagnosis was made according to the World Health Organization (WHO) criteria [8], and 14 B-ALL with t(12;21) (p13.2;q22.1), *ETV6-RUNX1*; 18 B-ALL with t(9;22) (q34.1;q11.2), *BCR-ABL1*; and 15 B-ALL not otherwise specified (NOS) were included. The B-ALL NOS consisted of specimens from 13 patients with more than two copies of the *IGH* gene including polysomy 14 and those from two patients with a large fraction of hematogone in complete remission (CR) status. The characteristics of the patients are summarized in Table 1. This study was approved by the institutional review board of St. Mary's Hospital, which is affiliated with The Catholic University of Korea (IRB No: KC15SISI0308).

2.2. Cytogenetics

Chromosome analyses were performed on BM specimens according to conventional cytogenetic protocols for G-banding technique. At least 20 metaphases were analyzed in each case, and the results were described according to the International System for Chromosome Nomenclature (ISCN) 2013 [9].

Fluorescence in situ hybridization (FISH) was used to confirm the presence of t(12;21) and t(9;22) using the LSI *ETV6/RUNX1* and *BCR/ABL1* dual-color, dual-fusion translocation probe (Abbott Vysis, Des Plaines, IL), respectively. Each signal was observed using an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan), and fusion was counted among 400 interphase cells.

2.3. Quantification of leukemia-specific fusion transcripts

The RNA was extracted from patients' BM samples using a High Pure RNA isolation kit (Roche Diagnostics, Mannheim, Germany), and the complementary DNA was synthesized using a Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics). The fusion transcript of *ETV6-RUNX1* and *BCR-ABL1* was measured via reverse transcription quantitative-PCR (RT-qPCR) using the Real-Q *ETV6-RUNX1* quantification kit (Biosewoom Inc., Seoul, Korea) and Real-Q *BCR-ABL1*quantification kit (Biosewoom Inc.), respectively. The samples were assayed on an ABI-Prism 7500 Real-Time PCR System (Applied Biosystems, Foster city, CA). The standards of both the target and reference (*ABL1*) are used to generate standard curves to determine the levels. The relative expression level of the fusion transcripts was calculated using the mean of normalized standard *ABL1* as a stable reference gene and was presented relative to control. Complete molecular response (CMR) was defined as undetectable levels of *BCR-ABL1* transcript with a sensitivity of 0.01%. The major molecular response (MMR) was defined as a *BCR-ABL1*:*ABL1* ratio of 0.1% or less on the International Scale for p210 *BCR-ABL1* or a 3-log reduction in transcripts for p190 *BCR-ABL1*, but not meeting criteria for CMR [10]. Relapse was defined by a recurrence of 5% or more blasts in a BM aspirate or by the presence of extramedullary disease.

2.4. MRD monitoring through flow cytometry

Immunophenotyping was performed at diagnosis with flow cytometry against CD3, CD41a, CD14, CD34, CD33, CD20, CD5, CD10, CD19, CD64, CD11c, CD13, CD117, CD56, CD2, CD7, HLA-DR, cytoplasmic CD22, cytoplasmic myeloperoxidase, cytoplasmic CD3, and cytoplasmic CD79a (BD Biosciences, San Jose, CA, USA). Leukemic blasts were gated and analyzed using the FACSDiva program (BD Biosciences). MRD was also assessed by flow cytometry in patients who did not possess leukemia-specific fusion transcripts ($n = 5$). At least 5×10^5 events were collected and analyzed to estimate MRD.

2.5. Analyses of Ig rearrangement

To draw up practical guidelines for the test process, we screened clonal *IGH* rearrangements using both fragment analysis and HTS at diagnosis. The methods for MRD monitoring were chosen on the basis of the initial results for RT-qPCR or HTS.

2.5.1. IGH clonality using fragment analysis and qPCR

Genomic DNA was extracted from BM specimens using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Clonal *IGH* rearrangement was assessed using IdentiClone™ IGH Gene Clonality Assay (InVivoScribe Technologies, San Diego, CA, USA) according to the manufacturer's instructions. The primers of the IdentiClone™ target not only the conserved framework of variable and joining regions (Tube A, B, C), but also the diversity and joining regions (Tube D, E).

Amplicon fragment was detected and analyzed via the ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and GeneMapper 3.2 software (Applied Biosystems), respectively. Clonal *IGH* gene rearrangement was suspected when one or two prominent peaks were within the valid size range.

The amplified clonal *IGH* fragment was sequenced using the corresponding primer. Next, we designed primers and probes of qPCR for each of the sequences. The standard curve was generated using a dilution series of five different concentrations of the standard (10^{-1} to 10^{-5}). The value for the slope of each standard curve fell between -3.1 and -3.9 with a correlation coefficient of ≥ 0.98. Clonal *IGH* rearrangement was quantified using this standard curve. All quantification experiments were triplicated.

2.5.2. HTS

We used the LymphoTrack® IGH FR1/2/3 assay panel (InVivoScribe Technologies) to assess the clonal *IGH* rearrangement via HTS. All experiments were performed according to the manufacturer's guidelines. When clonal *IGH* rearrangement was negative in the initial specimen after using the primers targeting the conserved framework region (FR) 1 within the variable and joining region, a test using FR2, FR3, and the IGK assay panel (InVivoScribe Technologies) was performed. Briefly, we amplified 50 ng of high-quality DNA with a single multiplex master mix containing primers after checking genomic DNA using the Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA). Next, we purified the amplicons using an Agencourt®AMPure XP system (Beckman Coulter, Brea, CA) and measured the amplicons with an Agilent 2100 BioAnalyzer (Agilent Technologies, Inc., Santa Clara, CA). The library DNA was denatured and loaded onto a Miseq reagent kit v2 (InVivoScribe Technologies).

2.6. Bioinformatic platforms for analysis of clonal Ig rearrangement and MRD

2.6.1. LymphoTrack®Dx Assay

The FASTQ files were analyzed using the LymphoTrack Software–MiSeq v2.3.1. The final reports provide seven results, including the Read Summary, VJ Sequence Frequency Graph, VJ Usage, VJ Usage Percent Graph, VJ Usage Raw Graph, VJ Sequence Frequency, and Unique Reads. Due to the amplification and sequencing error rates found within HTS technology, we merged reads and their frequencies before clonality determination. We added the frequencies of the sequences with the same V-J rearrangement but differed in less than or equal to two base pairs. The sequences also need to be within the list of the five most frequent sequences for merging sequences. When the total number of reads for each sample is $< 10{,}000$, the sample is regarded as not evaluable. If the total number of reads is between 10,000 and 20,000, the clonality is detected if the top merged sequence has 5% or more of the total reads and more than twice % reads for the third most frequent merged sequence. The clonality criteria of 5% decreases to $< 2.5\%$ when the total number of reads for each sample is $> 20{,}000$.

For MRD monitoring, the clone of the same sequence with the diagnostic sample was sought after chemotherapy. If any identical sequences to the initial clone are found, the amount of remnant clone is described as the % of total reads.

2.6.2. Vidjil platform

We also used an open-source bioinformatic algorithm, Vidjil, to analyze the clonal Ig rearrangement. The FASTQ files obtained using the FileExporter software were processed with Vidjil using its default parameters (www.vidjil.org, version 2015.01). Vidjil gathers the reads into clones on the basis of their V(D)J recombinations. Two reads belong to the same clone if they share a same 50 bp "window" overlapping the actual complementarity-determining region. Window detection is based on a fast bioinformatics method using k-mers indexing of the germline gene [11]. The Vidjil application shows all rearranged Ig segments as plots on the V and J segment map. The size of the clones is reported as the ratio, which is calculated from the number of clonal reads divided by that of the total. We called the reads surrounding the dominant clone as minute satellite sequences and then established the cut-off for clonality to be the same as that of a LymphoTrack®Dx Assay of 5% or greater [12,13]. The software is used to quantify the MRD during patient follow-up by displaying each Ig rearrangement as a line graph that tracks the clones detected in the initial diagnostic specimen.

The application also provides information of the heterogeneous immune repertoires as the ratio of clones per reads. A ratio close to 1.0 indicates each read corresponding to a distinct clone, whereas a ratio of 0.0 indicates only one single clone presented in all reads, in other words, no heterozygosity.

2.7. Statistical analysis

The relation between Ig clonal burden and other parameters was analyzed by the Pearson correlation coefficient. Prognostic significances of MRD affecting overall survival (OS) and relapse-free survival (RFS) was determined the Kaplan-Meier method, and comparisons were made with the log-rank test. P values of < 0.05 were considered statistically significant. Statistical work was done on SPSS ver. 12.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Identification of Ig clonality in B-ALL at the time of diagnosis

First, we assessed the possibility of a fragment analysis as a first-line clonality assay for *IGH* rearrangement. Among 47 initial specimens, 6 showed negative *IGH* clonality through a fragment analysis while showing a positive *IGH* clonality via HTS. Therefore, fragment analysis is not an optimal first-line study to detect Ig clonality.

HTS could detect Ig clonality in all included B-ALL at diagnosis. The average number of clonal *IGH* rearrangements was 1.7 per patient. The *IGH* FR1 assay was positive in 42 patients (89.4%). The *IGH* FR2 and FR3 assay was positive in all five FR1-negative patients. In one patient, *IGH* FR2 and FR3 showed two clones different from each other. We also identified an additional clone that was not detected by the *IGH* FR1 assay in two patients. Twenty-two patients (46.8%) possessed one *IGH* clone at diagnosis, and two, three, and four *IGH* clones were found in 18, 6, and 1 patient, respectively.

The type of clonal *IGH* rearrangements could be identified with HTS. *IGH* V3-J4 (23.4%) was the most commonly identified rearrangement, which was followed by V1-J6 (12.8%), V3-J5 (10.6%), V3-J3 (8.5%), V3-J6 (8.5%), and V4-J4 (8.5%). There was no difference in the *IGH* rearrangement profile (number and type) in patients with more than two *IGH* genes (polysomy14 including trisomy 14 or

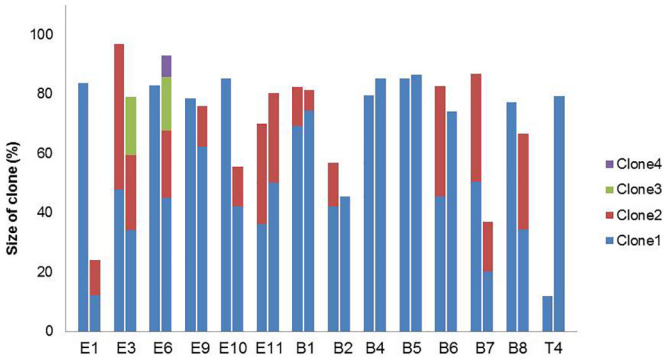

Fig. 1. Comparison of number and burden of clonal *IGH* and *IGK* rearrangements. Left bar indicates *IGH* clones and the right bar indicates *IGK* clones for each patient.

Table 2
IGH rearrangements found for diagnosis specimens using massive parallel sequencing.

No.	sex	age	Type	Blasts (%) in BM	No clone	*IGH* Assay	Clone1 (%)	Clone2 (%)	Clone3 (%)	Clone4 (%)	Total (%)
E1	M	3	*ETV-RUNX1*	*95*	1	FR1	V3-23*01-J4*02 (83.62)				83.62
E2	M	12	*ETV-RUNX1*	*90*	1	FR1	V1-3*01-J6*02 (80.23)				80.23
E3	F	2	*ETV-RUNX1*	*98*	2	FR1 + FR3*	V3-21*01-J4*02 (47.88)	V4-4-39*02-J4*02 (48.89)			96.77
E4	M	6	*ETV-RUNX1*	*89*	2	FR1	V3-21*01-J3*02 (48.37)	V3-66*01-J4*02 (30.47)			78.84
E5	M	6	*ETV-RUNX1*	*90*	1	FR1	V3-74*01-J6*02 (58.84)				58.84
E6	F	8	*ETV-RUNX1*	*85*	1	FR1	V5-51*01-J4*02 (82.76)				82.76
E7	M	3	*ETV-RUNX1*	*92*	3	FR1	V3-23*01-J6*02 (45.25)	V3-43*01-J6*02 (13.27)	V4-39*07-J6*02 (10.95)		69.47
E8	F	4	*ETV-RUNX1*	*81*	2	FR1	V3-30*04-J4*02 (42.42)	V2-5*01-J5*02 (32.76)			75.18
E9	F	8	*ETV-RUNX1*	*89*	1	FR1	V4-61*02-J4*02 (78.58)				78.58
E10	M	5	*ETV-RUNX1*	*97*	1	FR2	V1-2*05-J5*02 (85.25)				85.25
E11	M	5	*ETV-RUNX1*	*85*	2	FR1	V1-69*01-J6*02 (36.09)	V1-NL*01-J4*02 (33.94)			70.03
E12	F	6	*ETV-RUNX1*	*95*	3	FR1 + FR3*	V4-61*01-J5*02 (39.91)	V4-4*08-J5*02 (7.04)	V7-04*01-none* (16.18)		63.13
E13	M	3	*ETV-RUNX1*	*96*	2	FR1	V4-39*05-J4*02 (34.30)	V4-31*04-J4*02 (22.24)			56.54
E14	M	6	*ETV-RUNX1*	*96*	1	FR1	V3-9*01-J4*02 (43.02)				43.02
B1	F	51	*BCR-ABL1*	*73*	2	FR2 + FR3*	V3-15*07-J4*02* (69.31)	V2-70*01-J4*02 (13.06)			82.37
B2	M	70	*BCR-ABL1*	*27*	2	FR1	V3-13*01-J4*02 (42.19)	V4-4*03-J4*02 (14.73)			56.92
B3	M	66	*BCR-ABL1*	*95*	2	FR1	V3-7*01-J4*02 (49.19)	V5-10-1*03-J3*02 (35.95)			85.14
B4	F	59	*BCR-ABL1*	*98*	1	FR1	V3-15*04-J4*02 (79.51)				79.51
B5	F	56	*BCR-ABL1*	*99*	1	FR1	V4-4*03-J4*02 (85.23)				85.23
B6	F	18	*BCR-ABL1*	*95*	2	FR1	V3-30-3*03-J4*02 (45.41)	V3-30*06-J4*02 (37.10)			82.51
B7	M	42	*BCR-ABL1*	*91*	2	FR1	V3-13*04-J5*02 (50.44)	V3-21*01-J5*02 (36.22)			86.66
B8	F	18	*BCR-ABL1*	*93*	1	FR1	V1-2*02-J4*02 (77.19)				77.19
B9	M	49	*BCR-ABL1*	*98*	1	FR1	V3-23*01-J5*02 (81.47)				81.47
B10	F	64	*BCR-ABL1*	*95*	2	FR1	V3-71*01-J6*03 (56.04)	V3-74*01-J4*02 (23.38)			79.42
B11	M	52	*BCR-ABL1*	*90*	1	FR3	V1-c*01-J4*02 (85.80)				85.8
B12	M	9	*BCR-ABL1*	*99*	2	FR1	V3-9*01-J3*02 (25.42)	V3-33*06-J4*02 (23.95)			49.37
B13	M	13	*BCR-ABL1*	*97*	2	FR1	V3-49*04-J5*02 (32.64)	V3-33*06-J6*02 (18.98)			51.62
B14	M	21	*BCR-ABL1*	*96*	3	FR1	V4-59*08-J6*03 (39.69)	V3-64*01-J6*03 (11.02	V3-74*01-J1*01 (10.96)		61.67
B15	F	68	*BCR-ABL1*	*93*	1	FR1	V3-30*18-J4*02 (51.08)				51.08
B16	F	37	*BCR-ABL1*	*97*	2	FR1	V3-49*04-J5*02 (71.63)	V1-69*13-J5*02 (17.92)			89.55
B17	F	66	*BCR-ABL1*	*93*	1	FR1	V3-33*06-J4*02 (84.22)				84.22
B18	F	54	*BCR-ABL1*	*48*	1	FR1	V3-7*03-J3*02 (88.56)				88.56
T1	F	6	Trisomy14	75	2	FR1	V1-46*01-J6*02 (40.62)	V2-26*01-J6*02 (17.94)			58.56
T2	M	14	Trisomy14	98	4	FR1	V1-3*01-J6*02 (35.62)	V3-11*01-J5*02 (24.21)	V4-39*01-J4*02 (8.44)	V3-33*01-J4*02 (5.85)	74.12
T3	M	14	Trisomy14	92	2	FR1	V3-30*04-J4*02 (66.00)	V3-64*05-J4*02 (22.07)			88.07
T4	M	4	Trisomy14	94	1	FR2	V5-51*04-none (11.82)				11.82
T5	M	3	Trisomy14	88	1	FR1	V6-1*01-J4*02 (61.09)				61.09
T6	M	4	Trisomy14	98	1	FR1	V1-NL1*01-J6*03 (70.06)				70.06
T7	F	1	Trisomy14	97	2	FR1	V6-1*01-J3*02 (41.59)	V3-9*01-J6*02 (38.54)			80.13
T8	F	5	Trisomy14	99	1	FR1	V6-1*01-J4*02 (81.26)				81.26
T9	F	7	Trisomy14	99	3	FR1	V3-23*01-J4*02 (41.37)	V3-64D*06-J6*02 (28.84)	V4-4*02-J4*02 (10.78)		80.99
T10	M	4	Trisomy14	98	2	FR1	V3-7*03-J5*02 (21.81)	V3-21*02-J3*02 (19.54)			41.35
T11	M	4	Trisomy14	81	3	FR1	V1-3*02-J6*02 (27.99)	V1-2*05-J6*03 (11.76)	V2-5*01-J6*02 (2.90)		42.65
T12	F	5	Trisomy14	91	2	FR1	V3-49*05-J3*02 (37.59)	V6-1*02-J6*02 (12.6)			50.19
T13	M	16	Trisomy14	80	1	FR1	V4-4*01-J4*02 (55.46)				55.46
H1	M	3	46,XY	90	1	FR1	V4-31*03-J5*02 (78.02)				78.02
H2	F	8	46,XX	35	1	FR2	V5-51*04-none (9.47)				9.47

BM: bone marrow; FR: framework region.

tetrasomy 14). Table 2 summarizes the cumulative detection of *IGH* rearrangement. The *IGK* assay was positive in 70% of the patients and three (60%) of five *IGH* FR1-negative patients were positive (Fig. 1, Supplemental Table 1).

Diverse numbers (3–52) and sizes (0.0009%–3.987%) of the minute satellite sequences were observed around dominant clones using the Vidjil platform. The minute satellite sequences occupied approximately 10.0% of the *IGH* dominant clone (1.5%–30.6%). The dominant *IGH* clonal burden was significantly correlated with the number ($R^2 = 0.702$, $P < 0.001$) and sum ($R^2 = 0.626$, $P < 0.001$) of the minute satellite sequences (Supplemental Fig. 1).

3.2. Ig gene rearrangement for MRD monitoring

We investigated the MRD using the Ig clonality in B-ALL patients who had been monitored for *ETV6-RUNX1* and *BCR-ABL1* fusions during treatment. *IGH* FR1 assay alone covered monitoring in the majority of B-ALL (85.1%). The *IGH* FR2 and FR3 assays could be selected to evaluate the MRD in three and two patients, respectively. In three patients, two assays were needed to monitor all *IGH* clones detected in the BM at the time of diagnosis; FR1&FR3 ($n = 2$) and FR2&FR3 ($n = 1$).

A comprehensive measurement of the clonal burden is important for MRD monitoring. All diagnostic BM specimens in the study contained > 70% leukemic blasts, except for three cases (median 93%; range 27%–99%). The sum of the *IGH* clonal burden varied (median 78.02%, range 9.47%–96.77%). Among patients with *IGK* rearrangement, Vκ-Kde and INTR-Kde rearrangements made up 61.5%. When we compared clonal number of *IGH* and *IGK* rearrangements, most (85.7%) showed the same or more clones of *IGK* compared with *IGH*. The *IGK* clonal burden also varied (median 77.57%, range 24.07%–92.95%), but did not show a significant correlation with the *IGH* clonal burden. The sum of clonal burden of *IGK* was lower than *IGH* in 64.3% of patients (median difference 11.56%, 1.15%–59.55%). One showed a > 10% higher *IGK* clonal burden than *IGH* (79.32% vs. 11.82%).

3.3. MRD monitoring using Ig clonality

We validated the analytical performance including linearity and limit of detection of the *IGH* assay using a serially diluted specimen (10^{-1} to 10^{-5} dilutions) and demonstrated that the sensitivity was comparable to *IGH* qPCR and acceptable for MRD monitoring ($< 10^{-5}$).

There was a significant correlation between the proportion of leukemic blasts and *IGH* clonal burden ($R^2 = 0.898$, $P < 0.001$) (Fig. 2A). Quantitative data of *ETV6-RUNX1* and *BCR-ABL1* analyzed by fusion with FISH and RT-qPCR, respectively, correlated well with Ig clonal burden (Fig. 2B, C). All B-ALL with *ETV6-RUNX1* achieved and maintained complete remission (CR) for > 5 years. We performed MRD monitoring after induction chemotherapy (day 33) and did not detect an identical sequence to the initial Ig rearrangement in any patient. Nine of eleven (81.8%) B-ALL with *BCR-ABL1* achieved morphologic CR after consolidation chemotherapy. The Ig clonal burden (Fig. 3A, D) changed along with the quantity of fusion transcript (Fig. 3B, E). Patients who achieved better response than MMR (two CMR and two MMR) showed < 0.01% of Ig clones (three negative conversion and one 0.0078%). We calculated log reduction of Ig clonal burden and compared the results with the molecular response of *BCR-ABL1* (Fig. 3G, H). The *IGH* clone showed a similar response to *BCR-ABL1* fusion. We also compared the MRD value evaluated through HTS and flow cytometry in five patients. There was significant correlation between the two methods ($R^2 = 0.776$, $P = 0.005$). We detected a patient whose *IGH* clonal burdens were 0.05% and 0.06% at two serial time points after chemotherapy, and who relapsed at the third monitoring (0.17%), whereas the MRD value by flow cytometry was maintained at < 0.01% before relapse (1.3%).

In patients with more than two coexisting clones, each clone responded to chemotherapy independently. On the other hand, minute satellite sequences reacted along with their affiliated dominant clone after treatment (Supplemental Fig. 1). Any patient who did not achieve molecular remission showed less than a 3-log reduction of Ig clonal burden (median −1.38, range −2.43 to −0.66). In one patient, a new clone emerged at relapse, presenting a very low level (0.47%) in the initial specimen and increased during follow-up. We also observed a satellite sequence (0.17%) with one base difference that gained high proliferation activity so as to become a subclone (8.68%) at relapse (Fig. 4). In addition, a large fraction of the hematogone present in the remission status BM did not influence the MRD monitoring using Ig gene rearrangement.

3.4. IGH repertoire diversity

The *IGH* assay further provided information of clonal heterogeneity of *IGH* rearrangement in the investigated samples using the Vidjil algorithm. Data 0 means that a single clone was seen in all reads (no diversity), whereas data 1 indicated that each the read corresponds to a distinct clone (full diversity). The initial specimen showed a significantly lower *IGH* repertoire diversity compared with the follow-up specimen (median 0.051 vs. 0.138, $P < 0.001$), and most patients had increased diversity after treatment ($P < 0.001$) (Fig. 3C, F). Interestingly, two relapsed patients showed a gradual decrease in diversity before the overt relapse (Fig. 4A, C).

3.5. Outcomes according to MRD

We analyzed the prognostic significance of MRD by FISH or RT-

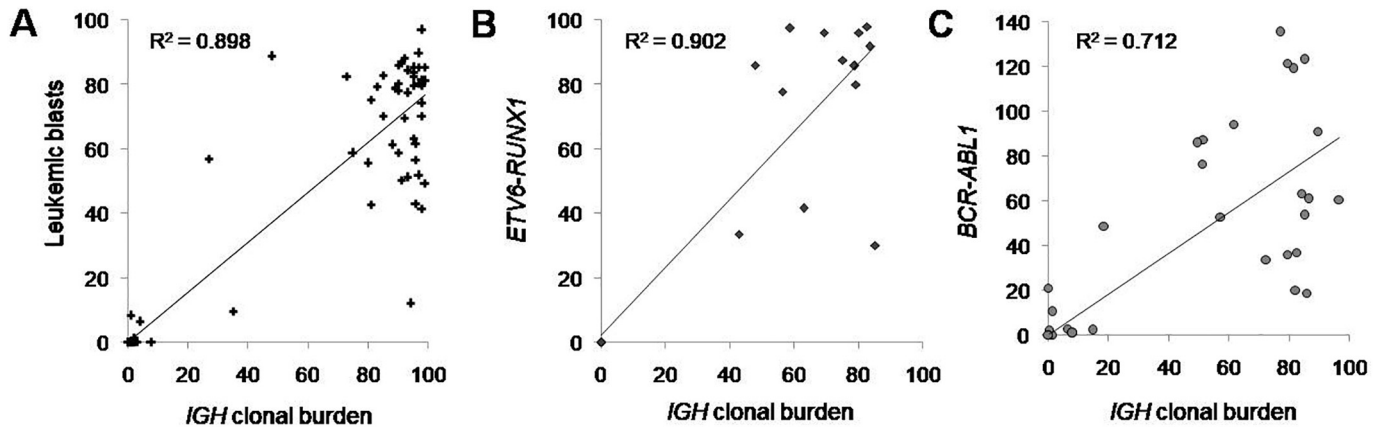

Fig. 2. Correlation between *IGH* clonal burden and proportion of leukemic blasts (A), *ETV6-RUNX1*(B), and *BCR-ABL1* (C) fusions.

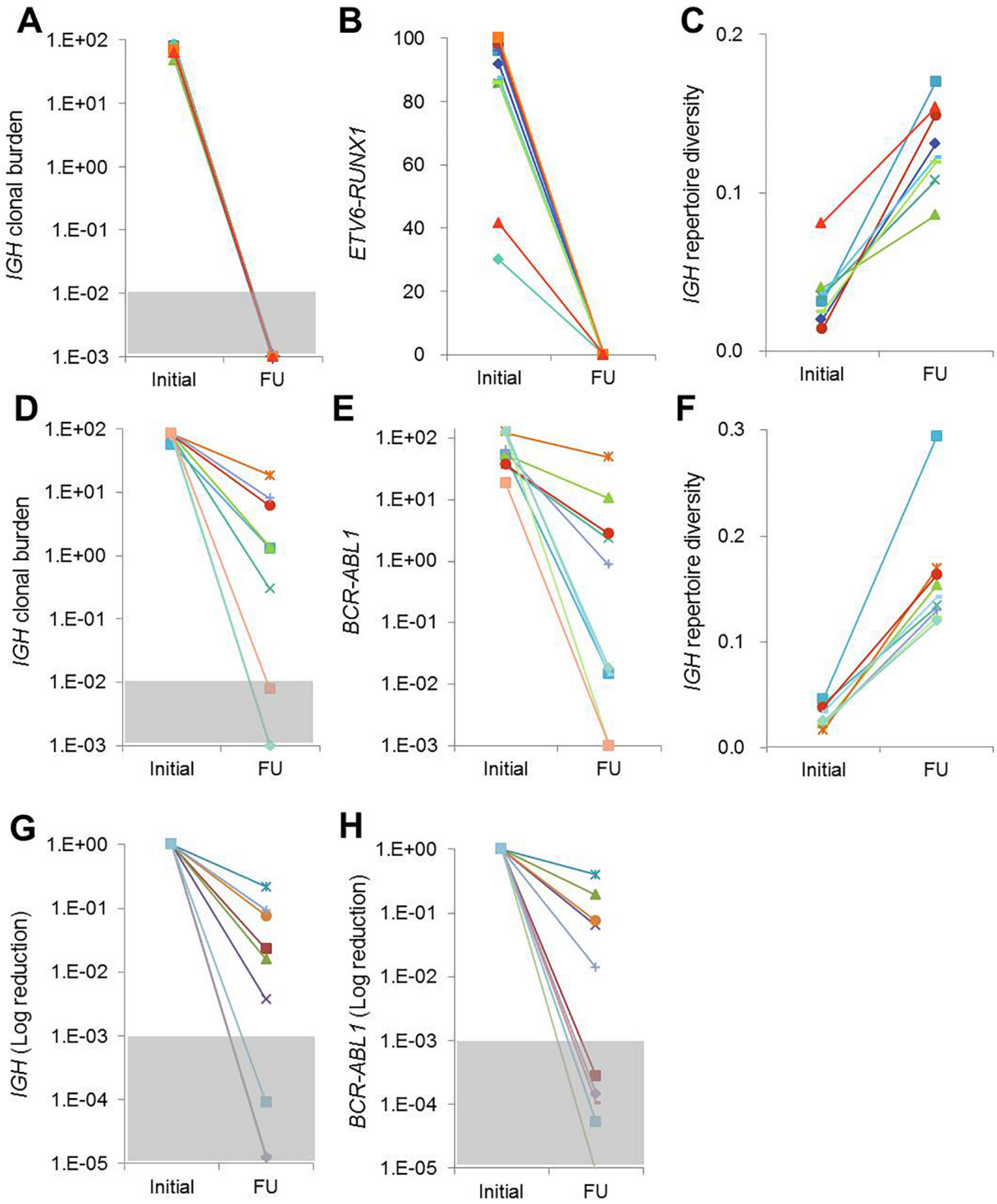

Fig. 3. Follow-up of patients with *ETV6-RUNX1* (A–C) and *BCR-ABL1* (D–H). *IGH* clonal burden, fusion transcript and *IGH* repertoire diversity at initial diagnosis and after induction chemotherapy (day 33). Graphs (G) and (H) are log reduction of *IGH* and *BCR-ABL1*, respectively. The shadow zone indicates better than major molecular response.

qPCR and HTS. Three patients died, and the median OS was 46.6 months (range 8.7–139.9 mo). Nine patients experienced relapse, and the median RFS was 42.2 months (range 0.7–139.9 mo). MRD by HTS was associated with reduced OS [hazard ratio (HR) 1.429, 95% confidence interval (CI): 0.952–2.143] and RFS (HR 3.333, 95%CI: 1.293–8.591) (Fig. 5A, B). As expected, MRD by FISH or RT-qPCR was associated with reduced OS (HR 1.750, 95%CI: 0.921–3.324) and RFS (HR 6.533, 95%CI: 1.059–40.307) (Fig. 5C, D).

4. Discussion

In this study, we aimed to develop a practical strategy for MRD monitoring in B-ALL using Ig clonality. There are several considerations when we choose an MRD marker. First, the analytic method should be applicable in a clinical laboratory and in most individuals [14,15]. Leukemia-specific genetic aberrations have been used for MRD monitoring. However, there are patients who do not reveal genetic aberrations. Considering these points, the Ig rearrangement has been

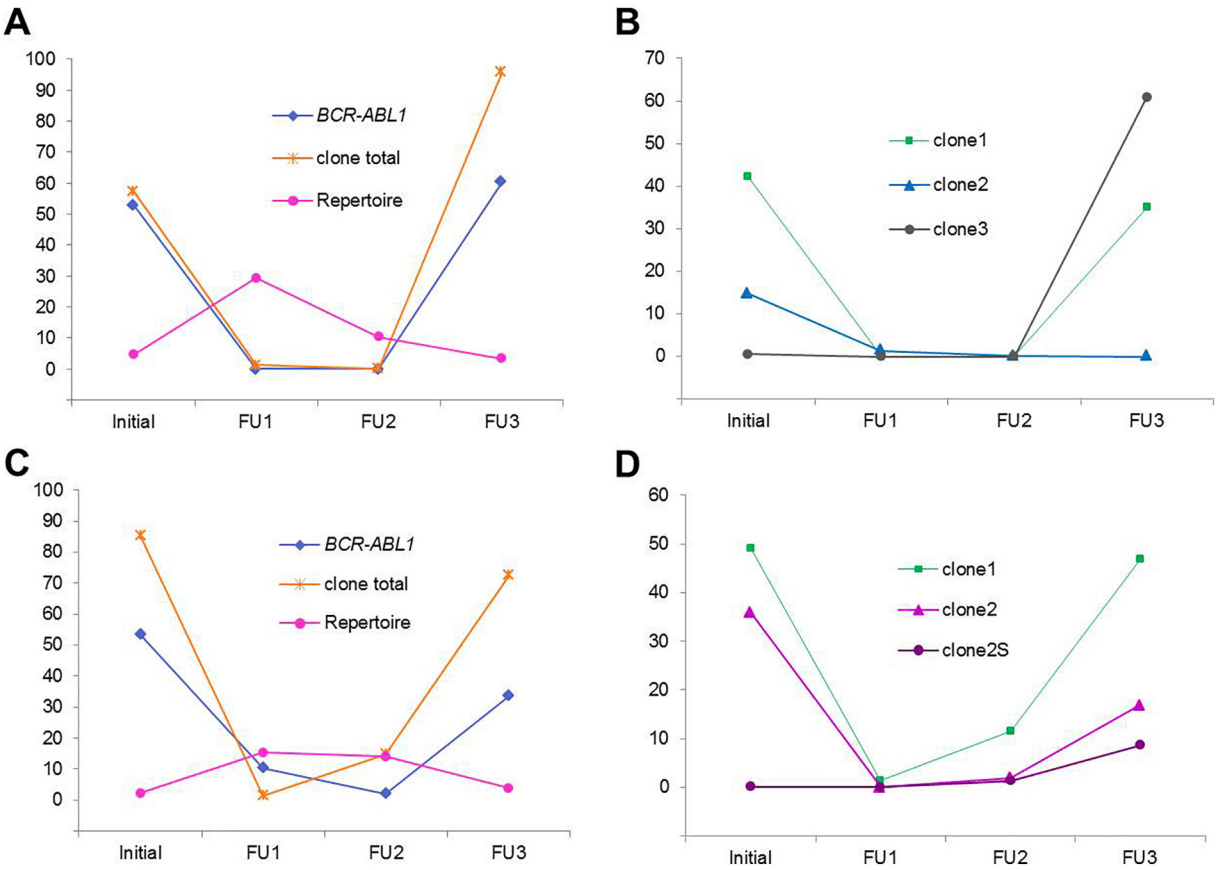

Fig. 4. Follow-up data in two relapsed patients with *BCR-ABL1* (A and B from patient B2, C and D from B3) for fusion transcript, *IGH* total clonal burden, and repertoire diversity (A, C). Changes in percentage of each *IGH* clone (B, D) over time (different colored lines are used for each individual clone > 5% at any of the indicated time points). Time points initial, follow-up (FU1), FU2, and FU3 refer to diagnosis, after induction (day 33), consolidation, and relapse for each patient. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

recommended as a universal MRD marker in B-ALL. Although qPCR using patient-specific Ig primers has been used for MRD monitoring, it is less practical regarding the workload and workflow complexity. HTS has been known to overcome some limitations of qPCR assays in ALL, including the limits of sensitivity, the presence of oligoclonality at the time of diagnosis, and difficulties in identifying markers in some leukemia subtypes such as hyperdiploid leukemias [16,17]. To improve the reliability of Ig clonality assay, targeting all three conserved frameworks of the V_H gene segment significantly reduced the risk of not detecting the presence of clonality due to sequence variation in the primer binding sites [18]. We investigated clonality profiles in B-ALL and showed that the FR1 detected clonal *IGH* rearrangement in the majority of patients with B-ALL. When combined with FR2 and FR3, we found appropriate clonal *IGH* rearrangements in all cases. The combination of three FR primers not only improved the detection rate of Ig clonality, but also measured clonal burden more closely to real leukemic burden. HTS was also able to detect clonal *IGK* rearrangements. We found an exceptional case with very high *IGK* clonal burden compared with *IGH*. Therefore, we postulated that the use of *IGK* rearrangements can be applied to Ig clonality testing, especially in cases with a low *IGH* clonal burden [19,20].

Post-induction MRD status is mandatory in a risk assessment study for ALL, and the molecular MRD response should be analyzed by methods that can define $< 10^{-4}$, $< 0.01\%$, or < 1 leukemic cell in 10,000 [21]. The results from this and the previous studies indicate that Ig clonality via HTS satisfied these conditions and showed a good correlation with leukemia-specific fusion genes and flow cytometry [22]. All patients with persistent remission had negative or $< 0.01\%$ MRD of Ig clonality after induction chemotherapy, and MRD by HTS was significantly associated with survival outcomes. The other benefit of the HTS in MRD monitoring is the ability to monitor multiple clones, including subclones [23]. An analysis of the diagnosis-relapse pair found that dominant clones present at diagnosis re-emerged at relapse. Our study and previous studies have recognized that a trace of Ig rearrangement already present in the initial specimen appeared as a clonal evolution at relapse [22,24]. Therefore, we should pay attention to the change of the individual clones that showed an increase in clonal size even though the sum of Ig clones decreases. In addition, because the clonal heterogeneity of Ig rearrangement may be different before and after chemotherapy as well as overt relapse, all the Ig gene rearrangements can be considered for MRD monitoring.

Furthermore, HTS provides information on the physiological B-cell repertoire during and after treatment, which has been shown to be helpful to monitor disease status [25]. The increased *IGH* repertoire diversity after treatment can be associated with a recovery in a normal B-lymphoid system. We observed that a decrease in diversity occurred before an overt relapse in two patients. Therefore, the decreased *IGH* repertoire diversity may implicate a deterred recovery proceeding to relapse.

Although several points should be clarified before we implement Ig clonality analyzed by HTS into clinical MRD testing [6], these results are very encouraging for the following reasons. An appropriate strategy makes it possible to identify almost all clones in B-ALL and to reduce errors missing important clones at monitoring. It added convenience in that changes in *IGH* gene number or presence of a large fraction of hematogone did not affect MRD monitoring via HTS. The other point is that there have been continuous efforts to upgrade bioinformatics pipelines to overcome the technical limitations of HTS. Each pipeline has

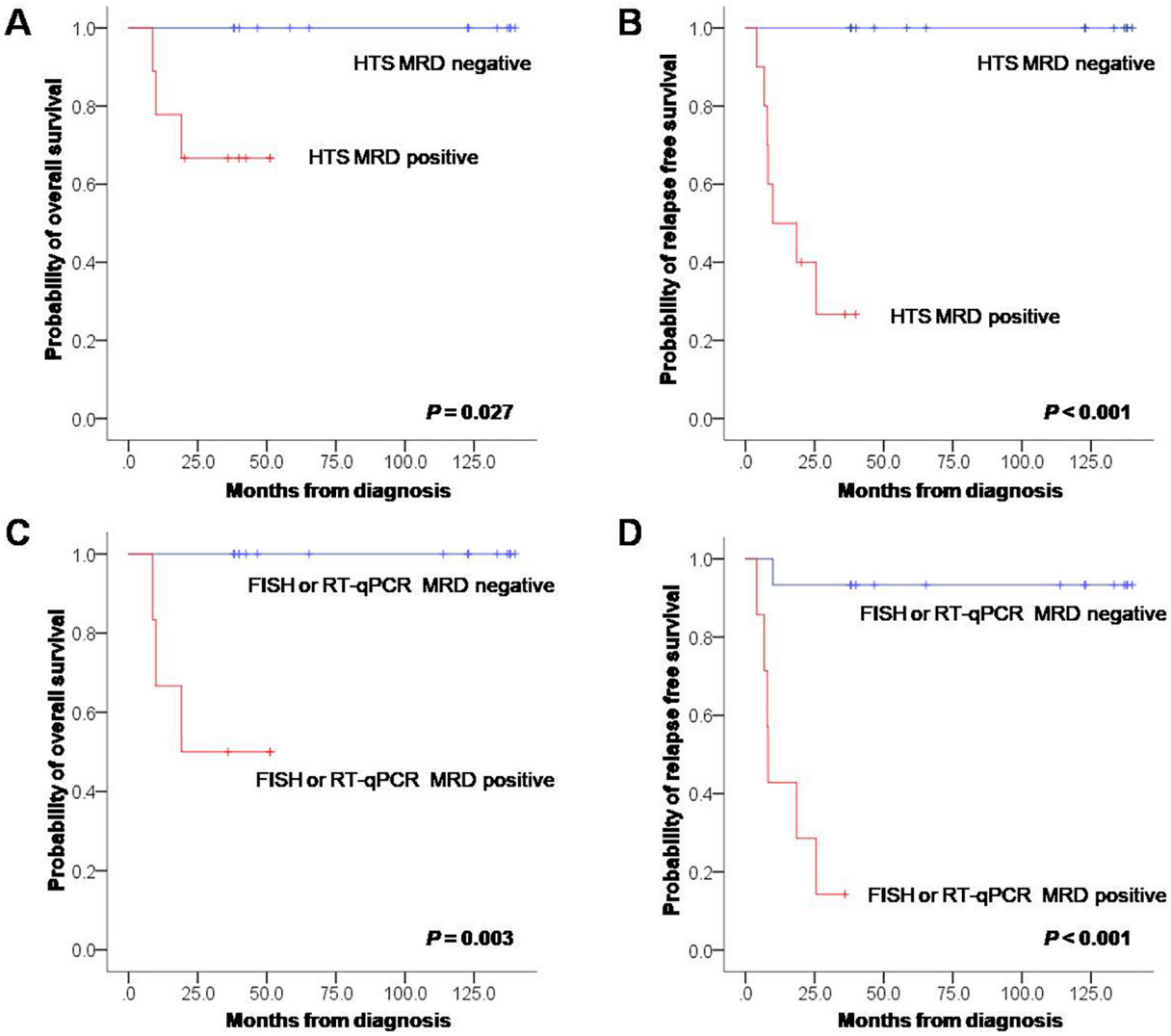

Fig. 5. Influence of minimal residual disease (MRD) evaluated by high-throughput sequencing (HTS) (A, B) and leukemia specific fusion detected by fluorescence in situ hybridization (FISH) or quantitative reverse transcription PCR (RT-qPCR) (C, D) on patients' outcomes.

developed its own method, such as merging highly similar sequences (Lymphotrack) and setting a 50 bp "window" overlap (Vidjil) [11] to include all minute satellite sequences. Whether or not the minute satellite sequences were caused by PCR and/or sequence artifacts [12], they ought to be considered to belong to a dominant clone because they showed the same response pattern to the affiliated dominant clone. This study and previous studies showed that the different pipelines perfectly match or give lightly different designations that are acceptable [26].

In conclusion, Ig clonality via HTS will be a promising MRD monitoring tool for B-ALL based on our proposed strategy to identify all significant clones, monitor individual clones, and determine repertoire diversity. Further studies to accumulate prospective data with clinical implication will help refine the strategy.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2018.10.037.

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Conflicts of interests

The authors declare no conflict of interest.

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