Consensus JH Gene Probes with Conjugated 3'-Minor Groove Binder for Monitoring Minimal Residual Disease in Acute Lymphoblastic Leukemia

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Several approaches for the detection of minimal residual disease (MRD) in childhood acute lymphoblastic leukemia (ALL) have shown the importance of determining the level of MRD precisely. In the present study, we tested a new real-time quantitative polymerase chain reaction (RQ-PCR) strategy with minor groove binder (MGB) technology for immunoglobulin heavy chain gene rearrangements by positioning a MGB probe at the germline JH segments and one of the primers at the downstream introns in combination with an allele-specific oligonucleotide (ASO) primer complementary to the VH-DH or DH-JH junctional region. A MGB probe forms extremely stable duplexes with single-stranded DNA targets, allowing the use of shorter probes for hybridization-based assays. Therefore, it shows positional flexibility. We have designed two novel consensus MGB JH germline probes for analyzing all of the germline rearrangements registered in the VBASE database, and demonstrated that the MRD was detectable with the probes in 17 cases of childhood ALL. The actual copy number for the targets and dynamic changes before and after treatment were almost identical between the JH MGB probe and conventional non-MGB probes in each patient. MGB technology will undoubtedly contribute to MRD-PCR studies of childhood ALL. (*J Mol Diagn* 2005, 7:121-126)

Large multi-center studies on the detection of minimal residual disease (MRD) in childhood acute lymphoblastic leukemia (ALL) have shown that it is important to determine precisely the level of MRD at an early point in morphological remission for discrimination between low- and high-risk patients.¹⁻⁴ Information about the kinetics of tumor cell reduction has allowed the recognition of

MRD-based risk groups showing significant differences in relapse rate.¹⁻⁴ An allele-specific oligonucleotide real-time quantitative polymerase chain reaction (ASO RQ-PCR) is suitable for this purpose because of its sensitivity, reproducibility and simplicity, and is currently being used in ongoing MRD studies.¹⁻⁴

Recently, a stepwise strategy for selection of MRD targets in childhood ALL was proposed.⁴ In half of all childhood ALL patients, MRD can be monitored by using an immunoglobulin kappa-deleting element (IGK-Kde) rearrangement which is more stable at relapse than other markers such as the immunoglobulin heavy chain (IgH) and T-cell receptor (TCR) genes.⁵ However, the remaining patients have to be examined using IgH or TCR markers.^{4,5} MRD assessment using rearrangement of the IgH gene has some advantages such as the high rate of evaluable patients and the large junctional regions in which tumor-specific primers can be set, making the ASO RQ-PCR strategy highly sensitive and specific.

Donovan et al⁶ designed seven IgH variable (VH) gene consensus probes in view of the fact that clonal IgH sequences in childhood ALL typically are without somatic mutation. However, their consensus VH probes did not completely match all of the germline sequences because of the diversity of the VH families. Therefore, approximately 20% of our childhood ALL patients could not be assessed using their probes.⁷

Recently, DNA probes with conjugated 3'-minor groove binder (MGB) groups have been developed and used for 5'-nuclease PCR assays.⁸ The hydrophobic binding of the MGB to the DNA helix increases the melting temperature (T_m) by 15 to 30°C, which allows the use of shorter probes with high specificity. Due to the high T_m requirements of PCR, non-MGB probes vary substantially in length from 14- to 40-mers depending on the G + C content of the amplified DNA fragment, whereas the MGB probes vary from 12- to 20-mers. Therefore, MGB probes show positional flexibility. In addition, the non-fluorescent quencher dye (NFQ, Epoch Biosciences, Bothell, WA) used in this study quenches the fluorescence of a wide range of dyes. The MGB and NFQ work in concert to eliminate fluorescence for non-hybridized probe mole-

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Figure 1. Schematic diagram of the probe/primer design for the JH ASO RQ-PCR strategy. In this strategy, the sequencing analysis is performed using FR1c/LJH/VLJH and/or FR2a/LJH/VLJH primers to determine the V-D-J sequences. The number of the JH gene segment is used to choose an appropriate primer out of six intronic primers. A TaqMan MGB probe was designed for the 3'-end of the JH gene segments in combination with an allele-specific oligonucleotide (ASO) primer complementary to the junctional region and a primer complementary to the intronic sequence of the JH gene segments.

cules while allowing much stronger fluorescence for hybridization probes.⁸

For examination of childhood ALL, we recently designed MGB consensus probes corresponding to all of the IgH variable region (VH) germlines registered in the V BASE database (<http://www.mrc-cpe.cam.ac.uk/DNAPLOT.php>).⁷ In comparison with non-MGB (21- to 27-mers), the MGB probes were shorter (16- to 18-mers) but had a high *T_m* (approximately 70°C). The MGB probes made it possible to assess MRD even in cases that would normally require the design of patient-specific conventional non-MGB probes⁶ (about 20% of total cases). However, 10 MGB probes designed by us have still been required to examine all ALL patients.⁷ The high cost and extensive labor involved in the use of many fluorogenically labeled probes hamper clinical application of the ASO-RQ PCR strategy for large-scale studies of MRD.

Verhagen et al⁹ have designed IgH joining region (JH) consensus probes with a set of ASO forward and intronic reverse primers (Figure 1) because the number of JH gene subfamilies is lower than those of the VH gene. Their JH strategy is simpler and more suitable because they used only three germline probes combined with six consensus intronic primers,⁹ thus covering almost all ALL patients. A lower number of probes is efficient for MRD monitoring in large series of childhood ALL patients. In the present study, we developed shorter JH probes using MGB technology, and here we describe the technical details, pre-clinical validation, and initial clinical application of these probes in childhood ALL patients.

Materials and Methods

Samples and DNA Isolation

A B-ALL cell line (BALL 1, obtained from the Cell Resource Center for Biomedical Research, Tohoku University, Sendai, Japan) was used for the pre-clinical validation study of our MGB probes.

Seventeen children with precursor-B-ALL were subjected to the initial clinical application. All of the patients were treated according to the ALL-Berlin-Frankfurt-Münster (BFM) 90 protocol. Bone marrow (BM) samples were collected at diagnosis and during the follow-up period. Permission for the study was obtained from the Institutional Review Board of Iwate Medical University School of Medicine, and written consent to participate was ob-

tained from all of the patients and/or their parents. DNA was isolated from the cell line, peripheral mononuclear cells and BM samples using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany).

PCR

The isolated DNA was amplified using three sets of semi-nested PCR to determine the V-D-J sequences, as described previously.¹⁰⁻¹³ Briefly, in the first set of PCRs, 200 to 300 ng of DNA was amplified during 30 cycles consisting of 94°C for 2 minutes, 60°C for 2 minutes and 72°C for 2 minutes, using the primer sets FR1c/LJH (FR1c: TGG (A/G)TCCG(C/A)CAG(G/C)C(T/C)(T/C)CNGG, LJH: TGAG-GAGACGGTGACC) or FR2a/LJH (FR2a: AGGTGCA-GCTG(G/C)(A/T)G(G/C)AGTC(G/AT)GG). Subsequently, 2 µl of the products of the first PCR was subjected to 20 cycles of a second set of PCRs, consisting of 94°C for 5 minutes, 63°C for 2 minutes, and 72°C for 2 minutes. The forward primers for the second PCR set were the same as those used in the first PCR (FR1c or FR2a), while the reverse primer was VLJH (VLJH: GTGACCAGGGTNCCTTGCC-CCCAG) in both sets. The final reaction volume for all PCRs was 50 µl, and the mixture contained 1 µl of a 10 µmol/L solution of each primer, 1.65 units *Taq*DNA polymerase, 4 µl of 25 mmol/L MgCl₂, 1 µl of 10 mmol/L dNTP, 5 µl of the recommended buffer (10X PCR buffer, Applied Biosystems, Foster City, CA), and distilled water.

Subcloning and Sequencing Analyses

The PCR products were analyzed by 2% agarose gel electrophoresis and stained with ethidium bromide. A clonal band of appropriate size (240 to 350 bp) was excised from the gel electrophoresis material and purified using a QIAquick Gel Extraction Kit (Qiagen). The product was ligated to pGEM-T Easy Vectors (Promega, Madison, WI) and transformed into DH5α-competent cells (Toyobo, Tokyo, Japan). We selected 30 subcloned colonies at random from each patient and purified plasmid DNAs using a PI-200 DNA automatic isolation system (Kurabo, Osaka, Japan). Cycle sequencing was performed using a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems) and an ABI PRISM 3100 DNA Sequencer (Applied Biosystems). The nucleotide sequences of each clone were aligned with the closest germline sequences derived from the V BASE database. The RQ-PCR assay was performed using an ABI PRISM 7700 Sequence Detector (Applied Biosystems). The reaction mixture contained 300 ng of template DNA, 200 nmol/L of each primer, appropriate concentrations of JH MGB probes, and 25 µl of TaqMan Universal PCR Master Mix (Applied Biosystems) in a final volume of 50 µl. The mixture was placed in 0.2-ml MicroAmp optical tubes with caps (Applied Biosystems). The sequences of the downstream intron primers were DPJH-1: CGCTATCCCCAGACAGCAGA, DPJH-2: GGTGCCTGGACAGAGAAGACT, DPJH-3: AGGCAGAAGGAAAGCCATCTTAC, DPJH-4: CAGAGTTAAAGCAGGAGAGAGGTTGT, DPJH-5: AGAGAG-GGGGTGGTGAGGACT, and DPJH-6: GCAGAAAA-

Table 1. JH Usages, ASO Primers, Annealing Temperature, and Results of the RQ-PCR Assays Using MGB and Non-MGB Probes in 17 Patients with ALL and an ALL Cell Line

Patient no.	J _H	Forward primer sequence*	Annealing temperature	Normalized values of PCR targets by MGB [†] probe (those by non-MGB [‡] probe) [§]			
				Initial diagnosis	15 days	1 month	3 months
1	J _H 6	AGAGATGGGCGAGTGGCTGGTTATA	60°C	31400 (38200)	16500 (17500)	1490 (580)	4300 (2200)
2	J _H 4	CAACAGTGTGCTGGGGAGAGACT	60°C	69000 (58600)	3.4 (-)	- (-)	- (-)
3	J _H 5	TGCCCTCTATGATAGTAGTGGTAACAGA	60°C	56900 (52000)	38100 (38000)	687 (880)	4500 (6300)
4	J _H 4	TTCCCCGAGAGACGACGAC	60°C	30200 (28400)	34800 (32000)	- (-)	- (-)
5	J _H 4	TGTGCGAGAGGAATGAGCAAGT	60°C	9050 (12600)	425 (450)	6.21 (-)	- (-)
6	J _H 4	AGCGGGCCTCGAAAACCTGGAA	62°C	10600 (9800)	- (-)	- (-)	- (-)
7	J _H 6	ACTGTGCACGGAACCCCC	60°C	41000 (39500)	- (-)	- (-)	- (-)
8	J _H 4	TACTAGACATATTGTGGTGGTGA [†] CTGCT	60°C	59000 (72000)	- (-)	- (-)	- (-)
9	J _H 6	CGAGAGTCCGCTTCGAGGA	60°C	5740 (6540)	14.2 (-)	- (-)	- (-)
10	J _H 6	CAAGAGATATGAGTATAAACTCCTAGTAGG	60°C	7500 (8800)	- (-)	- (-)	- (-)
11	J _H 3	AGAGAAGGATATTGTACTGGTGGCC	60°C	12800	-	-	-
12	J _H 6	GTGTATTACTGTGCGAGAGTAATACCCTT	60°C	23000 (25000)	- (-)	- (-)	- (-)
13	J _H 6	TAGATGAAACCCCCGGGCAG	62°C	29000 (32400)	- (-)	- (-)	- (-)
14	J _H 4	TCCTCTGAGAGATGAAACCCCC	60°C	16500 (13200)	-	-	-
15	J _H 3	TGTGAAAGGGGACCTATTGACTAATGCTT	58°C	8900	10090	5.21	-
16	J _H 2	TATCCCCGACGCCGAGA	60°C	32600	3600	-	-
17	J _H 4	TGGTTATTACTTAGCTCAGCTATCTTTGAC	60°C	7900 (5650)	1260 (650)	- (-)	- (-)
BALL 1	J _H 4	TTACTGTGCGAGAGGGACCC	60°C	-	-	-	-

*Underlined sequences represent randomly inserted nucleotides of the junctional region.

[†]The normalized values of targets are expressed as the ratio of the target copy number for each 10⁵ cell equivalent from the integrated standard curves of human β -actin, as previously described.¹⁴⁻¹⁶[‡]Non-MGB probes are not examined in patients with JH2 or JH3 segment, because Verhagen et al⁹ did not design for the patients.[§] -, not detected; blank, not determined.

CAAAGGCCCTAGAGT.⁹ Each ASO primer is shown in Table 1. In the initial step, AmpErase uracil-N-glycosylase was activated for 2 minutes at 50°C. This step was followed by activation of AmpliTaq Gold DNA polymerase for 10 minutes at 95°C. The cycling program consisted of 50 cycles of a two-step PCR comprising a 15-second denaturation step at 95°C and a 1-minute combined annealing/extension step at 58 to 62°C (Table 1). Assay-specific standard curves for each set of primers and the probe were constructed by plotting the threshold cycle (C_T) against the known copy number of each positive control plasmid that was used for determining each of the JH sequences. The plasmid concentration was determined spectrophotometrically, and the copy number was calculated. Plasmids were diluted in a precise series, ranging from 5 pg to 0.005 fg (from 2×10^6 to two copies). For normalization of each target, the copy number of β -actin was used as an internal control. The normalized values for the targets were expressed as the ratio of the target copy number per 10⁵ cell equivalents from the integrated standard curves for human β -actin (TaqMan β -actin Detection Reagents Kit; Applied Biosystems).¹⁴⁻¹⁶ In all RQ-PCR assays, peripheral mononuclear cells from 20 normal individuals were examined as negative controls.

Results

Designs of JH MGB Probes and RQ-PCR Conditions

Initially, we designed only one MGB JH probe (MJHU: 5'-GGTCACCGTCTCCTCA-3') to analyze all of the germline JH gene segments registered in the V BASE data-

base. However, one base substitution (C/T) was observed between a segment (JH2) and our probe sequence (Figure 2). As the presence of the mismatch between the target DNA and the MGB probe would have resulted in a T_m decrease of about 10°C, it was necessary to make another probe (MJH2: 5'-GGTCACTGTCTCCTCA-3'). In comparison with conventional non-MGB probes (24- to 27-mers),⁹ our MGB probes were shorter (16-mers) but had a high T_m (approximately 70°C) (Figure 2). Our improvement using MGB technology reduced the number of probes in comparison with the non-MGB strategy.

According to the sequence data for each patient, ASO forward primers were designed by Primer Express (Applied Biosystems) (Table 1). The differences of T_m between ASO forward and intronic reverse primers were controlled within 2°C. The sizes of the amplicons in our series were below 280 bp. ASO primers were fundamentally set on the VNDNJ region because they contained a larger number of tumor-specific sequences. In cases that lacked DH and whose forward primer T_m was uncon-

Figure 2. Alignment of JH family sequences registered in the V BASE database. The regions of the JH non-MGB probe designed by Verhagen et al⁹ and our JH MGB probe are boxed. **Dashes** indicate identity with the germline sequence of JH4a, and differences are indicated by capital letters.

trolled, we had to set them on the VNJ, VND, or DNJ region.

Recent experiments by our group and others had suggested that the undigested fraction of the MGB probe behaves like a hybridized probe and the fluorescence increases about 20% during PCR because of partial digestion of the probe during the late cycles of PCR.^{17,18} This "nuclease effect" depends on the probe sequence and the conditions of the PCR and usually accounts for 10 to 40% of the total signal.¹⁷ To decrease this fluorescence background, we examined the probe concentration at 20 to 200 nmol/L. We were able to improve dramatically the fluorescent background at 20 to 50 nmol/L in comparison with the concentration (200 nmol/L) used for the conventional non-MGB strategy (data not shown). We determined the probe concentration at 50 nmol/L.

Preclinical Validation of JH MGB Probes

Next, we compared the performance of our JH MGB (MJHU) probes with conventional non-MGB ($J_HQ_{1/4/5}$ in Ref 9) probes. To assess the sensitivity of a MJHU probe, we examined the BALL 1 cell line (JH4, Table 1) by the dilution method, as described previously.⁹ The cells were diluted by normal mononuclear cells: 10^1 to 10^6 tumor cells in a total of 10^6 cells. Amplification plots and standard curves for JH MGB (Figure 3A) and JH non-MGB (Figure 3B) are represented. The dynamic range of both assays using assay-specific plasmids encompassed at least 7 orders of magnitude (2×10^6 to 2 copies). There was no significant difference of the amplification plots between the MGB JH and non-MGB JH probes. The limit of detection in the RQ-PCR assay was one tumor cell in 10^5 background cells in both assays. For the MJH2 probe, DNA extracted from bone marrow samples (Patient 16 in Table 1) at diagnosis was serially diluted in polyclonal DNA to give a final concentration of 10^{-1} to 10^{-6} . Although the dynamic range obtained using the assay-specific plasmids encompassed at least 7 orders of magnitude (2×10^6 to 2 copies), the limit of detection by the MJH2 probe was 10^{-5} (Figure 3C). No positive signals were observed in any no-template controls or negative controls extracted from peripheral mononuclear cells.

The intra-assay reproducibility was evaluated using eight replicates at different points on the calibration curve. The coefficient of variation was 24% for the most dilute solution (two copies) and <5% for 20 to 2×10^6 copies.

Examination of Clinical Samples

We also assessed the clinical significance of our MJHU probe in comparison with the quantitative result obtained using the conventional non-MGB JH probes ($J_HQ_{1/4/5}$ and JHQ_6 in reference 9), in 14 of 17 cases of childhood ALL, including patients who had been examined in our previous study. IgH gene rearrangements consisted of one JH2, two JH3, seven JH4, one JH5, and six JH6 segments (Table 1). Each of the ASO primers and annealing

Figure 3. Amplification plots and standard curves obtained using the MGB (A and C) and non-MGB probes (B). **A:** Amplification plot and standard curve for the MJHU probe. Amplification plots were constructed using genomic DNA from an ALL cell line (BALL 1). The cells were diluted with normal mononuclear cells: from 10^1 to 10^6 tumor cells in a total of 10^6 cells. The C_T value for each dilution is indicated by an arrow (a–f). The standard curve was constructed using BALL 1-specific plasmid DNA (black dots, a–f). **B:** Amplification plot and standard curve constructed using a conventional TaqMan probe (non-MGB, $J_HQ_{1/4/5}$, see reference 9). Amplification plots were constructed using genomic DNA from an ALL cell line (BALL 1). The cells were diluted with normal mononuclear cells: from 10^1 to 10^6 tumor cells in a total of 10^6 cells. The C_T value for each dilution is indicated by an arrow (a–f). The standard curve was constructed using BALL 1 specific-plasmid DNA (black dots and line). Serial dilutions of tumor cells are represented by clear circles (a–f). **C:** Amplification plot and standard curve constructed using the MJH2 probe. Amplification plots were constructed using genomic DNA from bone marrow samples at diagnosis (Patient 16 in Table 1). DNA was serially diluted in polyclonal DNA to give a final concentration of 10^{-1} to 10^{-6} . The C_T value for each dilution is indicated by an arrow (a–f). The standard curve was constructed using patient-specific plasmid DNA (black dots and line). Serial dilutions of bone marrow DNA are represented by clear circles (a–f).

temperatures are represented in Table 1. The actual copy number for targets and dynamic changes before and after treatment were almost identical between the JH MGB and conventional non-MGB probes in each patient, and the sensitivities of both were also equal (Figure 4, Table 1).

Figure 4. Normalized copy number of target genes before and after induction therapy using immunoglobulin heavy chain (IgH)-allele-specific oligonucleotide real-time quantitative polymerase chain reaction (ASO RQ-PCR) assay in two cases. RQ-PCR analysis with the MJHU probe (continuous line) is compared with the conventional assay using non-MGB probes (dotted line). The arrow indicates re-induction therapy for bone marrow transplantation.

Discussion

Reduction in the number of primer/probe sets is helpful for standardization of the RQ-PCR MRD assay. Numerous improvements for PCR targets and primer designs have been proposed in previous RQ-PCR studies of ALL patients, whereas few studies have attempted to introduce new probe technology.^{1,2} The JH ASO RQ-PCR strategy originally developed by Verhagen et al⁹ is feasible because of the lower number of consensus probes and intronic primers. Our modifications using MGB technology contributed to reducing the number of probes in comparison with the original JH ASO RQ-PCR strategy.

Other new types of probes have recently been introduced for the RQ-PCR assay. Molecular beacon probe¹⁹ and Scorpion primer²⁰ both contain a stem-loop structure, which keeps a fluorochrome and a quencher together. On binding to a target sequence, they undergo a conformational change. Consequently, the fluorochrome and the quencher are separated and fluorescence is emitted. The lengths of the target recognition sequences usually range from 15- to 33-mer. Although this approach achieves a shorter length of probe/primer than conventional non-MGB probes, unhybridized (free) probe/primer remains in the hairpin conformation and is non-fluorescent during hybridization of the probe/primer with the target sequence. Therefore, using the stem-loop structure concept, the annealing temperature of PCR has to be relatively lower than that for linear probes. The lower annealing temperature increases the background fluorescence in the ASO-RQ-PCR assay. Considering the higher annealing temperature and shorter length of the target recognition sequences, MGB technology is currently most suitable for the ASO RQ-PCR assay using consensus germline probes.

IgH MRD-PCR targets involving JH segments often result in non-specific amplification of normal cells,² when the ASO primer contains a small number of tumor-specific sequences because of shorter junctional regions. If non-specific amplification of normal cells is observed, the annealing temperature has to be increased or shorter primers used. MGB technology would also be helpful for increasing the annealing temperature of PCR using relatively shorter primers. Afonina et al²¹ demonstrated that short primers (8- to 10-mers) conjugated to MGB could separately amplify viral sequences with a high degree of variability. We are now developing MGB-ASO primers targeting short junctional regions. In the near future, molecular staging using the MGB-ASO RQ-PCR assay may be of help in determining prognosis, and providing an early indication of recurrence in ALL patients.

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