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Original Article
Molecular detection of B-cell neoplasms by specific DNA methylation biomarkers

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Abstract: A novel, easy to perform PCR-based method employing specific DNA methylation biomarkers to detect B-cell neoplasms in a variety of B-cell lines and B lymphoblastic leukemia (B-ALL) patient specimens has been developed. This method detects as few as 5 B-ALL cells, or 1 B-ALL cell in 1,000,000 normal background blood cells using a single marker, *DLC-1* gene CpG island (CGI) methylation. By adding two additional markers *PCDHGA12* and *RPIB9*, over 80% of B-ALL cases were detected in patients' bone marrow and/or peripheral blood specimens. We have traced clinical B-ALL cases up to 10 years retrospectively and the *DLC-1* methylation is correlated with patient clinical status. Thus, this epigenetic-based molecular method demonstrates its potential use in the diagnosis of B-cell neoplasia, in addition to traditional approach such as clinical features, morphology, immunophenotype, and genetic analysis.

Keywords: Mature B-cell neoplasms, B lymphoblastic leukemia, DNA methylation biomarker

Introduction

While morphology assessment was the traditional approach for diagnostic pathology, an integrated system of morphology, immunophenotype, genetics and clinical features has become the standard of care in modern diagnostic hematopathology [1]. This is especially important in monitoring disease course and detecting minimal residual disease.

Delineating cell lineage using various modalities is an important step to categorize, classify and define a hematologic tumor. Immunophenotyping by either flow cytometry or immunohistochemistry is used in routine diagnosis in the vast majority of hematopoietic malignancies [2]. However, such a method has its own limitations. For example, recent study has shown that the down-regulation of the B-cell transcription factor *PAX-5* by DNA methylation could result in reprogramming of both precursor and mature B-cell neoplasms to monocytes or T-cells [3]. The plasticity of cell lineage may partially reflect the fact that many cancers including tumors of hematopoietic origin are derived from cancer stem cells (CSCs) or leukemic stem cells (LSCs) [4, 5].

Genetic abnormalities such as immunoglobulin gene rearrangement and chromosomal translocations detected by molecular analysis and molecular cytogenetics [such as fluorescent in situ hybridization (FISH)] and other techniques are increasingly utilized to determine cell clonality in B-cell neoplasia [6, 7]. However, genetic analysis may not be a perfect method to determine monoclonality or to determine malignancy. For instance, the chromosomal translocation t(14;18)(q32;q21), a hallmark for follicular lymphoma (FL), was detected in 75% of FL cases [8]. However, this translocation could be detected in up to 66% of healthy adults' peripheral blood with no evidence of FL when using a sensitive real-time PCR method [9].

Table 1. Summary of cell lines used

Name of cell line	Disease entity and cell line derived	Vendors
NALM-6 MN-60 SD-1	B lymphoblastic leukemia (B-ALL)	DSMZ (Braunschweig, Germany)
Jurkat	T lymphoblastic leukemia (T-ALL)	DSMZ
Mec-1 Mec-2 Wac-3	Chronic lymphocytic leukemia (CLL)	DSMZ
RL	Follicular lymphoma (FL) with t(14;18)	ATCC (Manassas, VA, USA)
Granta	Mantle cell lymphoma (MCL) with t(11;14)	ATCC
Daudi and Raji	Burkitt lymphoma (BL)	ATCC
DB	Diffuse large B-cell lymphoma (DLBCL)	DSMZ
RPMI 8226 NCI-H929 U266B1	Plasma cell myeloma (PCM)	ATCC
KG-1 KG-1a Kasumi	Acute myeloid leukemia (AML)	ATCC
KAS 6/1	PCM	Dr. Jelinek, Mayo Clinic, MN, USA

Thus, a novel, simple, easily accessible, and reliable method is needed in order to diagnose, monitor disease course, and detect minimal residual disease.

In the past decade, the role of epigenetic alterations in carcinogenesis, leukemogenesis, and lymphomagenesis has been increasingly recognized [10, 11]. The human epigenome consists of three inter-related elements: DNA cytosine methylation, histone covalent modifications, and RNA interference (RNAi). DNA cytosine methylation, the addition of a methyl group to the fifth carbon of cytosine within the context of CG dinucleotides, is the most important and well-characterized inheritable epigenetic mechanism [12]. Over 50% of human genes contain a CG rich stretch (CpG island or CGI) in their promoters or exon 1 regions. In normal conditions, these CGIs are free from methylation. In tumor cells, however, aberrant CGI hypermethylation is very common and the pattern of hypermethylation is not random but rather tumor type-specific [13]. Therefore, these tumor-specific hypermethylation loci can be utilized as biomarkers for detection of tumor [14-16]. During the past few years, our group has identified hun-

dreds of aberrant hypermethylated loci in acute lymphoblastic leukemia (ALL) and matures B-cell lymphoma using CpG island DNA microarray technology [17-20]. In this report, we describe a novel PCR-based method using 3 genes, *DLC-1, PCDHGA12,* and *RPIB9* selected from our top list of tumor-specific CGI methylation loci, to detect B-cell neoplasms. This epigenetic-based approach offers a new type of diagnostic modality that may be used in addition to traditional morphological, immunophenotyping and genetic approaches.

Materials and methods

Cell lines

Table 1 summarizes all cell lines used in the present study. These cell lines represent a spectrum of major types of B-cell neoplasms including acute lymphoblastic leukemia, mature B-cell neoplasms, and plasma cell myeloma. All cell lines were maintained in RPMI 1640 medium supplemented with 10% FCS and 100 µg/ml of penicillin/streptomycin at standard cell culture conditions. Cells in the exponential growth phase were harvested for DNA extraction or kept at -80º C until DNA extraction.

Table 2. Clinical profile and *DLC-1* methylation status in 31 B-ALL patients

| Patients	Gender/Age	Blast % in bone marrow	Karyotype	*DLC-1*
1	M/7	61	Complex	Pos
2	M/2	90	Complex	Pos
3	F/10	79	Complex	Pos
4	F/13	98	Complex	Pos
5	M/6	96	47, XY, +21	Pos
6	F/22	89	t(9;22)(q34;q11.2)	Neg
7	F/20	91	t(4;11), del(21)	Neg
8	M/3	96	Normal	Neg
9	M/7	50	N/A	Neg
10	F/4	77	del(X)	Pos
11	M/3	86	Normal	Neg
12	M/51	74	t(9;22)(q34;q11.2)	Neg
13	M/3	92	Hyperdiploidy	Pos
14	F/84	95	Normal	Pos
15	M/24	90	t(2;3), del (6)	Pos
16	M/23	70	N/A	Neg
17	M/43	70	Normal	Pos
18	M/49	90	Normal	Neg
19	M/42	90	Normal	Pos
20	M/2	60	N/A	Pos
21	F/23	84	N/A	Pos
22	F/11	90	Hyperdiploidy	Pos
23	M/33	80	N/A	Neg
24	M/20	50	N/A	Pos
25	M/26	90	del(Y)	Neg
26	F/15	64	Normal	Pos
27	M/62	70	Normal	Neg
28	M/8	87	Complex	Pos
29	F/3	95	Normal	Pos
30	M/6	94	Normal	Pos
31	F/6	94	Normal	Neg

Note: M:male; F: female; Pos: positive; Neg: negative. DNA methylation status of DLC-1 gene was determined by MSR-PCR in CGI region A.

Patient Samples and DNA Extraction

Bone marrow aspirates and peripheral blood samples were obtained from patients at initial diagnosis as well as at follow-up visits at the Children's Hospital and Ellis Fischel Cancer Center of University of Missouri Health Care (Columbia, MO), the University of California at Irvine Medical Center (Irvine, CA) and the University of Texas Southwestern Medical Center (Dallas, TX) in compliance with the local Institutional Review Board (IRB) requirements. The mononuclear cell fraction from bone marrow aspirates was isolated with Ficoll-Paque Plus medium (GE Healthcare Bio-Sciences Co., Piscataway, NJ) by gradient centrifugation and stored in liquid nitrogen until use. Peripheral blood samples in EDTA additive tubes were stored at -20ºC until use. Additionally, some bone marrow and blood smears from archived unstained slides were
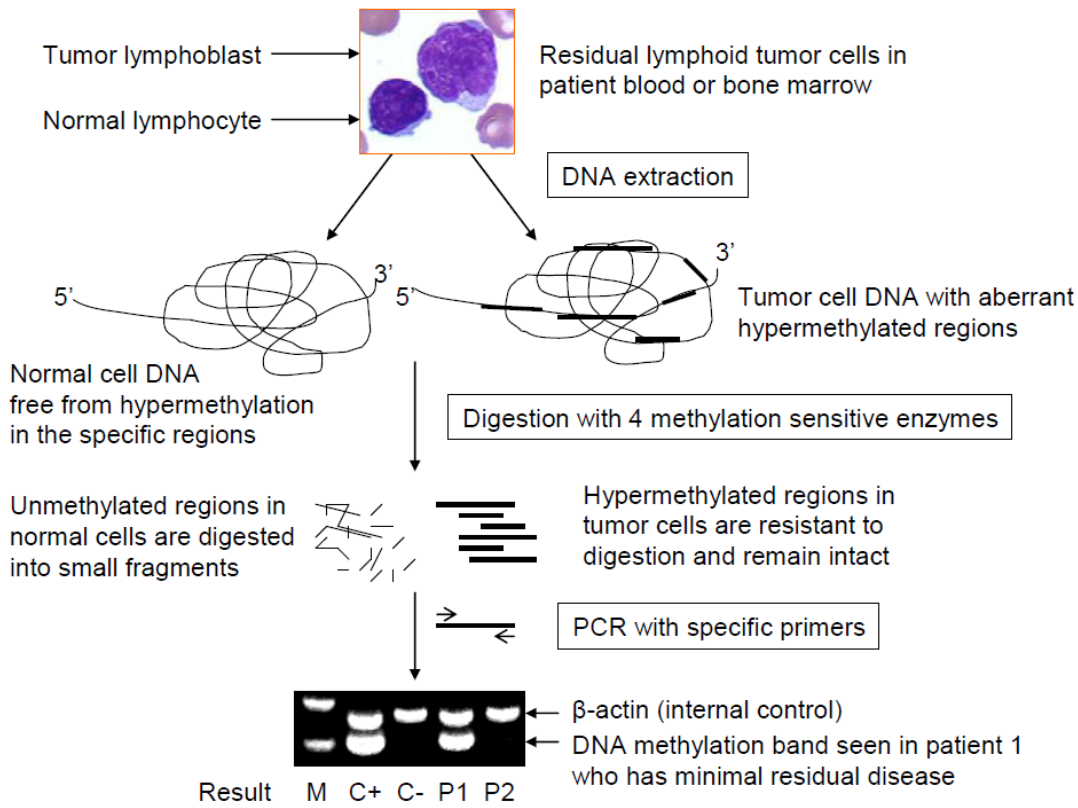
Figure 1. Design of MSR-PCR method. Genomic DNA extracted from patients' peripheral blood or bone marrow specimens is digested with four methylation sensitive enzymes. Specific hypermethylated regions in tumor cells are resistant to digestion, and are subsequently amplified by PCR. The same regions in normal blood or bone marrow cells are digested into small fragments and cannot be amplified. Thus, the PCR products (bands on the gel) represent the tumor cell population in the specimens. A restriction site-free region of the house-keeping gene β-actin is co-amplified as a PCR internal control. Multiple methylation sensitive enzymes and PCR target regions with maximal restriction sites are carefully selected to ensure complete digestion to prevent false positive result. M-molecular marker; C+, positive control with *M. SssI* methylase-treated normal human blood cell DNA or tumor cell line DNA ; C-, negative control with pooled normal human blood DNA; P1 and P2, patient samples with and without tumor cells.

scraped to retrieve cells. Genomic DNA was extracted from 18 cell lines and a total of 104 clinical specimens (60 bone marrows and 44 peripheral blood samples) from 60 B-ALL patients and 25 healthy volunteers or non-cancer patients. **Table 2** summarizes a series of 31 cases of bone marrow aspirates at initial diagnosis. Genomic DNA was isolated using the QIAamp DNA Blood mini kit (Qiagen, Valencia, CA). DNA concentration and purity were determined by a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Normal male and female genomic DNAs from pooled human peripheral blood were purchased from Promega (Madison, WI).

Multiple methylation sensitive enzyme restriction PCR (MSR-PCR), quantitative real-time methylation specific PCR (qMSP) and quantitative real-time MSR-PCR (qMSR-PCR)

MSR-PCR consists of three sequential steps: DNA extraction, DNA digestion and PCR (**Figure 1**). To prepare methylation-positive control DNA, genomic DNA from pooled normal human blood was treated with *M. SssI* DNA methyltransferase (New England Biolabs, Ipswich, MA), which methylates cytosine residues in all CG dinucleotides. In a typical digestion, the sample genomic DNA and *M. Sss I*-treated control DNA (250 ng) were incubated with 5U of methylation sensitive

Table 3. Primer and probe sequences

ID	Sequence	Orientation	Tm
DLC1-AF	5'-TAAAGAGCACAGAACAGGCACCGA-3'	Forward	60.4
DLC1-AR	5'-TGCTTGATGTGCAGAAAGAAGCCG -3'	Reverse	60.2
DLC1-BF	5'-TGTTAGGATCATGGTGTCCGGCTT-3'	Forward	60.2
DLC1-BR	5'-AGCGCTCCCTCGTTTCGATCTTTA -3'	Reverse	60.2
DLC1-FF	5'-AAATCCGGAGACTCTGCAGAAAGCG-3'	Forward	57.4
DLC1-WF	5'-GAAAGTGAACCAGGGCTTCC-3'	Forward	61.1
DLC1-WR	5'-TAAGGCCTGCGACCCAGA-3'	Reverse	62.9
PCDHGA12-AF	5'-ACTCACTTCTCCCTCATCGTGCAA-3'	Forward	60.1
PCDHGA12-AR	5'-ACCTCACTTCCGCATTGACTCCTT-3'	Reverse	60.3
RPIB9-F	5'-TCCAGGCTCCTTTCCTACATCCTT-3'	Forward	59.5
RPIB9-R	5'-GGAGGAACCTGATCACCGTGT-3'	Reverse	61.4
β-actin-AF	5'-GGCCGAGGACTTTGATTGCACATT-3'	Forward	60.2
β-actin-AR	5'-GGGCACGAAGGCTCATCATTCAAA-3'	Reverse	59.9
β-actin-BF	5'-GAGCTGGTGTCCAGGAAAAG-3'	Forward	59.8
β-actin-BR	5'-GCTGGAGGATTTAAGGCAGA-3'	Reverse	59.4
DLC1QF	5'- CCCAACGAAAAAACCCGACTAACG -3'	Forward	60.4
DLC1QR	5'-TTTAAAGATCGAAACGAGGGAGCG -3'	Reverse	60.2
MSP Q Probe	FAM/AAGTTCGTGAGTCGGCGTTTTTGA/BHQ1		60.8
TaqMan Probe	FAM/CCCTCGCGGTCCTCAACGCATCCTT/BHQ1		73.9

Note: ID, identification of sequences; Tm, annealing temperature of the primers and probes.

enzymes *AciI, HpaII*, and *HinP1I* (New England Biolabs, Ipswich, MA) in NEBuffer 4 in a final volume of 25 µl at 37°C for 16 hours. Then 10U of *BstUI* was added and digestion was continued for an additional 4 hours at 60°C. The enzymes were then inactivated at 65°C for 20 minutes and the digested DNA was stored at -20°C until use. In each digestion, normal human genomic DNA with and without enzymes were included as digestion controls. In a typical gel-based MSR-PCR, 40 ng of digested DNA, *DLC-1* (or *PCDHGA12* or *RPIB9)* primers (0.5 µM) and *β-actin* primers (0.25 µM) were mixed with Go-Taq Polymerase 2x green master mix (Promega, Madison, WI) in a final volume of 25 µl. The PCR was carried out in a PTC100 thermal cycler (MJ Research, Ramsey, MI) with a program of denaturing at 95°C for 30 seconds, annealing at 60°C for 60 seconds, and extension at 72°C for 60 seconds for 30 cycles with 2 minutes at 95°C for initial denaturation and 7 minutes at 72°C for final extension. Two sets of *β-actin* primers (either A or B) which amplify regions with no enzyme restriction sites in *β-actin* gene, were used as an internal control for the PCR. The PCR products were visualized on a 3% agarose gel containing SYBR Green 1 fluorescent dye after electrophoresis at 120 V for 30 minutes.

In the nested PCR, the digested DNA was first amplified with *DLC-1* primers FF/BR yielding a 383 base pair (bp) product. Then, an internal *DLC-1* primer set AF/AR (160 bp) was used to amplify an aliquot of the first PCR product in the second round of PCR. All PCR primer sequences, orientation, and annealing temperatures are listed in **Table 3**.

For qMSP, genomic DNA was treated with sodium bisulfite (EZ DNA methylation kit; Zymo Research, Orange, CA) and the real-time PCR was carried out in ABsolute QPCR mix (ABgene, Rochester, NY) in a SmartCycler System (Cepheid, Sunnyvale, CA) as previously described [17, 20]. The sequences of primers (DLC1Q) and probe (MSP Q Probe) are listed in **Table 3**. A positive result was defined when the ratio of *DLC-1* to *β-actin* signal is greater than 400. The results from MSR-PCR and qMSP were compared on the same DNA samples in **Figure 4A**.

For qMSR-PCR, the digested and undigested normal (digestion control) and digested B-ALL patient DNA samples were amplified at an iQ5 Real-time PCR detection system (BIO-RAD, Hercules, CA). In a typical qMSR-PCR, 20 ng of digested DNA, *DLC-1* B primers (0.25 μM), *DLC-1* TaqMan probe (0.125 μM) (IDT, Coralville, IA) were mixed with 2x iQ Supermix (BIO-RAD, Hercules, CA) in a final volume of 20 μl. The PCR program consists of 3 min of denaturation at 95°C followed by 40 or 45 cycles of 95°C for 15s and 60°C for 60s. To generate the standard curve, nearly whole CpG island of *DLC-1* gene was amplified using *DLC-1*w primers in GoTaq Polymerase 2x green master mix (Promega, Madison, WI). The PCR fragment was then purified with DNA Clean and Concentrator - 5 (Zymo Research, Orange, CA), quantified with NanoDrop 1000 spectrophotometer and used as template. The template was diluted from 10^8 copies to 1 copy per reaction at a dilution factor of 10. The standard curve was constructed with linear regression by build-in software of iQ5. For B-ALL patient bone marrow samples, 20 ng of digested DNA were amplified in triplicate under the same condition as well as negative and positive controls. The average copy number of each sample was calculated against the standard curve. Primer and probe sequences are listed in **Table 3**.

Results

Distinct DNA methylation patterns between leukemic cells and normal blood cells

First, we compared patterns of genomic DNA methylation of acute lymphoblastic leukemia cell lines with those of normal blood samples after digestion with methylation sensitive enzymes. As shown in **Figure 2A**, the overall DNA

methylation pattern differs between leukemia cell lines and normal blood cells. Comparing with a diffuse smear indicating much less methylation seen in normal male and female blood cell DNA (lanes 2 and 4), dense methylation in high molecular weight DNA fragments was clearly seen in all 4 leukemic cell lines (lanes 5-8). These densely methylated regions in leukemia cells might then serve as candidate biomarkers for further evaluation.

DCL-1, a candidate gene for methylation analysis

The genomic structure of the *DLC-1* CGI, an 824 bp DNA segment encompassing the promoter region, exon 1, and part of the first intron of the gene is shown in **Figure 2B**. As noted, regions A and B within the CGI were found to have many CG dinucleotides as well as multiple restriction enzyme recognition sites (10 sites in region A and 19 sites in region B), and therefore, were selected as candidate PCR targets for methylation analysis. The DNA digestion efficiency of these methylation sensitive enzymes was then examined in both regions. *DLC-1* methylation in regions A (upper panel) and region B (lower panel) of the CGI were shown in **Figure 2C**. Genomic DNA from normal blood samples (lanes 1, 2, 3, 5, 7, 9, 11) and B-ALL cell line NALM-6 (lanes 4, 6, 8, 10, 12) were digested with either a single enzyme or a combination, and then amplified with MSR-PCR. Methylation sensitive enzymes *HpaII* (lane 5) and *BstUI* (lane 9) gave complete digestion in both regions (no band seen) of normal blood cell DNA; *AciI* (lane 3) showed partial digestion (a faint band seen) in region A since only 50% digestion rate can be reached in NE-Buffer 4 for this enzyme, but complete digestion was achieved in region B since more *AciI* restriction sites exist in that region. *Hinp1I* showed no digestion in region A (lane 7 of upper panel), since there is no restriction site for *Hinp1I* in this region. The combination of four enzymes gave complete digestion in both regions (lanes 11 in both panels) of normal blood cell DNA samples. Except lanes 3 and 7 of the upper panel of region A, in no case did normal blood DNA show cleavable amplification, but NALM-6 DNA, cut by either a single enzyme or the combined enzymes (lanes 4, 6, 8, 10, 12), was amplified. The result of differential amplification in leukemia cells, but not in normal blood cells, was encouraging, which then led us
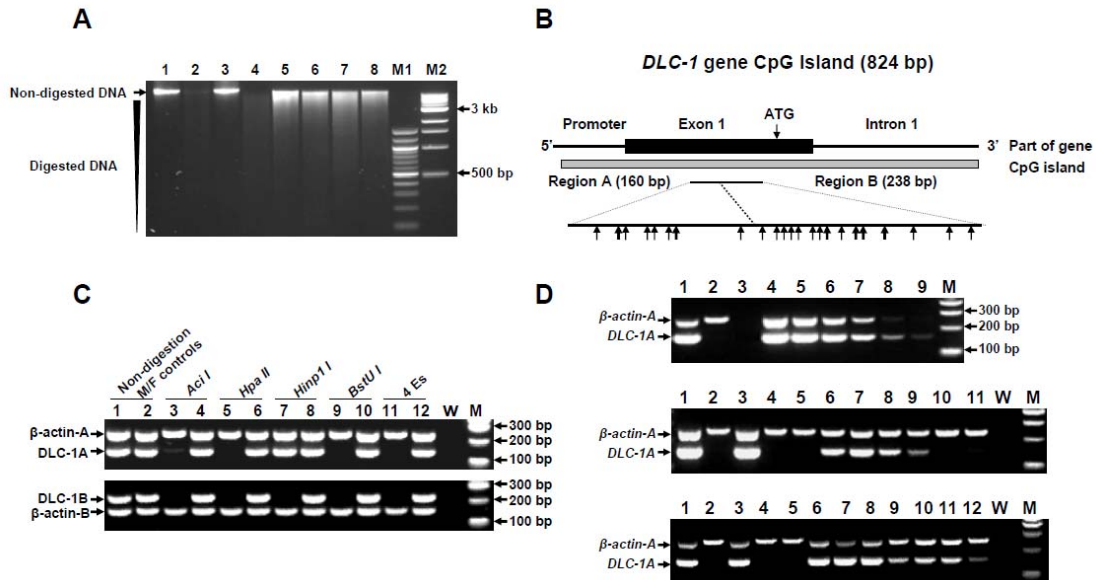
Figure 2. Establishment of MSR-PCR method. (A) Different DNA methylome (genome-wide methylation pattern) between normal blood and leukemic cells. Genomic DNA from normal (lanes 1-4) and ALL cell lines (lanes 5-9) give rise to different methylation patters when digested with 4 methylation sensitive enzymes with *AciI, HpaII, HinP1I,* and *BstUI* except lanes 1 and 3, in which no enzymes were added. Lane 1-2: normal male; Lanes 3-4: normal female; Lanes 5-8: four ALL cell lines (lane 5, NALM-6; lane 6, MN-60; lane 7, SD-1; and lane 8, Jurkat). 100 ng of digested DNA was separated by electrophoresis at 120 V for 60 min in 1% agarose gel and visualized with the florescent dye SYBR Green 1. The 100 bp (lane M1) and 1 kb (lane M2) DNA ladders were included. (B) *DLC-1* CpG island and the restriction map of PCR target regions. The island consists of an 824 bp at chromosome 8p21.3-22 (chr 8:13034462-13035285). Central regions A (160 bp) and B (238 bp) (black bar below the CpG island, restriction sites are indicated with arrows on the expanded line) with dense CG dinucleotides and multiple restriction sites were selected for PCR amplification. (C) Efficiency of DNA digestion by methylation sensitive enzymes. 250 ng of normal DNA from human blood (lanes 3, 5, 7, 9, 11) and B-ALL cell line NALM-6 (lanes 4, 6, 8, 10, 12) were digested with either a single enzyme or a combination (labeled above the lines). Lanes 1 and 2 are controls from normal male and female DNA digestion with no enzymes. W-PCR water control, M-100 bp DNA ladder. (D) Analytic sensitivity of MSR-PCR. Upper panel shows absolute sensitivity. After digestion with 4 enzymes, 80 ng of DNA from NALM-6 cell line was diluted in a 5x series starting from lane 4 and the targets of *DLC-1*A and β-actin-A were amplified with MSR-PCR. Lanes 1-2 were normal DNA without and with enzymes, respectively; Lane 3-water control. Middle panel shows relative sensitivity. A 10x serial dilution of DNA from NALM-6 was mixed with normal DNA from human blood to make a total of 250 ng DNA (lanes 7-11). Lanes 1-4 were DNA from normal male (lanes 1-2) and female (lanes 3-4) without enzymes (lanes 1 and 3) and with enzymes (lanes 2 and 4), respectively. Lane 5 contained 250 ng of normal DNA only. Lane 6 contained 250 ng of NALM-6 DNA only. The lower panel shows results from nested PCR. After amplification of a 10x dilution series of NALM-6 DNA with FF and BR primer pair in the 1st PCR, aliquots of PCR products (383 bp) were reamplified with an internal AF and AR primer pair in the 2nd PCR. Lanes 1-5, W and M were as same as described in middle panel. All experiments in Figure 2 were performed at least three times with the same results; a representative gel image is shown.

to examine the potential sensitivity of this assay.

Sensitivity of MSR-PCR

Analytic sensitivity can be divided into absolute and relative sensitivity [21]. Absolute sensitivity refers to the capability of detecting a minimal quantity of methylated target DNA in tumor cells. Relative sensitivity refers to the capability of detecting the smallest fraction of methylated tumor cell DNA in the presence of an excess amount of unmethylated normal cell DNA. The analytic sensitivity of MSR-PCR is shown in **Figure 2D**. The upper panel demonstrates the absolute sensitivity using 80 ng of NALM-6 DNA that was digested with the combination of 4 enzymes and subsequently diluted 5-fold in a
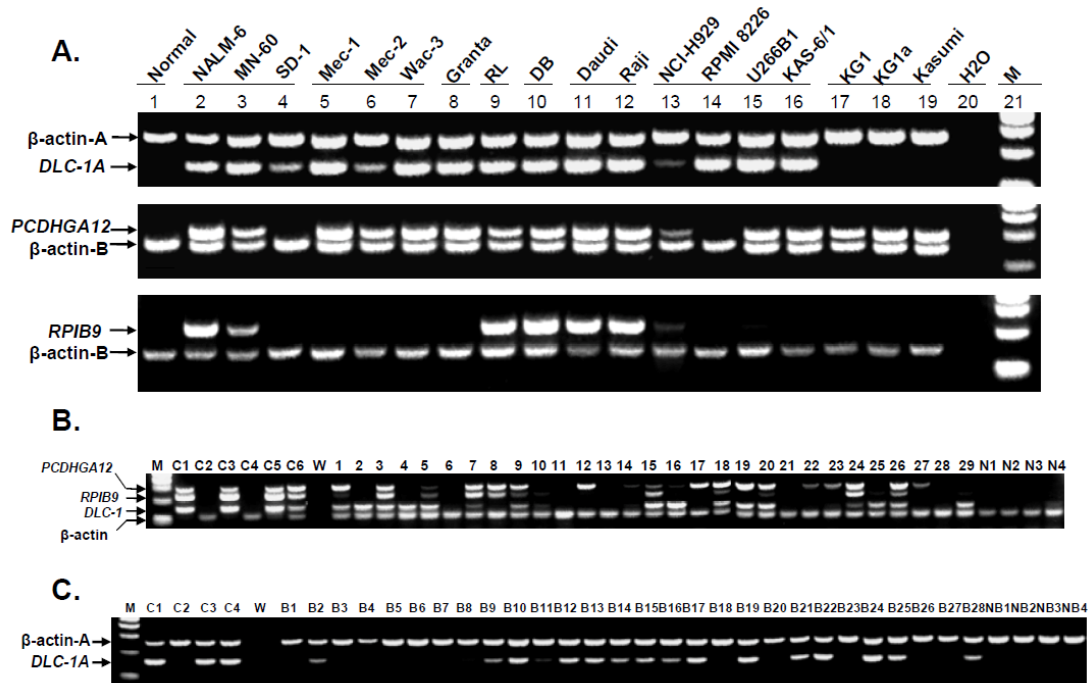
Figure 3. Validation of DNA methylation markers in B-cell tumor cell lines and B-ALL patient samples with enhanced sensitivity. (A) Cell lines. Genomic DNAs from normal blood (lane 1), 15 B-cell lymphoid tumor (lanes 2-16) and 3 AML (lanes 17-19) cell lines were subjected to MSR-PCR. The B-cell lymphoid cell lines are derived from B-ALL (lanes 2-4), CLL (lanes 5-7), MCL (lane 8), FL (lane 9), DLBCL (lane 10), BL (lanes 11-12), and PCM (lanes 13-16) (**Table 1**). The AML cell lines (lane 17-19) were used as controls. *DLC-1A* methylation (160 bp) and internal control β-actin-A (257 bp) are shown in upper panel. Methylation of *PCDHGA12* (310 bp) and RPIB9 (204 bp) are shown in middle and lower panels, respectively. (B) Triple markers of DNA methylation were assessed with a multiplex MSR-PCR in 29 B-ALL diagnostic bone marrow aspirates. Lane M: 100 bp DNA ladder; Lanes C1-C4: normal male (lanes 1 and 2) and female (lanes 3 and 4) blood DNA without (lanes 1 and 3) and with digestion (lanes 2 and 4); Lanes C5 and C6, positive controls using DNA from NALM-6 and *M. Sss I*-treated DNA; lane W: water; lanes 1-29: B-ALL patient samples; lanes N1-N4: normal individual bone marrow samples. Corresponding DNA methylation bands of 3 markers and internal control β-actin-A are denoted with arrows on the left side of the gel. (C) Peripheral blood samples from a cohort of 28 B-ALL patients at initial diagnosis (lanes B1-B28) and 4 normal individuals (lanes NB1-NB4) were subjected to MSR-PCR. Lane C1 and C2: normal human DNA without and with enzymes; lane C3 and C4: digested NALM-6 DNA and *M. Sss I* -treated DNA as positive controls; lane C5: water control.

series starting from lane 4. The density of the *DLC-1* methylation bands (160 bp) and *β-actin*-A (257 bp) bands decreased proportionately with each dilution. A weak *DLC-1* methylation band was observed at 0.0256 ng of genomic DNA, equivalent to ~5 leukemic cells (lane 9), and stronger bands at higher concentrations (lanes 4-8). Lanes 1 and 2 contain normal blood DNA with and without enzymes as digestion controls, and lane 3 contains water, instead of the DNA template, as PCR contamination control. The middle panel illustrates the relative sensitivity to detect tumor DNA at various levels mixed with normal DNA. A 10-fold serial dilution of NALM-6 DNA starting from lane 6 (250 ng NALM-6 DNA

only) was mixed with normal blood DNA to make a total of 250 ng DNA (lanes 7-11). After digestion, 40 ng of the DNA mixture was amplified with MSR-PCR. A faint *DLC-1* methylation band was seen with 0.25 ng of NALM-6 in 250 ng of normal DNA (lane 9) giving a relative sensitivity of 10^{-3} or 1 tumor allele in 1,000 normal cell alleles. The internal control *β-actin*-A band showed similar density in all lanes as expected since this gene is present in both tumor and normal cells. While this result was promising, we needed even higher sensitivity for an effective assay to identify residual leukemic cells in clinical samples. The relative sensitivity using a nested PCR was improved to 10^{-6}, or 1 tumor

cell allele in 1,000,000 normal cell alleles (lane 12 of lower panel). The density of *DLC-1* bands was slightly decreased while that of *β-actin* bands was increased with dilution indicating a competitive effect in multiplex PCR.

Validation of MSR-PCR on B-cell neoplastic cell lines and B-ALL patients

After having established a sensitive detection method using a B-ALL cell line, we proceeded to test a total of 18 leukemia/lymphoma cell lines (**Table 1**) and B-ALL patient samples with two additional markers, *PCDHGA12* and *RPIB9* (**Figure 3**). *DLC-1* methylation bands were visible in all 15 B-cell tumor cell lines (lanes 2-16), although there were weaker bands (lanes 4, 6 and 13) seen in SD-1 (B-ALL), Mec-2 (CLL) and NCI-H929 (PCM) cell lines. Methylation was not seen in the normal blood cell control (lane 1) and all 3 AML cell lines KG1, KG1a and Kasumi (lanes 17-19) (**Figure 3A**, upper panel). There was a similar methylation pattern for *PCDHGA12* in B-cell tumor cell lines, except for SD-1 (B-ALL, lane 4) and RPMI 8226 (PCM, lane 14) (**Figure 3A**, middle panel). In addition, *PCDHGA12* methylation was visible in all three AML cell lines (lanes 17-19). The CGI methylation pattern of *RPIB9* was very different from the other 2 genes (**Figure 3A**, lower panel). Methylation was seen only in 2 B-ALL (lanes 2 and 3) and 4 mature B-cell lymphoma cell lines that are all germinal center-derived tumors (FL, DLBCL, and BL, lanes 9-12). A very weak band was also seen in a PCM cell line (lane 13).

Subsequently, clinical bone marrow aspirates from 31 B-ALL patients at initial diagnosis were examined with MSR-PCR for *DLC-1* methylation. The methylation was detected in 61% (19/31) of B-ALL patients (**Table 2**, data not shown). CGI methylation of *DLC-1, PCDHGA12* and *RPIB9* was then examined in an additional 29 B-ALL bone marrow aspirates with a multiplex MSR-PCR showing a positive rate of 55% (16/29), 62% (18/29), and 31% (9/29), respectively. Taking three genes together, methylation was detected at least in one gene in 83% (24/29) of this series (**Figure 3B**, lanes 1-29), demonstrating this method is capable of detecting tumor cells in the vast majority of the B-ALL cases. Methylation was not detected in either 4 normal bone marrow controls (lanes N1-N4) or pooled normal male and female blood DNA (lanes C2 and C4). The digestion controls (C1-C4), positive controls (C5-C6) and water PCR control (W) showed expected patterns.

Next, we further examined if the method could detect leukemia cells in peripheral blood samples of B-ALL patients. *DLC-1* methylation was detected in 54% (15/28) of the cases (lanes B1-B28), but neither in 4 normal blood samples (lanes NB1-NB4) nor in pooled normal blood DNA (lane C2) (**Figure 3C**). *DLC-1* methylation was not detected in additional normal or non-cancer patient bone marrow (n=8) and blood (n=5) samples (Data not shown). Due to samples being collected from different locations at different times, most bone marrow aspirates and blood samples were not from the same patients. However, same *DLC-1* DNA methylation pattern was seen when both bone marrow and blood samples were collected from the same patients at the same time (n=12, also in **Figure 4**).

In order to develop a more sensitive and quantitative real-time PCR method (qMSR-PCR), a 763 bp fragment encompassing nearly whole region of CpG island of *DLC-1* gene was amplified by PCR using *DLC-1*w primers. The standard curve showed an adequate linearity from 10 to 10^8 copies per reaction (**Figure 5A**). Non-template control (water) or the dilution of 1 copy per reaction was not amplified even at 45th cycles. *DLC-1* DNA methylation in 40 digested DNA samples of B-ALL patient bone marrows was then determined under the same conditions. When the cut-off value was set in 10 copies per reaction, 21 of 40 (52.5%) samples were positive (**Figure 5B**) which is consistent with gel-based MSR-PCR method (**Table 2** and **Figure 3B**). The copy numbers in methylation positive patient samples calculated according to the standard curve were ranged from 20 to 39,849 copies with average of 4,592 copies per reaction.

Potential use of MSR-PCR as a tool in monitoring B-ALL patients

Our next goal is to decide whether this method could be potentially used to monitor the clinical course of B-ALL patients in both bone marrow and blood samples from the same patients. Bone marrow aspirates and peripheral blood samples including scraped cells from archived unstained slides (Ms) collected at different time points from 4 B-ALL patients were used. The MSR-PCR gel image along with the corresponding qMSP results is shown (**Figure 4A**). A chronologic clinical course of these 4 B-ALL patients is also shown (**Figure 4B**). In all cases, clinical
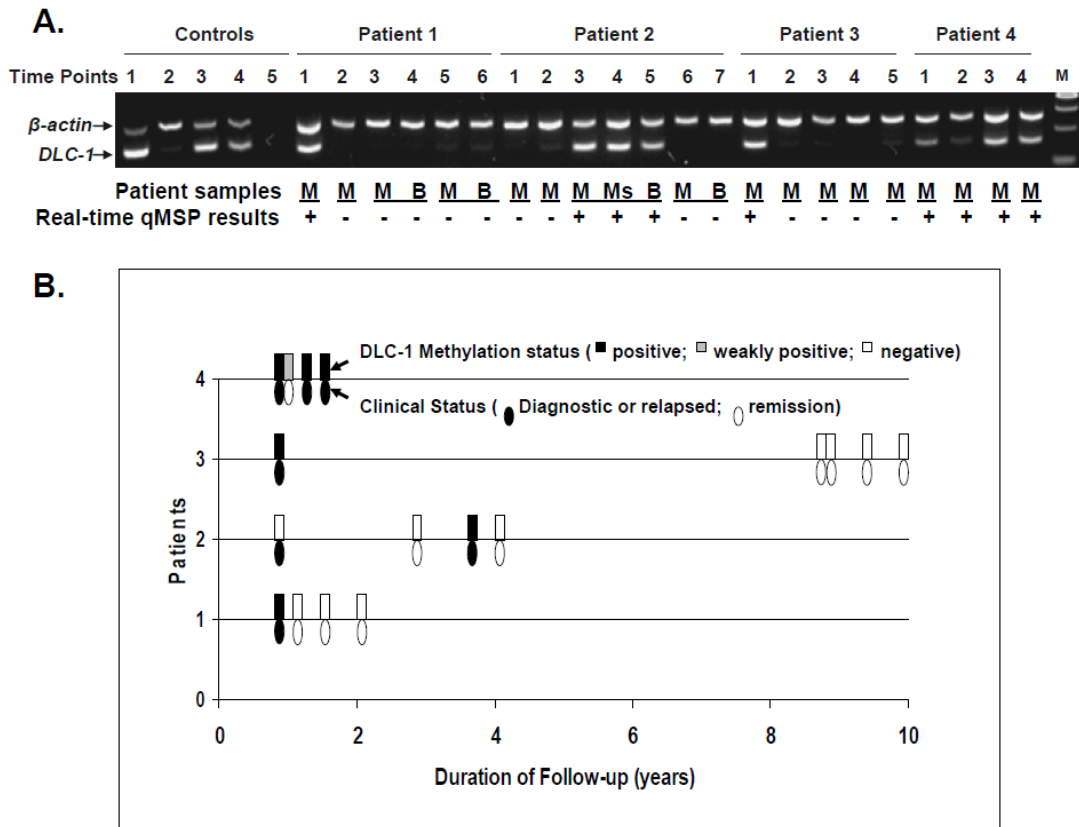
Figure 4. Correlation of *DLC-1* methylation with clinical status in 4 B-ALL patients. (A) DNA from bone marrow and/or blood samples collected at multiple time points from the same patient are subjected to MSR-PCR. Controls (lanes 1-4) were normal male blood cell DNA without and with digestion, NALM-6 cell line and *M.SssI*-treated DNA, respectively. Lane 5 was PCR water control. In patient samples, M denotes bone marrow; Ms, bone marrow slide; B, blood; Underlined M and B indicate that the bone marrow and blood samples were collected from the same patient at the same time. (B) Correlation of *DLC-1* methylation and clinical status during the period of patient follow-up (Y axis, patients; X axis, time course). Rectangles above the lines denote DLC-1 DNA methylation status; Ovals below the linen denote clinical status. Solid color indicates DNA methylation positive or patient was at diagnosis or relapsed; Empty shape indicates DNA methylation negative or patient was in remission. The positions of rectangle/oval indicate the time points of sample collection at diagnosis (the first one) and during follow-up visits.

remission or relapse was determined by a combination of bone marrow pathological examination, flow cytometry and clinical information. *DLC-1* methylation as detected by MSR-PCR and by qMSP [20] on the same samples was completely concordant (**Figure 4A**). The correlation between *DLC-1* methylation (rectangle, above lines) and clinical status (oval, below lines) of all 4 patients was observed (**Figure 4B**). As a general trend, *DLC-1* methylation was positive in diagnostic and relapsed specimens, but clearly negative in specimens when patients were in remission. Interestingly, in patient 2, *DLC-1* methylation was negative at initial diagnosis, but

became positive at relapse after 3.2 years, and then became negative in remission after chemotherapy. In patient 4, a weak methylation band (lane 2 of **Figure 4A**) was visible despite the patient was declared a morphologic and immunophenotypic remission. Subsequently, this patient relapsed in 6 months (lanes 3 and 4). The longest follow-up time period was 10 years (patient 3). In all cases, DNA methylation status in both bone marrow and blood samples was concordant at the same time point, indicating the possible utility of using blood samples, a less invasive procedure to monitor ALL patients rather than obtaining bone marrow aspirate or

biopsy.

Discussion

We describe in this report the development and validation of a novel method, namely, MSR-PCR, for detecting B-cell lineage neoplasia of immature and mature types. Although the sample is relatively limited, this proof of concept study demonstrates the simplicity and reliability of this epigenetics-based method in detecting B-cell neoplasms. Hence, the method described here might provide an additional diagnostic tool to identify malignant B-cells in the clinical setting.

Determining whether or not patients with acute leukemia are in true remission is sometimes problematic [22]. Therefore, it is of paramount importance to identify and detect MRD, defined as presence of submicroscopic leukemia cells that can be detected using more sensitive methods [23]. The presence of MRD in B-ALL patients is strongly associated with relapse [24]. The clinical significance of MRD in mature B-cell neoplasms has begun to be recognized [25]. MRD can be determined with several laboratory methods including molecular genetic diagnostic tests [26] and multiparameter flow cytometry [27]. Epigenetic DNA methylation methods may add additional laboratory modalities for MRD detection in B-cell malignancies.

While several methods have been developed for DNA methylation analysis, only a few have been successfully used in cancer detection. These DNA methylation methods can be divided into two categories: global genomic profiling and gene-specific detection. Among them, a cornerstone procedure, sodium bisulfite treatment of DNA that converts unmethylated cytosine to uridine, while leaving methylated cytosine unchanged, is the first step in many assays. Methylation-specific PCR [28], qMSP [29], and combined bisulfite restriction analysis (COBRA) [30] all require bisulfite treatment of DNA. While bisulfite sequencing remains the gold standard to demonstrate methylation levels and patterns with a single-base resolution [31], the bisulfite treatment degrades up to 95% of input DNA and generated 4 different strands [16, 32], thus dramatically reducing sensitivity and increasing the complexity for PCR amplification. To circumvent disadvantages of the aforementioned methods [28-31], we developed a method,

namely, multiple methylation sensitive enzyme restriction PCR (MSR-PCR), to detect DNA methylation without bisulfite treatment in clinical setting (**Figure 1**). Methylation sensitive enzymes are a group of DNA restriction endonucleases that cleave DNA at their recognition sites only when the cytosine of CG is not methylated. The enzymes do not cut the sites containing methylated CG dinucleotides. Although this feature has been utilized to study DNA methylation in developmental biology and in high throughput DNA methylation profiling [33, 34], a specific method for tumor cell detection in the clinical setting has not been established. Using multiple methylation sensitive enzymes in this method, unmethylated DNA of normal cells in patient specimens is digested into small fragments; whereas methylated DNA in tumor cells is resistant to digestion and remains intact. These tumor-specific densely hypermethylated regions, often present in CGIs, are differentially amplified by PCR (**Figure 1**). In contrast to scattered methylation patter in normal cells, the density of aberrant CGI methylation of selected functional genes including tumor suppressor genes in tumor cells is very high (10-19), the PCR target region cannot be cleaved even by a combination of restriction enzymes. To achieve the high specificity, the PCR target regions are carefully selected to contain as many cut sites as possible to ensure complete digestion to avoid false positive results (**Figure 2B** and **Figure 2C**). As a result, many cuts by multiple restriction enzymes in the target regions in normal DNA produce no amplifiable small DNA fragments (**Figure 1** and **Figure 2A**). Compared with other DNA methylation detection methods and other molecular tests, this method possessed several advantages. First, the method is simple and the whole procedure consists of three sequential steps: DNA isolation, digestion and a conventional multiplex PCR (**Figure 1**). Secondly, the method can be used with a variety of clinical samples including bone marrow aspirate, whole blood, buffy coat, mononuclear cells, plasma or serum, unstained slides, tissue biopsies, or paraffin blocks (data not shown). Thirdly, aberrant CGI methylation is a common phenomenon in cancers, especially in hematopoietic tumors [10, 11]. A few markers (3 in this study) can detect the majority of B-cell neoplasms by MSR-PCR (**Figure 3**). Thus, the method can potentially be used for a wide range of clinical applications in diagnosis and detection of residual leukemia/lymphoma tumor cells. Fourthly, the analytic sensitivity is
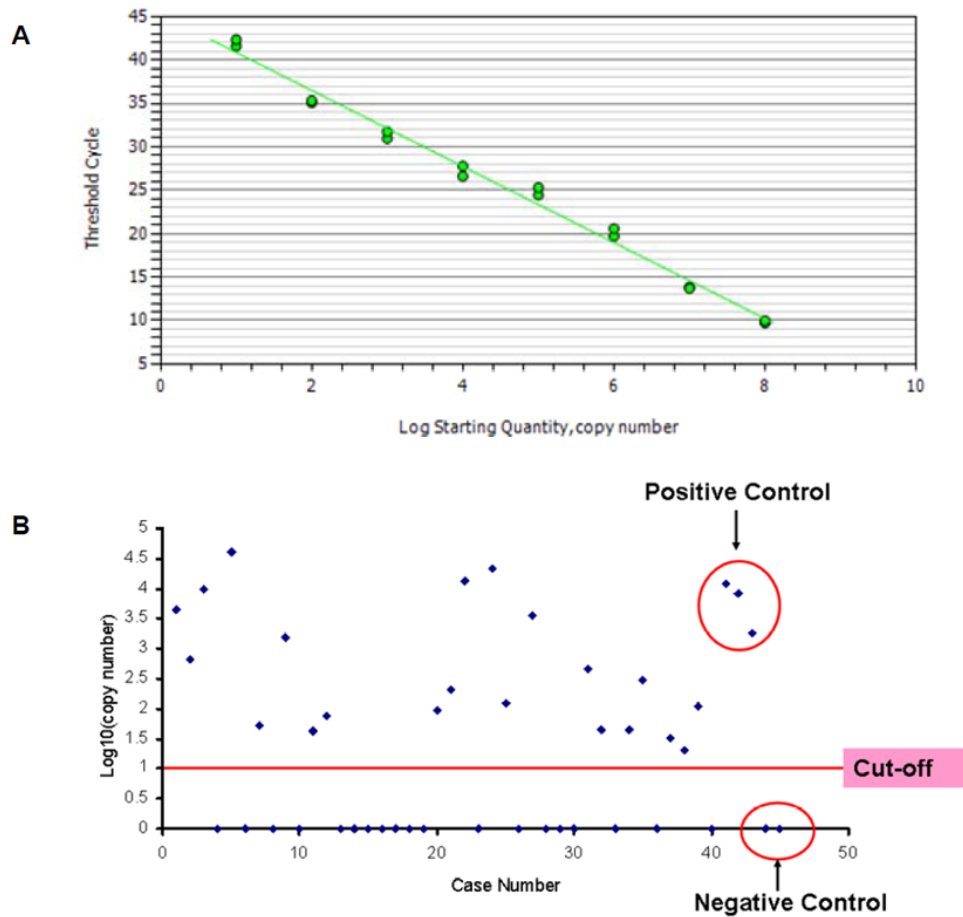
Figure 5. Development of qMSR-PCR method. (A) The standard curve of *DLC-1*, the linearity ranged from 10 to 10^8 copies per reaction with a R^2 value of 0.994 was obtained. (B) The distribution of the copy number of methylated *DLC-1* CpG island DNA in 40 B-ALL bone marrow samples. Positive controls included digested *M. Sss I*-treated normal male human DNA and NALM-6 cell line DNA, and non-digested normal male DNA; Negative controls included digested normal male and female human DNA. The copy number was calculated with the average of triplicate samples.

high since native genomic DNA, instead of bisulfite-treated DNA, is used as the input DNA. This method can detect as few as 5 leukemic cells in a single-step gel-based PCR (**Figure 2D**, upper panel). Depending upon needs in different clinical settings, this method can be modified to have two relative analytic sensitivity levels, 10^{-3} in a single-step PCR, and 10^{-6} in a nested PCR (**Figure 2D**, middle and lower panels), or a quantitative real-time PCR (**Figure 5**). The result was verified independently by a bisulfite-based qMSP method in B-ALL patient specimens (**Figure 4A**) [20]. Fifthly, the method can be performed as a multiplex PCR to detect methylation in multiple genes in a single tube (**Figure 3B**).

Thus the clinical sensitivity was increased to over 80% in B-ALL (**Figure 3B**), and potentially more by adding markers. With a single marker of *DLC-1* gene, the B-ALL patients can be followed in a long period of time and in peripheral blood samples (**Figure 4**). Finally, a *DLC-1* TaqMan probe-based real-time PCR (qMSR-PCR) has been developed to quantitatively determine leukemia cells in patient bone marrow specimen with a sensitivity of 10 copies per reaction which has opened a possibility for MRD detection (**Figure 5**). Agrawal et al. also used DNA methylation as biomarkers to detect MRD in AML and ALL, and found that increased methylation levels in p^{15} and *ERα* genes were closely

related to the risk of relapse demonstrating the clinical utility of DNA methylation as biomarker [35].

Like genetic abnormalities in cancer, not all leukemia/lymphoma patients carry the same epigenetic markers. It is critical to select markers that contribute to tumorigenesis, not just biological "noise" at the genetic and epigenetic levels. In this regard, we selected three DNA methylation markers that all play important roles in leukemogenesis and lymphomagenesis (17-20). Interestingly, DNA methylation of these three genes demonstrates different specificity in B-cell neoplasms (**Figure 3A**). The methylation of *DLC-1* and *PCDHGA12* was found in almost all B-cell lymphoid tumor cell lines as well as in most B-ALL patient samples, while *RPIB9* methylation appears to be only in precursor and germinal center-derived B-cell neoplasms (**Figure 3A** and **3B**). The *DLC-1* gene encodes a GTPase-activating protein that acts as a negative regulator of Rho signaling [36]. In cancer cells, *DLC-1* functions as a *bona fide* tumor suppressor gene to suppress tumor growth and metastasis [36]. CGI methylation of *DLC-1* results in the loss of its expression in many solid tumors and in B-cell neoplasms, thus it can be an invaluable cancer cell biomarker [17, 18, 20]. *RPIB9,* or Rap2 interacting protein 9, is another GTPase acting protein that regulates the activity of Rap2, a Ras-like GTPase protein [37]. In turn, Rap2 functions as an antagonist to Ras signaling pathways that stimulate cell proliferation [38]. *PCDHGA12* encodes a cell surface adhesion protein that plays important roles in cell-cell and cell-matrix interaction and tumor metastasis [39]. Methylation of *PCDHGA12* was demonstrated in both lymphoid and myeloid cell lines (**Figure 3A**), AML patient bone marrow aspirates, 5 major solid tumor cell lines and the patient samples (data not shown), indicating *PCDHGA12* is a potential "universal" tumor marker. Functionally, *DLC-1, RPIB9* and *PCDHGA12* proteins are linked in their roles by the Ras signaling pathways and cell adhesion. Loss of expression of these functional proteins by CGI methylation may be associated with the increase of tumor cell proliferation and tumor dissemination [36-39]. Finally, we noted that five pediatric B-ALL patients with a complex karyotype, an unfavorable prognostic indicator, all have *DLC-1* methylation (**Table 2**). Transcriptional inactivation of tumor suppressor genes including *DLC-1* by

CGI methylation may be significant in leukemogenesis and lymphomagenesis and may also serve as an independent prognostic factor [40].

In conclusion, we have developed a simple gel-based method as well as a real-time PCR format using selected specific DNA methylation markers to detect B-cell leukemia and lymphoma. This method was validated by an independent qMSP assay and in clinical B-ALL patient specimens. The high sensitivity and specificity as well as the simplicity and robustness of this method should allow broad clinical applications for residual tumor cell detection.

Acknowledgements

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