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Association of Clinical Status of Follicular Lymphoma Patients after Autologous Stem Cell Transplant and Quantitative Assessment of Lymphoma in Blood and Bone Marrow as Measured by SYBR Green I Polymerase Chain Reaction

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Molecular remission in the autograft and bone marrow after transplant are predictive of durable clinical remission in relapsed follicular lymphoma. Thus, a simple reliable method to quantify minimal residual disease (MRD) would improve prognostication in these patients. Fluorescent hybridization probes have been used in real-time quantitative polymerase chain reaction (RQ-PCR) to monitor MRD with a reproducible sensitivity of 0.01%; however, these techniques are expensive and require additional experiments to examine clonality. We describe a SYBR Green I detection method that is more universal, checks clonal identity, yields the same sensitivity for monitoring MRD, and is more economically attractive. Using this method to follow 14 follicular lymphoma patients treated with autologous stem cell transplantation, molecular markers were successfully defined for 12 patients. Median contamination of stem-cell grafts was 0.1% (range, 0 to 13%). Six patients with measurable graft contamination became PCR-negative in blood and bone marrow within 12 months after autologous stem cell transplantation. Three patients free of disease progression (median follow-up of 75 months) are in molecular remission. Increasing fractions of RQ-PCR-positive blood and bone marrow cells reliably predicted morphological and clinical relapse. In one case, both clinical relapse and spontaneous regression were reflected by changes in MRD levels. Thus, our RQ-PCR method reproducibly distinguishes different levels of MRD. ***(J Mol Diagn 2006, 8:40–50; DOI: 10.2353/jmoldx.2006.050050)***

Minimal residual disease (MRD) describes the presence of cancer cells, below the level of detection by standard light microscopy, which is ~1 in 100 cells. Using polymerase chain reaction (PCR) to assess MRD, the DNA molecular marker from one tumor cell can be detected in a background of DNA of up to 1 million normal nucleated cells. Sensitive PCR testing has been used to examine persistence or recurrence of disease in evaluating new treatment modalities and clearance of autologous stem cell products. Technologies to examine MRD are still evolving, and there is a lack of standard methodology in the tests used in clinical trials. Variability in the sensitivity and precision of the PCR assays can affect the frequency of false-positives and false-negatives and confuse clinical correlations. A quantitative PCR method that is economical and simple to implement with a variety of PCR targets is desirable to replace widely used conventional qualitative PCR.

There are currently two genetic features of follicular lymphoma (FL) cells that provide suitable targets for PCR monitoring of residual disease. The first is the t(14;18)(q32;q21) or *BCL2/JH* translocation characteristic of FL,[1] detectable in nearly all cases by fluorescent *in situ* hybridization.[2] However, the chromosomal breakpoints can occur over a 20,000-bp range. PCR reactions are best suited to amplify small 100- to 500-bp fragments; thus primers that target each breakpoint cluster region should be used to increase the number of patients with PCR-detectable translocations. Sixty percent of *BCL2* breakpoints are located at the major breakpoint region (MBR), 5 to 25% at the minor cluster region (mcr) 20,000 bp downstream of MBR, and other breakpoints have been found in clusters between MBR and mcr.[3–5] Although the *BCL2/JH* fusion sequence is characteristic of FL, many reports have shown that it is also present in low frequency [~0.1 to 100 per 1 million cells in peripheral blood (PB)] in normal individuals.[6] The method used to detect

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BCL2/JH should distinguish a false-positive result. A second and alternative PCR target for FL is the immunoglobulin heavy chain (IgH) gene rearrangement that is unique to the B-cell clone. In the subset of patients without PCR-detectable *BCL2/JH* translocation, tumor clones can be identified by PCR amplification of the uniquely rearranged variable-diversity-joining (VDJ) junction in the *IgH* gene using consensus VH and JH primers.[7] The unique VDJ product can then be sequenced to identify rearrangements by use of allele-specific oligonucleotide (ASO) PCR primers.

Although qualitative analysis of *BCL2/JH* with sensitive nested PCR[8] has been widely used, newer methods of quantification of MRD can be more informative. Several real-time quantitative PCR technologies are available and have recently been reviewed.[9] Fundamentally, fluorescent probes or PCR products are used to track the minimum number of PCR cycles required to generate measurable threshold amounts of PCR product as the reaction proceeds (real-time). The number of cycles to reach threshold is inversely related to the number of target templates in a sample.

Fluorescently labeled probes generate signal through hybridization of target PCR products and subsequent interaction with *Taq* polymerase. In these methodologies, each PCR amplification primer set has an associated specific probe oligonucleotide, which adds additional challenge to the design of the PCR reaction. Labeled probes are expensive to synthesize, so to be able to do quantitative assessments of a range of different breakpoint regions, a number of costly specific probes are required. Additionally, product analysis after PCR on agarose gels, capillary electrophoresis,[10] or by DNA sequencing is required to examine clonality. Methods have been developed for monitoring *BCL2/JH* with dual-labeled TaqMan (Applied Biosystems, Foster City, CA) hydrolysis probes for the *BCL2* gene.[10–14] Clinical studies incorporating these *BCL2* probes[14,15] limited analysis to patients with MBR breakpoints, thus possibly excluding results from up to 40% of study patients. A diagnostic kit that utilizes labeled hybridization probes to detect *BCL2/JH* translocations is available, but it is restricted for use on MBR region breakpoints (Roche Applied Science (Mannheim, Germany) catalog no. 3062651). A method using probes for the *JH* gene[16] required sequencing each patient *BCL2/JH* PCR product to determine the correct probe to use for quantitative PCR analysis. Although RQ-PCR using IgH-ASO strategies with consensus *VH* probes have been used for acute lymphoblastic leukemia patients,[17] due to the frequency of *VH* region somatic mutations in FL it has been more challenging to develop consensus probes to detect ASO-*IgH* in FL patients.[18] It is both expensive and time consuming to use patient-specific probes in a clinical study involving large numbers of patients.

Alternatively, the nonspecific fluorescent intercalating dye SYBR Green I can be used. SYBR Green I becomes excited when it binds to the double-stranded DNA amplicon, and the recorded fluorescence is a direct measurement of PCR product quantity. Additionally, the melting temperature of the fluorescent amplicon can be used to examine clonality in the same run. SYBR Green I is less costly because no labeled sequence-specific probes are required. A wide range of existing PCR reactions can readily be adapted to the SYBR Green I assay and monitored with the same reagents. Real-time PCR instruments with single color detection are generally less expensive than multicolor detection systems. SYBR Green I has been used to detect t(14;18) MBR translocations,[19,20] but these studies did not examine the value of these assays in long-term follow-up serial monitoring. In small trials, it can be essential to obtain molecular data on as many patients as possible in an economical way. In this study, we have implemented a SYBR Green I approach to detect t(14;18) with four different sets of primers and patient-specific clonal IgH enabling us to monitor the disease status of most of our patients. We compared previously generated qualitative nested PCR data to RQ-PCR for monitoring MRD in a clinical study of FL patients receiving autologous stem cell transplantation (ASCT) followed by maintenance treatment with interferon-α.

Table 1. Patient Characteristics

Category	Scale	Number
Age (years)	Median	48
	Range	35–56
Sex	Female	4
	Male	10
International Prognostic	Low	7
Index (IPI)	Low intermediate	6
score	High intermediate	1
Prior therapies	Median	1
	Range	1–3
Current status	Median follow-up	41
(months)	Range	9–75
	Median disease-free survival	42
	Median overall survival	Not reached

Materials and Methods

Patients

Adult patients aged 18 to 65 years with good performance status and less than or equal to three relapses of FL grade 1 or 2, according to the World Health Organization Classification[21] of lymphoma, were treated in this noncomparative, prospective, nonrandomized phase II clinical trial. All patients meeting eligibility requirements were included (Table 1). Patients were treated at the Toronto-Sunnybrook Regional Cancer Centre. The hospital institutional review board and ethics committees approved the trial, and informed consent was obtained for all patients. Patients received initial debulking chemotherapy with standard cyclophosphamide, doxorubicin, vincristine, prednisone or dexamethasone, cisplatin, and cytarabine in the event of previous anthracycline exposure. Stem-cell collection took place when bone marrow (BM) involvement with lymphoma was $<$15% as determined by histology. Stem cells were mobilized with 5 days of high-dose granulocyte colony-stimulating factor.

Table 2. Primers for t(14;18) Translocation Detection and the RAG2 Gene Control

Primer	Sequence	Reference
MBR (nested external)	5′-CAGCCTTGAAACATTGATGG-3′	[8]
MBR (nested internal)	5′-TATGGTGGTTTGACCTTTAG-3′	[8]
mcr (nested external)	5′-CGTGCTGGTACCACTCCTG-3′	[8]
mcr (nested internal)	5′-GGACCTTCCTTGGTGTGTTG-3′	[8]
MBR E	5′-TCCAGATGGCGAATGACCAG-3′	
MBR B	5′-GAGTTGCTTTACGTGG-3′	[20]
MBR H	5′-GCAACAGAGAACCATCCCT-3′	[26]
JH	5′-ACCTGAGGAGACGGTGACC-3′	[4]
Mcr	5′-CGTGCTGGTACCACTCCTG-3′	[4]
RAG 2 Forward (F1)	5′-CCTGAAGCCAGATATGGTC-3′	[23]
RAG 2 Reverse (R1)	5′-GTCCAATTCACAGCTGGGCT-3′	[23]

Patients achieving at least a 75% reduction in tumor bulk proceeded to high-dose therapy with standard cyclophosphamide, carmustine, and etoposide conditioning and ASCT. Immunotherapy after transplant consisted of subcutaneous interferon-α 3 million U/m^2 three times weekly beginning 3 months after ASCT for a period of 24 months. Response to therapy was assessed by serial clinical examination, blood counts and chemistries, BM aspiration and biopsy, and computed tomography (CT) scans of involved sites. These evaluations took place 8 weeks after ASCT, then every 3 months for the first year after ASCT, and every 6 months thereafter.

Sample Collection

Baseline samples of lymph node (LN), BM, or PB assessed as morphologically positive for disease involvement were analyzed by PCR as described below, for the presence of molecular markers for t(14;18) translocations.[22] If t(14;18) was not detectable, a minimum of 1% disease infiltration was desirable to identify a clonal IgH marker. The determination of baseline infiltration of the BM sample was achieved by combined morphological and immunohistochemical analysis, the latter using antibodies to CD20 and bcl-2. Quantities are expressed as a percentage of the visual stained lymphocytes. For patients in whom a baseline molecular marker was identified, serial molecular monitoring for MRD was performed on all BM and PB samples (before apheresis, before transplant, and after ASCT at specified 3- to 6-month follow-up intervals). The stem cell graft was similarly evaluated for the presence of occult disease.

DNA Preparations

DNA was extracted from fresh or frozen LN tissue, buffy-coated BM aspirates, or buffy-coated PB using Qiagen DNA mini kit (Qiagen, Valencia, CA). BM biopsy specimens were found to be unreliable for PCR analysis as yields were low and fixation and decalcification processes often degraded the DNA. Therefore, only BM aspirates were analyzed in follow-up samples. DNA concentrations were adjusted to be in the range of 80 to 140 ng/μL. The translation from DNA concentration to cell number is dependant on the total amount of amplifiable DNA in the sample. We have generated primers to amplify a 350-bp fragment of a highly conserved gene, *RAG2*,[23] as a control for both DNA quality and quantity.

Nested PCR Reactions

Precautions were taken to separate pre- and post-PCR work areas and to prevent potential contamination by PCR products. Negative controls included DNA from Jurkat T-cell lymphoma cell line, which lacked the B-cell-specific translocations. For a positive control of MBR translocations, OCI LY8 cell line[24] was serially diluted in Jurkat DNA. The minor cluster region (mcr)-positive control was patient LN DNA.

Nested PCR for t(14;18) followed by gel electrophoresis was performed with primers for the MBR and mcr of the *BCL2* gene, and *JH* of the immunoglobulin heavy chain gene as described previously.[8] Between 500 ng and 1 μg of patient sample was amplified with 0.5 U of Hot Star *Taq* polymerase (Qiagen), external MBR (or mcr)-*JH* primers and multiplexed with RAG2 primers (Table 2) in a 50-μl reaction volume. The reaction began with a 15-minute incubation at 94°C to activate the enzyme, followed by 25 cycles (1 minute at 94°C, 1 minute at 55°C, and 1 minute at 72°C). Subsequently, 5 μl of the PCR product was reamplified with internal MBR (or mcr)-*JH* primers for 30 cycles with an annealing temperature of 58°C. PCR products were analyzed on ethidium bromide-stained 2% agarose gels.

In patients for whom t(14;18) was not detectable, an attempt was made to determine clonal-specific IgH primers using the method described by Andersen and colleagues.[25] Essentially, *VH* framework I family-specific and JH consensus primers were used to amplify the clonal IgH fragment. This DNA was then sequenced and analyzed with IgBLAST, a software program specifically designed for comparing IgH DNA sequences available from the National Center for Biotechnology Information website (*http://www.ncbi.nlm.nih.gov*). From this analysis, patient allele-specific primers were designed with the sense primer located in *VH* framework 1 region and the anti-sense primer from the CDR3 region (Figure 1).

RQ-PCR Reactions

Amplification reactions were performed using QuantiTect SYBR Green PCR kit (catalog no. 204143, Qiagen) with

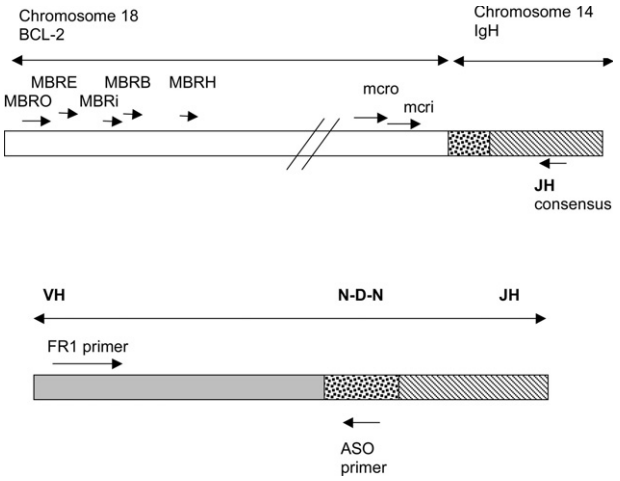

Figure 1. Primer locations and PCR strategies. The t(14;18) translocation is illustrated to indicate the locations of the BCL2 primers. All of these BCL2 primers (MBRO, MBRE, MBRi, MBRB, MBRH, mcro, mcri) are located 3′ to the *BCL2* gene exons on chromosome 18. The JH consensus primer anneals to all JH exons 1 to 6, but preferential amplification of t(14:18) amplicon occurs from the JH closest to the breakpoint. A rearranged IgH gene is illustrated to show the approach for ASO-specific amplifications. VH Framework1 and JH consensus primers are used to amplify a clonal IgH product. An ASO primer located in the N (inserted nucleotides)-D(diversity)-N region and a primer from the VH FR1 were designed based on the DNA sequence of the clonal IgH product.

each reaction containing 500 ng of sample DNA and 0.2 μmol/L to 0.3 μmol/L primers in a 50-μl volume. This kit was optimal for PCR products between 100 and 600 bp, so new primers for MBR were incorporated into the RQ-PCR protocols to amplify patient samples whose MBR-*JH* PCR product was larger than 500-bp as determined by agarose gel analysis. RQ-PCR primers, cycle conditions, and positive controls are listed in Tables 2 and 3 and mapped in Figure 1. Primers for each patient sample were selected based on previously generated positive results from qualitative PCR analysis. A 500-ng sample of patient DNA represents 8×10^4 cells. Patient samples were amplified with *RAG2* primers in a separate reaction to determine cell equivalents. The standard curve for this reaction was derived with 10-fold serial dilutions of DNA from Jurkat cells. Quantification of the MBR/JH for MBRE and MBRB primer sets was determined from a standard curve established with 10-fold serial dilutions of DNA from OCI LY8 diluted into *BCL2/JH*-negative Jurkat DNA. The positive control standard curves for MBRH, mcr, and ASO PCR reactions were generated using DNA from highly infiltrated patient LN DNA.

Thermocycling was conducted using a DNA Engine Opticon (MJ Research, Boston, MA) initiated by a 15-minute incubation at 94°C followed by cycles and annealing temperatures determined by each primer set as detailed in Table 3. A plate read was performed at each cycle after the 72°C extension. Each run was completed with a melting curve analysis to confirm specificity of amplification and also to verify clonal identity where possible. Cycle threshold (Ct) values were determined by the Opticon software using a fluorescence threshold manually set to 0.02 for all runs. Data were exported to Microsoft Excel for further analysis. For both nested and RQ-PCR, molecular markers were confirmed by testing more than one tissue or sample time point. All follow-up samples were analyzed under the same conditions accompanied by baseline samples as additional positive controls. All patient samples were analyzed in triplicate.

Results

Molecular Markers

Molecular markers were determined for 12 of 14 patients. Using the various BCL2 primers, *BCL2/JH* translocation was detected with MBRB (eight cases), MBRH (one case), and mcr (two cases) for a total of 11 patients (78%). For one of the three other patients, we were able to develop patient-specific ASO-*IgH* primers. We were unable to detect BCL2/JH or clonal *IgH* in the baseline LN samples of two patients.

Qualitative PCR Analyses of Patients by Nested PCR

An example of nested PCR monitoring of patient 009 with the MBRB/JH primer set is shown in Figure 2. The upper band corresponding to a product of *RAG2* amplification demonstrates that template DNA in the reaction is of sufficient quality and quantity for the assay in all samples. The positive control OCI LY8 cells in this experiment were detected at 10^{-5}, but the assay showed sensitivity as low as 10^{-6} approximately once every three runs. Baseline BM was amplified to demonstrate that the assay detected this particular breakpoint and to show that the unique patient PCR product size was reproducible in BM and PB samples taken at 42 months after transplant. Only one of the duplicate patient samples was amplified in each

Table 3. Real-Time PCR Reaction Conditions

Reaction	Primers	Cycle conditions		Positive controls
		Annealing (°C)	No. of cycles	
1	MBR E + JH	63	45	Ly8 cell line [23]
2	MBR B + JH	63	45	Ly8 cell line
3	MBR H + JH	58	45	Pt lymph node DNA
4	mcr + JH	60	37	Pt lymph node DNA
5	RAG2 F1 + RAG2 R1	58	45	Jurkat's cell line

The reactions began with a 15-minute incubation at 95°C to activate HotStarTaq DNA polymerase. This was followed by the indicated number of cycles of 20 seconds at 92°C, 20 seconds at the appropriate annealing temperature, 20 seconds at 72°C, followed by plate read. The melt curve was performed at the end of the amplification reactions; from 65.0 to 95.0°C; read every 0.2°C; hold for 0.01 seconds between reads.

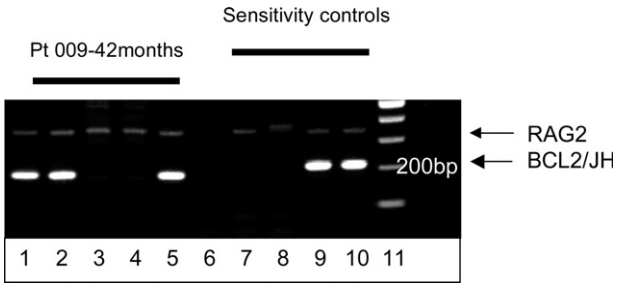

Figure 2. Qualitative nested PCR analysis of patient 009 at 42-month follow-up samples. **Lane 1**, baseline BM sample demonstrating size of BCL2/JH translocation product; **lane 2**, BM sample with PCR product identical in size; **lane 3**, duplicate analysis of BM with no amplification; **lanes 4** and **5**, blood sample amplified in one of two reactions to produce same size product as baseline; **lane 6**, no template DNA; **lane 7**, negative control DNA from Jurkat cells; **lanes 8**, **9**, and **10**, 10-fold increasing concentrations of LY8-positive control DNA with 200-bp BCL2/JH PCR product. **Lane 11** is 100-bp marker. DNA quality control, 350-bp RAG2 PCR product from the first round of PCR, is visible in patient sample lanes where BCL2/JH did not amplify.

case, indicating a low concentration of target, approaching the theoretical limit of sensitivity of the assay. Although the experiment suggests that there are less lymphoma cells in some samples (where only one of two duplicate reactions amplified), it was not otherwise possible to quantify the difference in lymphoma cell burden between sample time points in this assay. In nested PCR, the result was indicated as positive or negative only.

In patient 004, a t(14;18) translocation could not be detected, but it was possible to amplify the clonal-specific *IgH* rearrangement from a frozen biopsy sample of heavily infiltrated LN tissue, using *VH* FR1 consensus primer [cctcagtgaaggtctcctgaagg] and the *JH* consensus primer. The PCR product was isolated on agarose gel, purified using Qiaex II kit (Qiagen), and the DNA sequence was determined. From the sequence data, a *VH* FR1 forward primer (5′-tccctgtgcagcctctggat-3′) and ASO reverse primer (5′-catgccatgatgatctgtccct-3′) were determined to yield a 280-bp amplicon. PCR conditions were optimized to achieve amplification of a 10,000-fold dilution of the baseline LN sample. Serial dilution of the LN samples was used for the positive control in monitoring follow-up samples from patient 004. Similar to the results achieved with control cell line LY8 described above, a band representing the target amplification was visualized at each dilution of the positive control but not in a negative control (PB from a normal donor) (results not shown).

SYBR Green RQ-PCR: Quantification Curves

To determine whether the proportion of lymphoma cells in PCR-positive samples could be better documented, each patient sample was reanalyzed with appropriate primers for the SYBR Green I assay. Amplification of patient 009 samples with primer set MBRB-JH as an example, illustrates how the analysis was done. Figure 3a shows a display of MBRB-JH amplifications of the OCI LY8 cell line diluted in 10-fold steps to 10^{-5} in negative cell line DNA. The standard curve in Figure 3b of log quantity versus cycle threshold (Ct) shows an R^2 value of 0.999, indicating that the dilution series is very accurate. Theo-

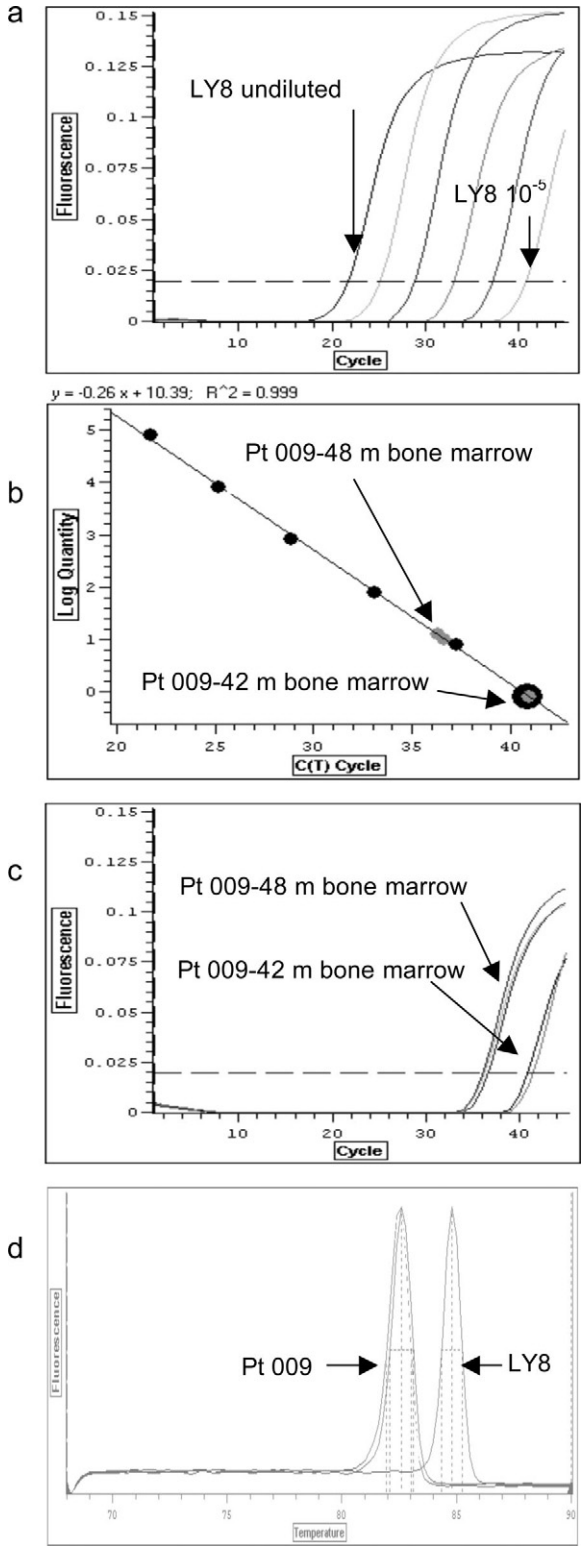

Figure 3. RQ-PCR analysis of patient 009 with primer set MBRB/JH. **a:** Tenfold serial dilutions of LY8 cell line calibration standard. The highest concentration produces detectable PCR at 22 cycles, whereas the lowest requires ~41 cycles. **b:** The log quantity of sample is plotted versus cycle threshold (Ct) to generate the standard curve from which unknown samples are quantified. **c:** Follow-up samples from patient 009 at 42 months amplified at 40 cycles, whereas triplicate samples from 48 months required only 36 cycles, indicating ~10-fold higher concentration. **d:** Melt peaks generated from plotting −dI/dt versus temperature, show coincident peaks from baseline and follow-up patient 009 samples at 82.8°C while the control LY8 cell line peaks at 84.8°C.

retically, the Ct difference between 10-fold diluted samples with 100% efficiency of amplification should be 3.3, consequently the slope of a standard curve of Ct/(log copies) should approach −3.3. The slope of the graphed line is −0.26, which when inverted to express Ct/(log fluorescence) equals −3.8.

SYBR Green RQ-PCR: Sensitivity

The sensitivity of the real-time assay was reproducible at 10^{-4} but often reached as low as 10^{-5} (Figure 3c). Triplicate analysis of BM and PB at 42 months after transplant from patient 009 revealed contamination at the lowest levels of sensitivity—approximately a single copy per reaction. These results confirm those obtained with the nested PCR analysis on the same samples shown in Figure 2. Not all reactions showed amplification of the target, as would be expected in the case of close to single copy number according to Poisson distribution laws. Subsequent triplicate samples at 48 months showed a lower Ct, reflecting an increase in PCR-positive cells. The SD for triplicate samples was less than 20% (data not shown), and the overlapped triplicate tracings on the graph in Figure 3c further illustrate this. Interrun variations were less than one cycle. After normalization, the calculated contamination was less than 10 lymphoma cells per million nucleated cells in BM and blood at 42 months after transplant. By 48 months, these numbers had increased to 180 and 800 lymphoma cells per million cells, respectively.

SYBR Green RQ-PCR: Melt Curve Analysis

As in the nested PCR, the reactions were specific and generated strong signals for PCR products of the desired targets. Melt curves can be used to determine the specificity of the amplified product. After the last cycle of PCR amplification, the sample is heated in 0.2°C increments from 70 to 90°C. Fluorescence is recorded with each increment in temperature. Initially fluorescence is high because all of the PCR products are double-stranded so SYBR green binds to them. When the PCR products reach melting temperature (T_m), they become single stranded and rapidly lose SYBR green fluorescence. The detection system calculates the negative of the first derivative of the fluorescence intensity versus temperature, and the resulting graphic display shows a sharp peak at the T_m. Similar to the agarose gel approach described above, it was possible to document the specificity of the amplified patient product by the visualization of a single peak. In fact, because T_m is also dependant on GC base content, melt peaks can sometimes distinguish PCR products that are not separated on standard gels. Diverse peaks with different T_m values or plateaus indicate nonspecific products. The graph of melting temperature analysis in Figure 3d confirms the specificity for the patient 009 clone in serial samples and also distinguishes the patient sample from the LY8-positive control. Primer-dimer peaks were expected to have melt temperatures near 70°C. These low-temperature peaks were rarely observed and did not appear in samples showing peaks from target molecules. Follow-up samples showing late RQ-PCR amplification sometimes revealed melt temperature peaks that did not match those from the baseline sample and were not reproducible in triplicate reactions. Much like the interpretation of PCR products of unexpected sizes visualized on agarose gels, these products were interpreted as nonspecific. Reactions with nonreproducible, nonspecific peaks were regarded as negative. As was demonstrated with MBRH-JH primers and cell line LY8, all primer sets and corresponding PCR conditions were optimized as documented in Table 3 to generate a single melt peak in corresponding positive control samples (data not shown). Melting temperatures of t(14;18) amplicons from each patient, listed in Table 4, were sometimes useful in establishing the uniqueness of each patient's amplified clonal product. For each patient, a median temperature was calculated from multiple samples and runs of the baseline sample. The variation from this median temperature was at most at 0.2°C, which is within the precision of the instrument.

Table 4. Melting Temperatures of PCR Products

Patient	Melt temperature of PCR products (°C)	Median (°C)	Deviation from median
002	84.4	84.4	0.2
	84.4		
	84.2		
009	82.8	82.8	0.2
	82.8		
	82.6		
011	84.8	84.8	0.2
	84.8		
	84.6		
015	84.4	84.4	0.0
	84.4		
	84.4		
032	84.0	84.0	0.0
	84.0		
	84.0		
019	81.2	81.2	0.0
	81.2		
	81.2		
020	82.8	82.8	0.0
	82.8		
	82.8		
110	84.8	85.0	0.2
	85.0		
	85.2		
014	80.8	81.0	0.2
	81.0		
	81.2		
004	82.0	82.0	0.2
	82.2		
	82.0		
021	83.2	83.2	0.0
	83.2		
	83.2		

Amplification Specificities and Efficiencies with SYBR Green I RQ-PCR

Amplification reaction conditions with all primer sets were optimized (Table 3) to yield single melt curve peaks with

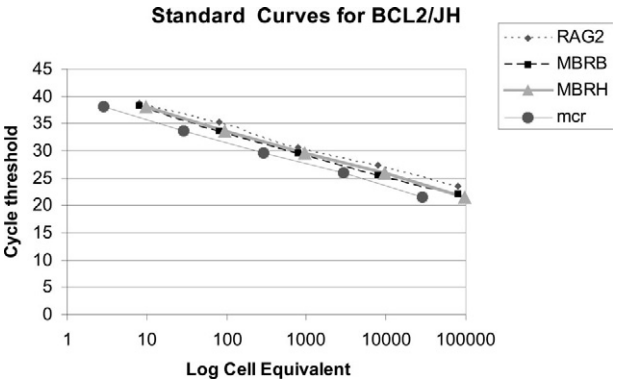

Figure 4. Standard curves of RQ-PCR BCL2/JH and RAG2 reactions. The log of initial cell equivalents is plotted versus cycle threshold for each RQ-PCR reaction. All curves have slopes of close to −3.9 and r^2 values equal to 0.998, indicating similar amplification efficiencies in each reaction. The PCR primers and conditions for each reaction are listed in Tables 2 and 3.

serial dilutions of the positive controls as has been demonstrated with the MBRB-JH primer set and patient 009 (data not shown). The standard curves for MBRB, MBRH, mcr, and RAG2 reactions, generated with the corresponding controls indicated in Table 3, have similar slopes as shown in Figure 4. For patient 004, 10-fold serial dilutions of diagnostic LN DNA were RQ-PCR amplified with ASO-specific primers to generate a calibration curve. As well, a PCR product of this reaction was gel purified with Qiaex II (Qiagen) and quantified with a spectrophotometer. Tenfold serial dilutions of the PCR product were then amplified to generate a calibration curve based on copy number of the target. The resulting standard curves are compared in Figure 5. These results demonstrate that a PCR product from a baseline tissue can also be used to generate a positive control and calibration curve for patient-specific primer sets.

Each patient's MBRB-JH PCR product will be different in length and may therefore have a different PCR efficiency than the product obtained from the LY8 cell line. We wanted to determine the significance of PCR product size on amplification efficiency and quantification. To do

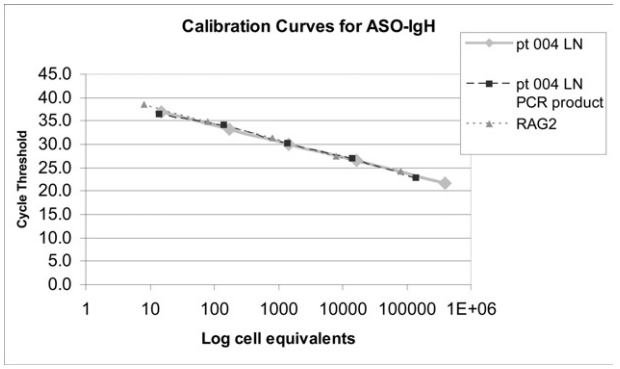

Figure 5. Standard curves for RQ-PCR for ASO (patient-specific) reactions and RAG2. The log of initial cell equivalents is plotted versus cycle threshold for patient 004 ASO RQ-PCR reactions. A curve generated from dilutions of patient 004 LN DNA is similar to a curve generated from dilutions of purified, quantified PCR product. In all three curves, slopes are −3.5 and $r^2 = 1.0$. This demonstrated that PCR products with known copy number can be used to generate a valid standard curve for the quantification of ASO-specific reactions.

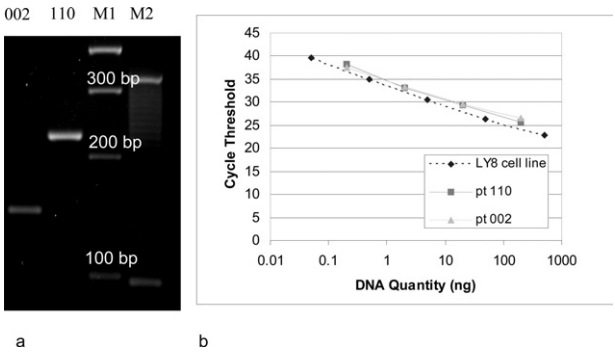

Figure 6. Small and large amplicons are RQ-PCR amplified with similar efficiencies. MBRB-JH primers were used to amplify the BCL2-JH junctions in highly infiltrated baseline BM samples from patients 002 and 110. **a:** The resulting products were sized against 100-bp ladder (M1) and 10-bp ladder (M2) on a 3% agarose gel and estimated to be 150 bp and 230 bp in length. LY8 amplicons were 210 bp. **b:** Tenfold serial dilutions (200 to 0.2 ng) of the patient samples and the LY8-positive control cell line were RQ-PCR amplified. Resulting cycle thresholds were plotted against log DNA concentrations and showed $r^2 = 0.99$ for all three dilution sets. The slopes for LY8, patient 110 and patient 002 were −4.2, −4.1, and −3.7, respectively, demonstrating similar amplification efficiencies.

this, we performed detailed analysis on samples from two patients whose PCR products differed in length by ~80 bp. In agarose gel analysis, shown in Figure 6a, patient 002 PCR product was ~150 bp in length and patient 110 PCR amplicon was ~230 bp long (LY8 amplicon was ~210 bp as seen in Figure 2). Highly infiltrated BM aspirate samples from patients 002 and 110 contained similar percentages of tumor cells as quantified with LY8 control cell line. RQ-PCR with MBRB-JH primers was performed on 10-fold serial dilutions (from 200 ng to 0.2 ng) of these samples. Cycle threshold versus log of DNA concentration showed good correlation ($r^2 = 0.99$) (Figure 6b). The slopes of the curves of log quantity versus cycle threshold are similar for LY8 control and both patient samples, indicating that all of the amplification efficiencies were similar. These results imply that the technique produced reliable quantification measurements for both small and large amplicons amplified by the MBRB-JH primers.

Serial PCR Monitoring

Serial data were obtained on all 12 patients with nested PCR and RQ-PCR, and the results are reported in Figure 7. For nearly all time points in which nested PCR was positive, we were able to obtain quantitative data (see Figure 7 legend for detail). One patient (no. 020) had inadequate baseline sample remaining for RQ-PCR so only the nested PCR result is shown. Patient 021 samples from stem cell product, 9-month, 12-month, and 30-month samples were negative by RQ-PCR but positive by the more sensitive nested PCR assay in some replicates. In contrast, patient 014 was PCR-positive by nested PCR and RQ-PCR in pretransplant samples, but after transplant only RQ-PCR detected *BCL2/JH*. In this patient, the 1-kb nested PCR product of *BCL2/JH* was sequenced. A more 3′ primer, MBRH,[26] was paired with *JH*, generating a product through RQ-PCR that was smaller (300 bp) and likely more efficiently amplified.

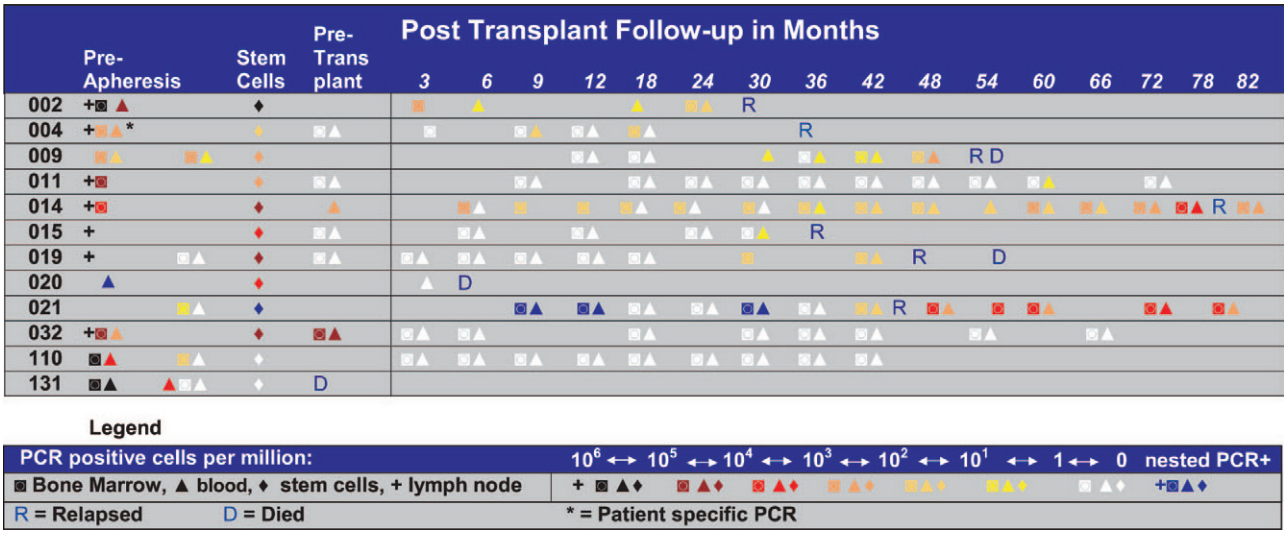

Figure 7. Serial analysis of 12 patients with molecular markers. All patient samples were initially analyzed with qualitative nested PCR as described in Materials and Methods. The samples were then reanalyzed with RQ-PCR. All samples shown as PCR-negative were negative by both techniques. All samples that were positive with nested PCR were quantifiable with RQ-PCR with the exception of some samples for patient 021 (detail in text) shown only as nested PCR+. The calculated percentages of contamination were converted to cell equivalents per million cells and rounded to the nearest log for ease of representation. Each log concentration is represented by a separate color. Clinical relapse occurred, as indicated with R, only in patients with increasing proportions of PCR-positive cells.

Assessment of Stem Cell Graft Contamination by SYBR-Green I RQ-PCR

Table 5 lists the calculated contamination of stem cell collections. Median *BCL2/JH* contamination of stem cell grafts was 0.1% (range, 0 to 13%). Patient 002, with the highest level of contamination, had no follow-up samples that were PCR-negative. Patients reinfused with both high and low levels of graft contamination were able to achieve molecular remission, and in the cases of patients 011 and 032, for a longer period of time than patient 021 who had very low levels of PCR positivity in the graft.

Clinical Correlations with SYBR-Green I RQ-PCR

Before transplant, no patient had achieved complete response. The best response was complete response unconfirmed in patient 014, with all others diagnosed as partial response. After transplant, most were able to achieve PCR negativity, including patient 032 who had high residual disease quantity before treatment with cyclophosphamide, carmustine, and etoposide. In those who were PCR-positive, residual disease quantities were low for a number of months or years. At a median follow-up of 75 months (9 ~ 91), 10 of 14 patients are alive. Of these, three are free from progressive disease at 72, 66, and 36 months. Median progression-free survival is 42 months; median overall survival has not been reached.

Measurements of RQ-PCR *BCL2/JH* in BM aspirates at baseline and follow-up time points were consistent with lymphoma cell contamination estimates based on morphology of the BM biopsies. Preapheresis samples with highly infiltrated BM showed high levels of PCR-detectable disease in BM aspirates in patients 002, 011, and 032. Exact quantification of FL cells in the BM biopsy would not correspond exactly to RQ-PCR data. When measuring *BCL2/JH* copies by PCR, the numbers were calculated based on cell equivalents of DNA from all cell types, not just CD20+ cells. As previously found,[27] BM aspirates contained fewer lymphoma cells than the biopsies. This can be due to dilution in PB and sampling deficiencies. It was not possible to obtain exact numbers of lymphoma cells in BM aspirates (there are no FL-specific markers for flow cytometric measurements) for correlation to RQ-PCR data. In PCR-positive follow-up samples with very low RQ-PCR, the corresponding BM biopsies were often morphologically negative. Increasing fractions of *BCL2/JH*-positive blood and BM reliably predicted morphological and clinical relapse. In all time points in which the BM samples were PCR-negative, the BM biopsy was morphologically negative. The three patients, 011, 032, and 110, without molecular evidence of FL are also free of clinical disease progression.

Patient 014 had a long-term, low-level persistence of tumor cells in BM and blood, with no clinical symptoms for 70 months after ASCT. At 76 months there was a

Table 5. Quantitative MRD in Stem Cell Collections

Patient no.	Percent PCR-positive CE in stem cell collections
002	13.0
004	0.01
009	0.15
011	0.8
014	1.15
015	0.19
019	1.000
020	0.10
021	0.000
032	1.240
110	0.000
131	0.000

10-fold increase in the quantity of PCR product in both BM and blood. Radiological evidence of relapse was observed 3 months later (CT scan data not shown). In this interesting case, the patient underwent spontaneous disease regression again documented by radiology (CT scan data not shown), which was paralleled by a 10-fold decrease in molecular evidence at 82 months.

Discussion

We have optimized and compared two different methodologies for evaluating MRD in patients with low-grade non-Hodgkin lymphoma. We have found that qualitative PCR is highly sensitive, can detect relatively large fragments and allows distinction of translocations from different patients if size differences are large enough to be resolved by gel electrophoresis. The main drawbacks are that little can be said about tumor burden and quantity changes in MRD in the sample.

On the other hand, we found that the real-time quantitative PCR (RQ-PCR) method was highly reproducible and could also distinguish different levels of MRD in samples such as BM, blood, and apheresis product. The method was flexible and applicable to multiple *BCL2* breakpoints, as well as ASO-specific amplification for patient 004. We were able to quantify residual cells in nearly all samples previously only indicated as positive by nested PCR (Figure 7). Our molecular technique seemed accurate in monitoring the volume of disease as shown in the case of patient 014 in which clinical regression was paralleled with a logarithmic decrease in PCR positivity. We were also successful in distinguishing many patient samples with melt curve analysis (Table 4).

In one patient (002) with a very high level of PCR-positive graft, molecular remission was not achieved. It is interesting to note that some patients who were reinfused with lower level PCR-positive grafts were able to achieve molecular remissions after transplant. In the case of patients 011 and 032 (with 800 and 12,400 PCR-positive cells per million, respectively), this remission has even exceeded the time of patient 021 who had levels of PCR positivity in stem cell graft that were less than 1 to 10 copies per million (too low to be detected by RQ-PCR). Hirt and Dolken[14] have also reported that some patients with the lowest levels of t(14;18)-positive cells (seven cells per million) in autologous grafts relapsed within the first 2 years of transplantation, whereas patients who received up to 100 times more t(14;18)-positive cells (700 cells per million) in their BM preparations were still in remission after almost 4 years. Factors in addition to stem cell contamination thus may also influence relapse. However, our results show that quantitative PCR is more informative than qualitative PCR in evaluating stem cell purging effects.

A significant factor in effective molecular monitoring may be the choice of tissue. Using nonquantitative PCR monitoring, both paired PB and BM samples were found to be informative in this study and others.[28,29] In some studies[30,31] BM was more sensitive than PB, and our RQ-PCR serial monitoring results in Figure 6 show that in many paired samples, higher quantities were detected in BM than in PB. Shortened FL cell survival in the peripheral circulation due to the absence of stromal cells to provide survival signals may account for the decreased numbers of FL cells in blood. There may also be more resistance in the BM compared to PB. In an RQ-PCR evaluation of 86 FL patients, a low level of *BCL2/IgH+* cells in BM was the best predictor for achievement of complete clinical and molecular response.[31] Sampling of only PB may be inaccurate and lack prognostic value.

Very low levels of tumor cell contamination in PB or BM may not be predictive of clinical relapse. It may be that at low levels the immune response may control the disease for variable periods. However, once logarithmic increases in PCR-positive PB or BM occur, clinical relapse may be imminent, as demonstrated by patients 009, 014, 015, and 021. This was also found by Hirt and Dolken.[14] The quantitative detection of circulating t(14;18) cells during follow-up of patients with FL after ABMT reflected the clinical course of the disease. Relapses were associated with increasing numbers of circulating t(14;18)-positive cells, and continuous complete remissions were associated with stable (within one magnitude) levels of contamination. In another recent report by Ladetto and colleagues,[32] RQ-PCR and sequencing were used to analyze molecular recurrence in autografted patients who had initially achieved molecular remission after transplant. Clinical relapse followed in patients who showed a sharp increase in the number of clonal cells on follow-up samples within 2 years after transplant. In contrast, some patients showed persistent PCR positivity due to rearrangements unrelated to the original clones that was not accompanied by clinical evidence of progressive disease by radiology, histology, or flow cytometry.

The analysis of more study patients with RQ-PCR could be accomplished if more t(14;18) translocations could be detected and used as molecular markers. The work of van Dongen and colleagues[5] has produced sets of primers and controls that have been designed to amplify a wider range of t(14;18) breakpoints. It may be possible to optimize some of these primers for use in SYBR Green I assays and thus expand the utility of this approach for clinical study patients in an economical way. As well, when necessary we have shown that it is possible to monitor patient-specific IgH products with SYBR Green I.

The more widely used fluorescent probe techniques generally detect tumor cells at 10^{-4} reproducibly but are sensitive to a level of 10^{-5}.[9,33] Our SYBR Green approach was both specific (as determined by single peak in the melt curve analysis) and achieved the same detection frequencies as the published reports using fluorescent probes. As in an RQ-PCR method using BCL2 probes,[10] we were able to quantify the amplification of both small 150-bp and large 230-bp fragment with one standard curve for each PCR primer set. Patients with BCL2 breakpoints producing PCR products much smaller (less than 80 bp) or larger than 500 bp (as in patient 014) require more specific primer sets for quantification both in our assay and in probe-based RQ-PCR.[16] Large PCR product size was also found to decrease amplification efficiency in our nested PCR

protocol, in which in the case of patient 014, we obtained negative results at low-level tumor burden with the standard nested PCR method.

Elimination of false-positive results is important. Products from very low-frequency cells could represent clonal lymphoma cells, normal nonmalignant cells bearing the t(14;18) translocation, or contamination from a sample from a different patient. With qualitative PCR analyses it is impossible to know when signal represents low-frequency events. With probe technologies, the PCR product can only be analyzed through manipulations after PCR such as gel electrophoresis, capillary electrophoresis,[10] or DNA sequencing. Capillary electrophoresis and DNA sequencing are the most precise methods to verify recurrence of the original tumor clone, but require additional expensive equipment. However, with our SYBR Green method, we have shown that we can analyze both the frequency and predict clonality in one instrument. If necessary, clonality can be further verified by DNA sequencing. Our method is a suitable economical alternative to probe technologies.

In conclusion, real-time quantitative PCR was more informative in our hands than qualitative PCR in evaluating the efficiency of stem cell purging and molecular recurrence. A limitation of our study is the small patient population analyzed. Further experience with larger numbers of patients is required to verify these preliminary results. Although the study described here has closed to accrual, we are currently evaluating this SYBR Green I RQ-PCR technology in other stem cell transplant trials. We have also expanded the assay to include *BCL1/JH* detection in mantle cell patients.

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