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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.