NCCN Task Force Report: Molecular Markers in Leukemias and Lymphomas

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Key Words

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Abstract

The introduction of targeted therapies has revolutionized treatment and improved outcomes in patients with leukemias and lymphomas. However, many patients experience relapse caused by the persistence of residual malignant cells. Cytogenetic and molecular techniques are increasingly being used to assess and quantify minimal residual disease (MRD). The emergence of advanced technologies has led to the discovery of multiple novel molecular markers that can be used to detect MRD and predict outcome in patients with leukemias and lymphomas. Gene expression signatures that predict clinical outcomes in patients with non-Hodgkin's lymphoma have been identified. In chronic myelogenous leukemia, molecular monitoring has become more important in assessing response and detecting resistance to therapy. In acute leukemias, several new markers have shown potential in prognostication and monitoring treatment. In leukemias and lymphomas, microRNAs have been identified that may be useful in diagnostics and prognostication. To address these issues, the National Comprehensive Cancer Network (NCCN) organized a task force consisting of a panel of experts in leukemia and lymphoma to discuss recent advances in the field of molecular markers and monitoring MRD. (*JNCCN* 2009;7[Suppl 4]:1–34)

Overview

Oncogenesis is complex and impacts diverse cellular processes, such as cell signaling, cell cycle, differentiation, and programmed cell death, and the tumor microenvironment. Molecular markers (proteins, cDNA, mRNA, or microRNA) reflect these perturbations and can be identified in tumor tissue, lymph nodes, bone marrow, peripheral blood, or other bodily fluids. Within

malignant cells, molecular markers can be expressed either directly or indirectly through various mechanisms, such as activation of oncogenes, inactivation of tumor suppressor or DNA repair genes, activation of anti-apoptotic genes, or inactivation of pro-apoptotic genes.¹ Because molecular markers are often associated with clinical prognosis, their measurement can be useful in clinical management to estimate a patient's chance of survival. Furthermore, molecular markers help identify individuals who are unlikely to benefit from standard therapies and therefore may choose to pursue investigational alternatives.

In hematologic malignancies, dysregulation of gene expressions occur predominantly through 2 mechanisms:² 1) reciprocal translocations between chromosomes, leading to the formation of fusion genes and corresponding chimeric proteins; and 2) genomic rearrangements juxtaposing proto-oncogenes with immunoglobulin heavy chain (IgH) or T-cell receptor (TCR) genes, resulting in the dysregulated expression of structurally normal or truncated proteins. The best example of the former mechanism is the formation of *BCR-ABL* fusion gene and its fusion protein p210, resulting from the t(9;22) translocation in patients with chronic myelogenous leukemia (CML). Examples of the latter mechanism include t(14;18) translocation involving *BCL2* and *IgH* genes in follicular lymphoma and some diffuse large B-cell lymphoma (DLBCL), and t(11;14) translocation involving *BCL1* and *IgH* genes in mantle cell lymphoma (MCL).³ Other genetic abnormalities, such as chromosomal deletions and amplifications, result in gene expression alterations that may be used as molecular markers.

Although recent advances in treatment options have improved outcomes, currently available therapies

are not curative in many patients with leukemia and lymphoma. Disease often relapses because of the persistence of low numbers of malignant cells that were not eradicated by previous therapies. Disease that is undetectable using conventional methods is referred to as minimal residual disease (MRD). MRD detection during or subsequent to treatment using reliable molecular markers is important because it provides biologic insight into the natural history of the disease. MRD status may theoretically allow early intervention with more effective treatment options, which may translate into improved outcome (e.g., prolonged time-to-progression).⁴⁻⁶ Recent clinical studies have shown that MRD eradication using new treatment modalities has significantly improved the duration of response and long-term survival in patients with hematologic malignancies.

Some molecular markers are anticipated to be critical for maintenance of the malignancy and would be potential targets for the development of novel treatment strategies. These targeted therapies may significantly improve the outcomes of patients with less treatment-related toxicity, leading to an era of individualized medicine.¹ Molecular markers can also help identify subgroups of patients with similar risk profiles that are important in evaluating the effectiveness of therapy. Furthermore, molecular marker profiles may define which patients are eligible for treatment uniquely tailored to their particular disease biology.

Many molecular markers have been identified over the years through careful study of tumor biology. The emergence of novel genome-wide technologies has greatly accelerated the identification of molecular markers associated with the underlying biology of hematologic malignancies and prognostic importance.⁷ Molecular markers are increasingly being used as measures of response in clinical trials.⁸ Molecular markers associated with prognosis have also been used increasingly to stratify prospective randomized trials to ensure balanced assignment of patients according to risk group to the different treatment arms. Prognostic molecular markers facilitate the comparison of results from different clinical trials through characterizing different patient populations according to risk.

This report focuses on the methods used for identifying molecular markers and detecting MRD, their prognostic significance, and practical issues

regarding their incorporation into routine clinical practice for leukemias and lymphomas, as discussed at the meeting on November 17, 2008.

Molecular Markers in CML

CML is characterized by the presence of the Philadelphia chromosome (Ph chromosome) resulting from t(9;22). This translocation leads to the fusion of the *BCR* gene on chromosome 22 and the *ABL* gene located on chromosome 9.⁹ CML has classically been categorized as 3 different phases (chronic, accelerated, and blast) based on clinical and laboratory findings. However, gene expression profiling (GEP) suggests that the bulk of genetic changes in progression occur during transition from chronic to accelerated phase.¹⁰ Untreated chronic phase CML (fortunately now rare) will eventually progress to advanced phase disease in 3 to 5 years.¹¹

Imatinib is the standard first-line therapy for patients newly diagnosed with chronic phase CML, based on its successful induction of durable responses in most patients.¹²⁻¹⁵ However, some patients with chronic phase disease either have primary resistance to imatinib or develop resistance, typically from the acquisition of point mutations in the *ABL* tyrosine kinase domain of the *BCR/ABL* gene. Once disease progresses to advanced phase, responses to imatinib are far less frequent than in the chronic phase, and are not generally durable.¹⁶ More potent tyrosine kinase inhibitors (TKIs), such as dasatinib and nilotinib, have been effective in patients with imatinib-resistant or -intolerant chronic phase CML.^{17,18}

A better understanding of the mechanisms of disease progression and resistance to TKIs might lead to the development of curative treatment options for patients with CML. MRD monitoring and mutational analysis during the course of treatment are essential for timely implementation of treatment interventions to overcome resistance or prevent disease progression.^{19,20}

Monitoring Disease Response in CML

Given that all cases of CML harbor the Ph chromosome (and thus, the *BCR-ABL* gene), sensitive monitoring can be used to establish treatment response. This section briefly summarizes methods for monitoring the most clinically significant treatment milestones for patients with CML undergoing TKI therapy.

Hematologic Response: Complete hematologic re-

sponse (CHR) is defined as complete normalization of blood counts with no immature blood cells; leukocyte count less than $10 \times 10^9/L$; platelet count less than $450 \times 10^9/L$; and lack of splenomegaly. Partial hematologic response indicates the presence of immature blood cells; platelet count less than 50% of pretreatment count but more than $450 \times 10^9/L$; or persistent splenomegaly (but $< 50\%$ of pretreatment).

Most patients will experience CHR at 3 months after initial treatment with imatinib. If no CHR occurs at 3 months, patient compliance to imatinib therapy should be evaluated. Treatment interruptions and non-adherence to imatinib are associated with a lower probability of achieving cytogenetic or molecular response. Bone marrow cytogenetics should be repeated to confirm the diagnosis of chronic phase CML.

Cytogenetic Response: Cytogenetic response is determined by the decrease in the number of Ph-positive metaphases, using conventional cytogenetic analysis (CGA) or fluorescence in situ hybridization (FISH; Table 1).^{21–24} Complete cytogenetic response (CCyR) indicates that there are no Ph-positive metaphases. Partial cytogenetic response indicates that 1% to 35% of cells still have Ph-positive metaphases. Major cytogenetic responses (MCyR) include both CCyR and partial cytogenetic responses. However, these categories are only relevant if at least 20 metaphases are evaluated.

Cytogenetic response is an important prognostic factor in CML treatment. Results from the International Randomized Study of Interferon Versus STI571 (IRIS) trial showed that CCyR is associated with less risk for events such as progression to advanced phases of CML and loss of hematologic and cytogenetic responses.²⁵ At median follow-up of 60 months, progression-free survival was better for patients who experienced CCyR or partial cytogenetic response at 12 months than for those who did not have a MCyR at 12 months (97%, 93%, and 81%, respectively; Figure 1A).²⁵ The updated results also confirmed that overall and event-free survival rates at 6 years were significantly better for patients who experienced a CCyR at 6, 12, and 18 months and thereafter.²⁶

After 1 year of imatinib therapy, de Lavallade et al.²⁷ also identified cytogenetic response as the major prognostic factor for overall and progression-free survival. In the retrospective analysis of data from phase II studies of dasatinib in patients with imatinib-resistant chronic phase CML, progression-free survival

was better for those who began taking dasatinib after losing MCyR on imatinib than for those who received dasatinib after loss of hematologic response.²⁸ Although not a randomized trial of early versus late intervention for the loss of imatinib response, this analysis suggests that cytogenetic monitoring and early detection of relapse may improve outcomes in patients with imatinib-resistant chronic phase CML.

Early cytogenetic response to second-generation TKIs can also predict survival and guide subsequent therapy. Tam et al.²⁹ examined this issue in patients with chronic phase CML receiving nilotinib or dasatinib after imatinib failure. After 12 months of treatment, patients experiencing MCyR had a significant survival advantage over those with minor cytogenetic response or CHR. Estimated 1-year progression to accelerated or blast phase were also significantly different between the groups (3% vs. 17%, respectively). Similar observations were made in a recent analysis of pooled clinical trial data of dasatinib treatment in patients with imatinib-resistant/intolerant CML.³⁰ Cytogenetic response at 12 months was highly predictive of progression-free survival at 24 months, but did not have any effect on overall survival. These results suggest that patients experiencing no cytogenetic response at 3 or 6 months after treatment with dasatinib or nilotinib may be considered for alternative therapies.

Molecular Response: Molecular response is measured by the decrease in the *BCR-ABL* transcript levels in the peripheral blood or bone marrow using reverse transcription polymerase chain reaction (RT-PCR; Table 1).^{31–34} Major molecular response (MMR) indicates a 3-log reduction or greater of *BCR-ABL* chimeric mRNA. Complete molecular response occurs when no *BCR-ABL* chimeric mRNA is detectable.

Several studies have reported the prognostic significance of molecular response as reviewed by Jabbour et al.³⁵ In the IRIS trial, the estimated progression-free survival rate at 24 months was 100% for patients with CCyR and at least a 3-log reduction in the *BCR-ABL* transcript level at 12 months, compared with 95% for those with CCyR and a *BCR-ABL* reduction of less than 3-log at 12 months (Figure 1B).²⁵ Press et al.³⁶ also showed that failure to achieve at least a 2-log reduction in *BCR-ABL* mRNA at the time of CCyR or a 3-log reduction any time thereafter is an independent prognostic factor for progression-free survival. In a recent report, Press

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Table 1 Options for Monitoring Response in CML

Test	Target	Tissue	Sensitivity (%)	Comments
CGA	Ph chromosome	BM	1–10	• Confirm diagnosis of CML • Detects karyotypic abnormalities other than Ph chromosome (i.e., clonal evolution) • Gold standard for determination of cytogenetic response
FISH (interphase or hypermetaphase)	Juxtaposition of <i>BCR</i> and <i>ABL</i>	PB and BM	0.5–5	• Confirm diagnosis of CML • Detects Ph-positive cells, which are undetectable using CGA • Routine monitoring of cytogenetic response in patients who are clinically stable • Routine measurement of minimal residual disease • Useful until Ph chromosome levels are < 5%–10%, but not useful for monitoring further reduction in Ph-chromosome levels • Not validated in IRIS trial
RT-PCR	<i>BCR-ABL</i> mRNA	PB and BM	0.0001–0.001	• Most sensitive method for the measurement of minimal residual disease • Useful for monitoring of molecular response after the patient has achieved CCyR • Strong correlation between results obtained from PB and BM, allowing minimal residual disease monitoring without the need to obtain BM aspirations • Techniques and use of various internal controls are different among institutions and laboratories • Assay and reporting have not been standardized

Abbreviations: BM, bone marrow; CCyR, complete cytogenetic response; CGA, conventional cytogenetic analysis; CML, chronic myelogenous leukemia; FISH, fluorescence in situ hybridization; PB, peripheral blood; RT-PCR, reverse transcription polymerase chain reaction.

et al.³⁷ also reported that a minimal half-log increase in the *BCR-ABL* or loss of MMR predicts shorter relapse-free survival in patients who were in complete cytogenetic remission on imatinib therapy (Figure 2).

Molecular responses also predict the duration of CCyR. Cortes et al.³⁸ reported that major and complete molecular remissions were associated with more durable cytogenetic responses. A significantly lower portion of patients (5% with MMR and 4% with complete molecular remission) lost the CCyR, compared with 37% who did not reach these levels of molecular response.

The GIMEMA study group reported similar findings.³⁹ In patients who received imatinib after interferon failure, those who attained MMR at 12 months and those who experienced a MMR at first CCyR remained in complete cytogenetic remission

for a longer period. Only 3% of these patients lost their CCyR compared with 30% of patients who did not attain this level of molecular response.³⁹

Although early molecular response is a predictor of durable long-term remission rates and progression-free survival, some studies suggest that it does not predict a long-term survival advantage. In patients experiencing CCyR at 12 or 18 months, achievement of molecular response at these points did not affect progression-free or overall survival.^{27,40} Marin et al.⁴¹ also confirmed that patients who did not have an MMR at 18 months had a higher chance of losing CCyR, this did not translate into a difference in progression-free survival.

Kantarjian et al.⁴² recently analyzed the significance of rising *BCR-ABL* transcript levels as deter-

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Figure 1 Rate of progression to the accelerated phase or blast crisis based on cytogenetic response after 12 months or molecular response after 18 months of imatinib therapy. From Druker BJ, Guilhot F, O'Brien SG, et al. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N Engl J Med* 2006;355:2413; with permission.

mined with quantitative RT-PCR. Of 258 patients with chronic phase CML on imatinib therapy, 116 in CCyR experienced an analytically significant increase in *BCR-ABL* transcript levels on at least 2 occasions. Only 13 of these patients experienced CML progression. Of these 13, 11 patients had either lost an MMR or never achieved it and had a greater than 1-log increase in the *BCR-ABL* transcript level, suggesting that caution should be used when interpreting rising

BCR-ABL levels in patients with CCyR.

Early molecular response to second-generation TKIs can also predict survival and guide subsequent therapy. Branford et al.⁴³ and Milojkovic et al.⁴⁴ showed that measuring *BCR-ABL* transcript levels at 3 months after the switch could predict response to second-generation TKIs, thus providing further information on the value of continuing treatment with these agents.

Figure 2 A half-log increase in BCR-ABL RNA levels predicts shorter relapse-free survival. A) Cumulative rate of relapse-free survival (Kaplan-Meier method) for subjects with increasing BCR-ABL RNA levels (> 0.5 log; $n = 42$) versus those with stable or declining BCR-ABL RNA ($n = 48$). From Press RD, Galderisi C, Yank R, et al. A half-log increase in BCR-ABL RNA predicts a higher risk of relapse in patients with chronic myeloid leukemia with an imatinib-induced complete cytogenetic response. Clin Cancer Res 2007;13:6140, with permission. B) Loss of major molecular response (MMR) predicts shorter relapse-free survival. Cumulative rate of relapse-free survival (Kaplan-Meier method) for the subset of patients ($n = 76$) experiencing MMR, split into those with ($n = 33$) and without ($n = 43$) subsequent loss of MMR (to < 2.5 -log drop). From Press RD, Galderisi C, Yank R, et al. A half-log increase in BCR-ABL RNA predicts a higher risk of relapse in patients with chronic myeloid leukemia with an imatinib-induced complete cytogenetic response. Clin Cancer Res 2007;13:6141, with permission.

Several studies have examined the implications of monitoring BCR-ABL after hematopoietic stem cell transplantation (HSCT). Radich et al.⁴⁵ reported that patients who had early PCR-positivity (6 or 12 months after HSCT) had a 42% risk for experiencing disease relapse compared with only 3% in patients who tested PCR-negative. This study also showed that early PCR-positivity is associated with more aggressive disease and a high risk for relapse. The prognostic significance of monitoring BCR-ABL transcripts after allogeneic HSCT was also confirmed in subsequent trials.^{46,47} Thus, monitoring MRD after HSCT may help identify the subgroup of patients who may benefit from alternative treatment options.

Currently, no specific guidelines exist for changing therapy based on rising BCR-ABL transcripts detected with RT-PCR. However, close monitoring is essential for these patients. Changes of therapy based solely on a rising BCR-ABL level should occur only in the context of a clinical trial. Table 2 provides NCCN recommendations for monitoring MRD.

Mechanisms of Resistance to Imatinib

Primary hematologic resistance (failure to experience hematologic response within 3–6 months of

initiation of treatment) is very rare in patients with newly diagnosed chronic phase CML. Primary cytogenetic resistance (failure to experience any level of cytogenetic response at 6 months, MCyR at 12 months, or CCyR at 18 months) is evident in 15% to 25% of patients. Secondary resistance is defined as the subsequent loss of response after initial response to imatinib. Mutations in BCR-ABL kinase domain are the main mechanism of resistance to imatinib in the chronic phase. Recent findings suggest that secondary resistance to imatinib is multifactorial, and several other mechanisms of resistance independent of BCR-ABL kinase domain mutations have been reported.⁴⁸

Pharmacologic Mechanisms: Alpha-1-glycoprotein binds to imatinib, and excessive binding has been shown to alter its pharmacokinetics.⁴⁹ Imatinib plasma levels have been shown to correlate with response to therapy in patients taking a standard dose of imatinib.^{50,51} In the IRIS study, maintaining an imatinib trough level at or higher than 1000 ng/mL was important for achieving CCyR.⁵⁰ Early dose adjustments might improve clinical responses in patients with low plasma levels of imatinib. However, Ault et

al.⁵² recently reported that plasma levels of imatinib had no correlation with response to therapy in patients on different dose schedules. The clinical value of monitoring plasma levels of imatinib remains to be clearly defined.

Aberrant expressions of drug transporters also contribute to resistance through altering the intracellular concentration of imatinib.⁵³ Overexpression of the multidrug resistance gene *MDR1* decreases the intracellular concentration of imatinib, leading to *BCR-ABL* gene amplification, which confers resistance to imatinib.⁵³ Higher activity of the human organic cation transporter-1 (OCT-1) is associated with excellent molecular response irrespective of dose, whereas response was highly dose-dependent in patients with low OCT-1 activity.^{54,55} However, cellular uptake of dasatinib or nilotinib seems to be independent of OCT-1 expression.^{56,57} Thus, patients with low-OCT-1 expression might have better outcomes with dasatinib or nilotinib.

The cytochrome P450 enzymes, CYP3A4 or CYP3A5, predominantly metabolize imatinib in the liver. Drugs that induce CYP3A4/5 enzyme levels are known to decrease the therapeutic concentrations of imatinib.⁵⁸ CYP3A4/5-inducing drugs, such as anticonvul-

sants and steroids, should be used with caution in patients receiving imatinib, and appropriate alternatives should be explored to maximize treatment outcome.

BCR-ABL Kinase Domain Mutations: The most common mechanism of resistance is the reactivation of *BCR-ABL* activity. This occurs most often through mutations in the *ABL* tyrosine kinase domain of *BCR-ABL* gene, and less frequently through *BCR-ABL* gene amplification or increased *BCR-ABL* gene expression.^{59,60} In patients who achieved very low levels of *BCR-ABL* transcripts, those who subsequently experienced a more than 2-fold increase in *BCR-ABL* levels had a higher risk for emergence of *BCR-ABL* mutations than those with stable or decreasing *BCR-ABL*.⁶¹

Point mutations in the *ABL* kinase domain are emerging as the most frequent mechanism of resistance. In a large study of 319 patients with chronic phase CML, Khorashad et al.⁶² found that kinase domain mutations were the only independent predictors of the loss of CCyR, and these patients had a higher risk for progression than those without a mutation. Some studies have linked mutations in the ATP phosphate-binding loop (P-loop) to poor prognosis and high risk for progression in patients

Table 2 NCCN Recommendations for Monitoring Response and Mutational Analysis

Test	Indication
Cytogenetics	• BM cytogenetics at diagnosis to establish disease stage. If collection of BM is not feasible, FISH on a PB specimen using dual probes for the <i>BCR</i> and <i>ABL</i> genes is an acceptable method for confirming the diagnosis of CML • At 6, 12, and 18 months from initiation of therapy to assess response to imatinib, until CCyR. If CCyR is experienced at either of the earlier time points, then cytogenetics do not need to be repeated • After CCyR, every 12 months to look for newly acquired cytogenetic changes (optional) or anytime <i>BCR-ABL</i> increases
RT-PCR for <i>BCR-ABL</i> transcript levels	• At diagnosis to establish baseline <i>BCR-ABL</i> level and every 3–6 months after CCyR is reached after imatinib therapy, to monitor major molecular response (≥ 3-log reduction) or increase in <i>BCR-ABL</i> • If a rising level of <i>BCR-ABL</i> transcripts (1-log increase) with a major molecular response is present, RT-PCR analysis should be repeated in 1 to 3 months; if no major molecular response occurs, then BM cytogenetics is recommended • Quantitative RT-PCR monitoring (every 3 months for 2 years, then 6 months for 3 years) after cytogenetic remission after allogeneic HSCT
<i>ABL</i> tyrosine kinase domain point mutation analysis	• Before initiation of treatment for advanced-phase disease • If there is inadequate initial response (failure to experience CHR at 3 months, minimal cytogenetic response at 6 months, or MCyR at 12 months), any sign of loss of response, or an increase in <i>BCR-ABL</i> transcript levels

Abbreviations: BM, bone marrow; CCyR, complete cytogenetic response; CHR, complete hematologic response; CML, chronic myelogenous leukemia; FISH, fluorescence in situ hybridization; HSCT, hematopoietic stem cell transplantation; MCyR, major cytogenetic response; PB, peripheral blood; RT-PCR, reverse transcription polymerase chain reaction.

receiving imatinib,^{63–66} but other studies could not confirm this finding.⁶⁷ However, dasatinib induced comparable MCyR rates in imatinib-resistant patients irrespective of the presence of P-loop or other mutations.^{17,68,69} Branford et al.⁶⁶ observed that although patients with accelerated phase CML had a higher incidence of P-loop mutations, the difference in frequency of mutation was significant between early chronic and accelerated phase, compared with that between accelerated and late chronic phase.

The presence of T315I mutation confers the highest resistance to imatinib, dasatinib, and nilotinib.^{70,71} Jabbour et al.⁷¹ reported that survival of patients with T315I depends on disease stage. Unsurprisingly, survival is similarly poor among patients in accelerated and blast phase with or without the T315I mutation.

BCR-ABL–Independent Pathways of Resistance:

Additional chromosomal abnormalities, a process known as *clonal evolution*, develop in approximately 80% of patients with CML. Clonal evolutions are considered a feature of advanced phase CML, and patients with clonal evolution are more likely to harbor *BCR-ABL*–independent mutations. In patients with chronic phase CML treated with imatinib, O'Dwyer et al.⁷² identified clonal evolution and failure to achieve MCyR by 6 months as adverse prognostic factors for relapse. Jabbour et al.⁶⁷ also reported that development of clonal evolution is associated with worse survival; mutational status had no impact on survival. However, clonal evolution is associated with a better prognosis when considered the only criteria for disease progression compared with other accelerated features.^{73,74}

Together, these data suggest that mutational analysis would be helpful in identifying subgroups of patients that demand careful monitoring, because these patients are at a higher risk for progression, and second-line TKIs are available for patients who become resistant to imatinib. Table 2 provides NCCN recommendations for mutational analysis.

CML Stem Cells: CML is one of the best examples of a “stem cell” malignancy, first described by Fialkow et al.⁷⁵ more than 3 decades ago. More recently, increasing data have attempted to better define the clinical implications of CML stem cells in hopes of developing more selective treatments for these malignancies.^{76–79}

CML stem cells seem to share many common characteristics with their normal hematopoiesis-maintaining counterparts. These characteristics include the ability to undergo orderly differentiation and to self-renew. Moreover, the CML stem cell population is relatively small, is normally maintained in a quiescent state, and has relatively high expression of both aldehyde dehydrogenase (ALDH) and ATP-binding cassette transporters and low expression of OCT-1.⁸⁰ Altogether, these characteristics contribute to cytotoxic drug resistance, which traditionally targets rapidly cycling cells, and resistance to TKIs whose reduced intracellular concentrations might limit their full impact. This important population of cells is clearly distinctive from the bulk of the tumor and helps explain the clinical responses to imatinib. Imatinib is able to quickly stabilize and eliminate most of the tumor in patients with newly diagnosed chronic phase CML, yet is unable to render patients completely free of disease because most still have measurable disease even after many years of treatment.

Interestingly, the clinical responses observed in patients treated with interferon are different. Formerly standard care for medically managing patients with CML, interferon commonly resulted in hematologic stabilization and a gradual reduction in Ph+ hematopoiesis over months to years. Unfortunately, complete cytogenetic and molecular remissions were relatively rare. Nevertheless, when these responses were seen and sustained, not only was discontinuing interferon possible, but many patients remained without recurrence many years off therapy. The dynamics of these responses suggest that in patients with CML, both the bulk of the tumor and the early progenitor population may be differentially sensitive to imatinib versus interferon. This may explain the difference in clinical outcomes between these agents.

Available evidence supports the clinical observations noted earlier: imatinib is effective against differentiated progenitors, but primitive CML stem cells seem resistant to it.^{81–83} At significant concentrations, imatinib seems to suppress the proliferation of primitive stem cells but does not eradicate residual CML stem cells completely.⁸² Furthermore, a differential sensitivity to the effects of imatinib appears to exist within the stem cell compartment, with the inhibitory effect of imatinib in vitro being much more pronounced against the mature CD34+CD38+lin-

stem cells than the primitive CD34+CD38-lin- stem cells.⁷⁷ Angstreich et al.⁸⁴ showed that imatinib was more active against differentiated CML progenitors and that interferon was more active against the primitive CML progenitors,⁸⁴ and raised the question of whether concurrent or sequential use of these agents might optimize the activity of these 2 drugs against their respective targets. The toxic side effects associated with long-term use of interferon are of concern.

The fact that the primitive CML stem cell population has a distinct phenotype (CD34+, CD38-, lin-) compared with the more differentiated progeny (CD34+, CD38-, lin+) may provide unique targets for novel therapies. However, this approach is also likely to have challenges that are not necessarily intuitive. As an example, the importance of the BCR-ABL gene rearrangement, which defines the biology of CML, may have little impact on the functionality of primitive CML stem cells. Data suggest that this CML-defining signal may be either minimally expressed or absent in primitive CML stem cells. Trials performed more than a decade ago showed that primitive progenitors with BCR-ABL rearrangement do not express the fusion protein (P210) and BCR-ABL mRNA.⁸⁵ However, this difference is not distinguished by real-time quantitative PCR assay (using K562 CML cell line as the standard), which showed that BCR-ABL expression was relatively equal between different types of CD34+ progenitors. This finding suggests that BCR-ABL is likely not the best marker for distinguishing or targeting the population of primitive CML cells.

Many new markers have now been identified in early CML progenitors, and these may offer better opportunities to identify and study the primitive stem cell population.⁸⁶ The clinical implications of 2 examples, proteinase 3 (PR3) and preferentially expressed antigen of melanoma (PRAME), are briefly touched on in the following sections.

PR3: One gene that has generated a great deal of interest in CML is the *PR3* differentiation gene. High expression of *PR3* at diagnosis, along with low expression of CD7 and high expression of neutrophil elastase (ELA2), has been identified as a potentially important predictor of longer survival in patients with chronic phase CML.⁸⁷ In patients with advanced phase CML, higher expression of *PR3* and ELA2 in CD34+ progenitors before HSCT is associated with lower incidences of relapse-related death, improved

disease-free survival, and overall survival.⁸⁸ Radich et al.¹⁰ also reported that decreased expression of ELA2 was associated with disease progression. The fact that *PR3* is expressed at higher levels in the more differentiated progenitors compared with early ones may explain the inconsistent clinical findings. **PRAME:** Overexpressed in various solid tumors, *PRAME* is a good prognostic marker of clinical outcome and is generally not found in normal tissues.⁸⁹ Recent reports have shown that *PRAME* is overexpressed in acute and chronic leukemias.⁹⁰⁻⁹² CD34+ cells in advanced phase CML had a higher *PRAME* expression than those in chronic phase.⁹² In an extensive GEP study of patients with CML, Radich et al.¹⁰ showed that up-regulation of *PRAME* is one of the significant prognostic factors associated with disease progression. *PRAME* was expressed in almost all of the patients in blast crisis and only at very low levels in 50% of patients in chronic phase. A trend was also seen toward increased expression in patients with poor response to imatinib, suggesting that *PRAME* expression in patients newly diagnosed with chronic phase CML is an indicator of poor treatment response.⁹³

Together, *PR3* and *PRAME* help illuminate the molecular underpinnings of CML and may offer some rationale for developing targeted agents beyond imatinib. Although no one pathway seems sufficient, combination strategies effectively targeting pathways active in the early stem cell progenitors may begin to indicate an eventual cure for CML.

Conclusions

Imatinib and other TKIs targeting BCR-ABL are very effective in treating the bulk of the tumor mass and produce dramatic clinical responses. However, the FDA-approved TKIs do not seem to completely eradicate all CML cells.

Targeting the residual tumor population may hold promise in improving the responses seen with imatinib alone. However, the development of these therapies has been limited partly by biologic properties that this residual population of cells share with normal hematopoietic stem cells.

New findings revealing molecular characteristics of the CML stem cell population continue to contribute to the understanding of cancer biology and may provide targets for new drugs and approaches to improve clinical outcomes.

Ideally, cancer stem cell-targeted therapies should be used in patients when they achieve MRD.

CML may be an ideal cancer to focus on the impact of imatinib on achieving MRD in most patients. Integrating the concept of CML stem cells into drug development and clinical trial designs may have the potential to completely “cure” patients with CML.

Molecular Markers in Acute Leukemias

Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is associated with variable clinical outcomes, and therefore molecular markers could help stratify patients⁹⁴ and predict relapse. However, none of these molecular markers has reached the same level of agreement as *BCR-ABL* in CML. This report concentrates on the role of the molecular markers in detecting MRD, and hence predicting relapse. Molecular markers in AML can be grouped into 3 categories: fusion genes, gene mutations, and aberrantly expressed genes. The clinical implications of some of the most common markers from each group are discussed briefly.

Fusion Genes: *PML-RAR α* (*promyelocytic leukemia gene/retinoic acid receptor alpha*), *CBF β -MYH11* (*core binding-factor beta gene/myosin heavy chain 11 gene*), *AML1* (*acute myeloid leukemia 1*)-*ETO*, and *MLL* (*mixed-lineage leukemia gene*) are the 4 most common fusion gene transcripts that have been used as targets for detecting MRD using PCR-based assays and to predict clinical outcome after chemotherapy or transplantation in patients with AML.

***PML-RAR α* :** The translocation between chromosomes 15 and 17 fuses the *RAR α* gene on chromosome 17 with the *PML* gene on chromosome 15, resulting in the fusion transcript *PML-RAR α* . This transcript is found in almost all patients with acute promyelocytic leukemia (APL) and its presence after remission is predictive of relapse.

***CBF β -MYH11*:** The fusion transcript *CBF β -MYH11* is a fusion of the *CBF β* gene at chromosome 16q22 with the *MYH11* gene at chromosome 16p13 presenting as *inv(16)/t(16;16)*. Amplification of this fusion transcript using qualitative RT-PCR in bone marrow and peripheral blood has been used to detect MRD and assess the risk for relapse in patients with *inv(16)/t(16;16)*. However, use of the qualitative assay resulted in conflicting results,⁹⁵ suggesting that quantitative RT-PCR may be a more useful tool.

***AML1-ETO*:** The *AML1-ETO* fusion transcript results from the *t(8;21)*, which fuses the *AML1* (also

RUNX1) gene on chromosome 21 with the 8 to 21 (*ETO*) gene on chromosome 8. This fusion transcript can be detected with nested RT-PCR in most patients experiencing long-term remission after conventional chemotherapy or autologous or allogeneic HSCT.⁹⁶ The discrepancy between clinical cure and the presence of MRD found with nested RT-PCR suggests that this qualitative assay was too sensitive. ***MLL*:** The *MLL* gene on chromosome 11q23 is involved in approximately 10% of AML and associated with adverse outcome.⁹⁷ *MLL* can be aberrantly expressed, either from partial tandem duplication or partnering with more than 50 different fusion genes. A subset of patients with aberrant *MLL* may experience long-term remission after conventional chemotherapy. Thus, monitoring MRD would be of great value during follow up of these patients.

Prognostic Significance of Fusion Genes: The predictive value of postconsolidation quantitative PCR in patients with APL has been shown in several prospective trials.⁹⁸ The technique has to be able to detect a *PML-RAR α* -positive cell among 103 to 104 normal cells. However, physicians should refrain from basing clinical decisions on earlier postinduction, molecular results because these did not correlate with outcome.

Molecular remission has become the therapeutic objective in APL and is used as a surrogate marker for survival. Furthermore, molecular monitoring is also used after completion of treatment because molecular relapse precedes morphologic relapse. However, the frequency of monitoring is still not established. Some groups tend to use the pretreatment clinical characteristics to guide the timetable for molecular monitoring (e.g., more frequent monitoring for patients with hyperleukocytosis at presentation).

Higher copy numbers of *CBF β -MYH11* assessed using RT-PCR at diagnosis had a significant correlation with high percentage of bone marrow involvement, and appeared to correlate with shorter response duration in 1 group but not in others.^{99,100} Furthermore, patients experiencing complete remission after chemotherapy with 10 or more copy numbers of *CBF β -MYH11* had a significantly shorter response duration and higher risk for disease relapse.⁹⁹ In others, a log rise in transcript level at any stage relative to the remission level was used to predict relapse.^{100,101} The recent finding of *c-Kit* mutations, especially those affecting exon 17, as a predictor for higher re-

lapse risk and shorter survival may explain the early relapse in some patients.^{102,103} No studies have yet analyzed the role of MRD measurement in patients with inv(16)/t(16;16) without *c-Kit* mutation.

Marcucci et al.¹⁰⁴ showed the feasibility of RT-PCR for the quantitative detection of *AML1-ETO* fusion transcript in patients with t(8;21)-associated AML at diagnosis and after high-dose cytarabine and anthracycline-based therapy.¹⁰⁴ All of the 5 patients who had at least 1000 copies of *AML1-ETO* fusion transcript at diagnosis showed a 2- to 4-log reduction in the transcript levels after induction chemotherapy; 1 patient, who had high transcript levels after remission induction chemotherapy, eventually experienced disease relapse. Similar to the studies in inv(16)/t(16;16), a log rise in *AML1-ETO* transcript level at any stage relative to the remission level heralded relapse.^{100,101} These studies confirm the role of quantitative RT-PCR in defining relapse risk. However, the recent finding that *c-Kit* mutations have a negative impact on outcome in t(8;21) AML may explain the early relapse experienced by some of the patients.^{102,103}

Several groups described monitoring MRD in patients with AML with *MLL* aberrations.^{105–108} The use of real-time PCR to quantify *MLL* partial tandem duplication revealed that at least a 2-log reduction 2 months and beyond after the start of therapy was associated with a longer survival, suggesting that measuring MRD in this patient population can be used prognostically.¹⁰⁵ However, molecular relapse preceded clinical relapse by only 35 days. Similarly, for *MLL-AF9* resulting from t(9;11)(p22;q23), patients with negative quantitative PCR test results had lower cumulative incidence of relapse and better overall survival than those with at least 1 positive measure.¹⁰⁸ As in patients with *MLL* and partial tandem duplication, the interval between molecular and clinical relapse was short (4–6 weeks). Therefore, these patients would have to be monitored very closely.

Gene Mutations: Mutations of FMS-like tyrosine kinase 3 (*FLT3*) receptor are detected in approximately 30% of patients with AML.¹⁰⁹ The presence of this mutation, either in the form of internal tandem duplication (ITD) or a point mutation in the activation loop, is associated with a high risk for relapse in patients with normal karyotype, whereas the mutation does not affect the complete response rate.¹¹⁰ In patients with normal karyotype AML, the presence of *FLT3* receptor mutation correlates

with higher risk for relapse compared with *FLT3*-negative disease.

FLT3 was used to detect MRD, concentrating on patients with normal karyotype AML for whom no other marker is available.¹¹¹ However, not all patients who present with *FLT3* ITD/point mutation maintain this aberration at relapse, suggesting that *FLT3* aberrations are unstable.^{112–114} Therefore, using *FLT3* in following up patients for MRD and predicting relapse may be misleading.

Insertion mutations in exon 12 of the nucleophosmin (*NPM1*) gene is a characteristic feature of a large group of patients with AML with normal karyotype.⁹⁴ Its main role in predicting better outcome is reserved for patients with normal karyotype AML whose cells do not carry *FLT3* aberrations.^{115,116} Several groups explored the validity and prognostic significance of MRD detection using quantitative PCR in patients with *NPM1*.^{117,118} After patients experienced complete remission, the presence of detectable mutations predicted relapse if no further chemotherapy was given. Furthermore, failure to achieve a 2-log reduction after consolidation therapy predicted shorter overall and relapse-free survival.¹¹⁹ Interestingly, *NPM1* mutations are more stable than *FLT3* aberrations, and therefore are more suitable for monitoring MRD.¹¹⁹

Aberrant Gene Expressions: For patients with no specific marker, the Wilms' tumor 1 (*WT1*) gene, located on chromosome 11p13, has been suggested as a marker for MRD because it is detected in 75% to 100% of patients with AML.¹²⁰ *WT1* gene transcripts have been reported to be useful molecular targets for MRD detection using RT-PCR-based assays by some experts, but not others.^{121–129} In one study, patients with fewer than 1000 copies of *WT1* after induction and second consolidation chemotherapy had significantly better disease-free and overall survival.¹²³ Interestingly, when comparing *WT1* and *NPM1* during follow-up in patients with AML, *WT1* decreased rapidly after induction but maintained residual levels after treatment in patients experiencing complete remission, whereas *NPM1* showed mild reduction after induction but was undetectable in long-term survivors.¹³⁰

Acute Lymphoblastic Leukemia

The Ph chromosome resulting from t(9;22) is detectable in approximately 20% to 30% of adult patients with acute lymphoblastic leukemia (ALL), with the

incidence rising to more than 50% in patients aged 50 years or older, and is associated with adverse prognosis.¹³¹ Both fusion proteins of the *BCR-ABL* gene (p210 and p190) are equally expressed in adult patients with Ph+ ALL, and the presence of p190 may help distinguish between CML in lymphoid blast crisis and Ph+ ALL.

The most common method to detect MRD in ALL relies on the clonal rearrangement of the immunoglobulin (*IG*) and *TCR* genes, whose junctional regions are unique to the leukemic clone.¹³² Thus, the presence of rearranged genes is typically screened using PCR primers matched to the opposite sides of the junctions, to the V and J regions of various *IG* and *TCR* genes. Once an apparently clonal rearrangement is detected, the PCR products are then used for direct sequencing of the junctional regions of the *IG/TCR* gene rearrangements, which is then used to design junctional region-specific, or *allele-specific*, oligonucleotides.¹³²

Most (>95%) B-lineage ALL (B-ALL) cases have *IG* gene rearrangements.^{133,134} Interestingly, cross-lineage *TCR* gene rearrangements also occur in up to 90% of B-ALL cases.¹³⁵ *TCR* genes are rearranged in most cases of T-lineage ALL (T-ALL).¹³⁶ Cross-lineage *IG* gene rearrangements occur in approximately 20% of T-ALL.¹³³ Despite these caveats, monitoring MRD with PCR using either *IG* or *TCR* gene rearrangements is feasible and commonly used. As with fusion transcripts in AML, the most frequently used technique is real-time quantitative PCR.¹³⁷

In adult ALL, one group showed that 23% of B-ALL lacked a clonal marker for PCR analysis.¹³⁸ Another group showed that 10% lacked this marker,¹³⁹ with 7% having none detected and 3% having clonal markers that were not suitable for MRD quantification. These results show that it's feasible to detect MRD through *IG/TCR* gene rearrangements in adult ALL.

The *BCR-ABL* fusion gene transcript has been used as a specific target for detecting MRD in adult patients with Ph+ ALL.^{140,141} Detection of *BCR-ABL* transcripts after chemotherapy or transplantation is associated with a high risk for relapse.¹⁴² Stirewalt et al.¹⁴³ identified MRD detection and p190 transcript expression posttransplant as risk factors for relapse. In a more recent study, rapid molecular response after chemotherapy in combination with imatinib was not associated with more favorable prognosis in pa-

tients with Ph+ ALL.¹⁴⁴ Secondly, although a single observation of elevated MRD predicted relapse, allogeneic HSCT seemed to be able to overcome that adverse effect.

Ikaros zinc finger transcription factor (*IKZF1*) plays an important role in differentiation and proliferation of all lymphoid lineages (T- and B-cell). Deletions of *IKZF1* gene are found in Ph+ ALL and lymphoid blast crisis but not in myeloid blast crisis or chronic phase CML.¹⁴⁵ Recently, Paulsson et al.¹⁴⁶ reported that the *IKZF1* gene was deleted in 27% of adult patients, compared with only 9% of childhood cases. The deletions are associated with resistance to TKIs, worse clinical outcomes, and higher relapse rates. Mullighan et al.¹⁴⁷ also reported that the deletion of *IKZF1* is found in 83.7% of patients with *BCR-ABL*-positive ALL. The deletions are acquired as CML transforms from chronic phase to lymphoid blast crisis.

IKZF1 gene, located on chromosome 7p12, can give rise to isoforms with and without DNA binding properties. All isoforms have 2 C-terminal zinc finger motifs but differ in the number of N-terminal motifs, which dictates the sequence specificity and DNA affinity.¹⁴⁸ Ikaros proteins with fewer than 3 N-terminal zinc finger motifs cannot bind to DNA, and play a dominant negative role in the transcription. *IK6* is a dominant negative isoform because it lacks all of the 4 N-terminal zinc finger motifs. In patients with Ph+ ALL treated with dasatinib and imatinib, *IK6* isoform was dominant in 49%.¹⁴⁹ The expression of *IK6* also correlated with the *BCR-ABL* transcript levels, and overexpression was associated with resistance to both dasatinib and imatinib. *IKZF1* expression provides insight into the evolution of the disease and might be valuable in identifying patients destined to progress from chronic phase to lymphoid blast crisis.

Conclusions

Advances in the understanding of the molecular biology of leukemia have led to dramatic changes in monitoring and treatment. In CML, molecular monitoring has now become a fundamental method in defining patient response and detecting resistance. Molecular markers of progression will probably be available soon, and inroads to understanding the biology of progression will spawn innovative treatments for advanced phase CML. In acute leukemia, the past few years have seen the development of many new markers that are relevant in prognostica-

tion, and potentially in monitoring and treatment. Like CML, treatment algorithms in acute leukemia probably will be defined by the distinct molecular biology of the individual disease.

Molecular Markers in Non-Hodgkin's Lymphoma

The management of patients with non-Hodgkin's lymphoma (NHL) continues to pose challenges because of its clinical, pathologic, and molecular heterogeneity. Clinical prognostic indices, such as the International Prognostic Index (IPI), Follicular Lymphoma International Prognostic Index (FLIPI), and the recently reported Mantle Cell Lymphoma International Prognostic Index (MIPI), are powerful tools for establishing prognosis at diagnosis.^{150–152} The clinical prognostic indices are based on easily obtained patient characteristics, such as age, performance status, stage of disease, serum lactate dehydrogenase (LDH) levels, and the number of extra nodal sites involved. Clinically based prognostic indicators remain the powerful prognostic tools available; however, considerable variability in outcomes remains within each risk category. Molecular markers provide additional prognostic information that can further augment the usefulness of clinically based risk stratification.

Methods of Detection of Molecular Markers

Immunophenotypic Analysis: Immunophenotypic analysis (IPA) can be performed using either immunohistochemistry or flow cytometry. Both methods rely on the availability of antibodies that can identify the protein of interest. The choice between immunohistochemistry and flow cytometry depends mainly on the expertise, type of tissues received, and resources available to the hematopathologist. In some cases, flow cytometry and immunohistochemistry are complementary diagnostic tools.¹⁵³

IPA is the most widely used method for detecting molecular markers that are proteins in lymphoid malignancies. The major advantage of immunohistochemistry is its capacity to analyze formalin fixed paraffin-embedded (FFPE) tissues and simultaneously provide morphologic analysis. However, variability in tumor preservation, fixation techniques, detection systems, and assay interpretation may result in discordant observations. Standardization of tissue processing and immunohistochemical assay is

essential to ensure the reproducibility of results and concordance in the findings from different laboratories.¹⁵⁴ The Lunenburg Lymphoma Biomarker Consortium (LLBC) has shown that semiquantitative immunohistochemistry is feasible and reproducible, but that the rates of concordance between laboratories depend on the particular biomarker.¹⁵⁴

Flow cytometry immunophenotyping (FCI) is also used to identify the expression of prognostic markers in NHL subtypes, in addition to its well-established use in diagnosing lymphomas.^{155,156} Flow cytometry can be performed on peripheral blood, bone marrow aspirates, fresh tissue biopsies, and all types of body fluids. The advantages of flow cytometry include its ability to simultaneously examine multiple parameters of single cells; analyze antigen with antibodies that cannot be used on FFPE tissue; provide quantitative analysis of the expression level; and work with small samples. Flow cytometry is very valuable for determining clonality in primary diagnosis and in staging studies.¹⁵⁷ The major disadvantage of flow cytometry is its requirement of fresh, unfixed tissue samples for analysis and the lack of morphologic information.

Cytogenetic Analysis: CGA and FISH are used in certain circumstances to identify the specific chromosomal translocations and copy number alterations that are used as prognostic markers in particular NHL subtypes.¹⁵⁸ CGA has to be performed on cultures of viable cells. In some cases CGA is uninformative because of low mitotic indices and poor chromosome morphology.

FISH is used as an alternative method to identify chromosomal abnormalities that are not detectable by CGA or when CGA is not performed.^{159,160} FISH can be performed on peripheral blood specimens, bone marrow aspirates, or fixed or sectioned tissue samples. Unlike CGA, FISH can be performed both on interphase and metaphase cells. Interphase FISH is particularly useful when metaphase cytogenetics may be difficult in the largely quiescent cells of some lymphoid malignancies, and has been used to detect t(14;18) translocation, *BCL6* gene rearrangements, and many other abnormalities.^{161,162} The major limitations of FISH include its inability to detect more than a few genetic abnormalities simultaneously. The advent of newer methods, such as multicolor FISH and spectral karyotyping, allow simultaneous visualization of the entire karyotype.¹⁵⁸

Comparative genomic hybridization (CGH) is an alternative technique used to detect genomic imbalances.¹⁶³ The unique advantage of CGH is its capacity to screen the entire genome for DNA copy number changes without using living cells. Array-CGH is performed on arrayed sequences of genomic DNA instead of metaphase chromosomes. Currently, DNA arrays for CGH can be constructed with very high-density coverage of the genome, resulting in a very high resolution platform for genome-wide detection of chromosomal abnormalities.^{7,164}

PCR: PCR assays have been used to detect some of the common genetic abnormalities associated with lymphoid malignancies and to quantitate target gene expression for the development of gene expression-based prognostic models. Molecular diagnostic analyses can be performed on a small amount of tissue and on archival paraffin-embedded material.

In some instances, it is preferable to perform PCR on complementary DNA (cDNA) rather than genomic DNA, a procedure called *RT-PCR*. In *RT-PCR*, mRNA is first transcribed to cDNA, which is used as a template for subsequent PCR amplifications. Quantitative *RT-PCR* is a sensitive method for detecting mRNA levels. PCR assays can be used to identify translocations that are strongly associated with certain lymphomas when the breakpoints are sufficiently clustered, such as the major and minor break point cluster regions involved in the t(14;18) translocation.^{3,165,166} However, PCR analysis is not suitable for detecting *BCL6* gene rearrangements because of the involvement of a large number of translocation partners. *RT-PCR* is often applied in the setting of novel fusion genes, such as the *NPM-ALK* seen in anaplastic large cell lymphoma. In these cases, normal RNA processing excises introns and enables the use of primers for the coding regions of the 2 partner genes.

In real-time PCR, the amplified DNA is quantified as it accumulates in the reaction after each amplification cycle.¹⁶⁷ This methodology allows the combination of DNA amplification and product detection into 1 homogenous assay thus eliminating the need for post-PCR processing. Real-time PCR is the method of choice for performing quantitative PCR. The major advantage of this assay is its high sensitivity and accuracy and wide dynamic range for quantifying DNA and transcript levels and fast turnaround time.

GEP: GEP is a powerful tool to identify multiple molecular markers simultaneously in malignant lymphomas.¹⁶⁸ GEP is usually performed with a microarray using RNA isolated from tissues, labeled with a detectable marker, and allowed to hybridize to gene-specific probes on the array. The fluorescence intensity on each gene-specific probe corresponds to the level of expression of the corresponding transcript. Supervised and unsupervised methods are used for data analysis. Quantitative real-time *RT-PCR* is considered a standard assay for validating microarray data.

The Microarray Gene Expression Data Society developed standards for data reporting, known as minimal information about a microarray experiment (MIAME).¹⁶⁹ Concern has been expressed about the reliability and reproducibility of GEP experiments and the feasibility of cross-platform comparison of results. With improvement of microarray technology, studies that are carefully performed under strictly controlled conditions are highly reproducible using the same array platform.^{170,171} Cross-platform comparison is more difficult; however, recent large multicenter studies have found that the results are reasonably comparable even across different platforms.^{170,171} It is now recognized that although the measurement of individual genes may have substantial variations, the comparison of groups of genes is much more robust.¹⁷² These gene groupings are referred to as *gene expression signatures* and represent coordinated gene expression involved in specific biologic processes, such as proliferation or signaling. Therefore, properly and carefully performed microarray studies represent a useful approach for GEP.

Gene signatures identified by GEP may include the following groups:

- Signatures dividing tumors into biologic subgroups (e.g., subgroups of DLBCL) based on cell of origin (germinal center–derived vs. activated B-cell–derived) or based on certain functional characteristics, such as high level of BCR activation or oxidative phosphorylation.
- Signatures representing certain biologic functions of the tumor, such as proliferation or NF- κ B activation.
- Signatures with relevance to host/tumor interaction, such as angiogenesis, expression of major histocompatibility complex (MHC) molecules, or stromal cell composition.

- Signatures predictive of response to therapy and outcome.

Although GEP looks promising, it is not yet ready for widespread clinical use because the methodology has not been standardized, findings require further validation, and optimal platforms have not been determined.

DLBCL

DLBCL is the most common aggressive subtype of NHL worldwide. The relative incidence has substantial geographic variation; for example, DLBCL accounts for approximately 30% of cases of NHL in the United States and Europe, but 46% of cases in Hong Kong and 59% in the United Arab Emirates. DLBCL is characterized by various genetic abnormalities. Recurring chromosomal translocations occur in 50% of cases. The identification of specific chromosomal translocations and the corresponding protein expression has led to many novel molecular markers that have the capacity to predict outcome in patients with DLBCL.^{173,174}

GEP: GEP has been used to identify 3 biologically and clinically distinct subtypes of DLBCL: germinal center B-cell (GCB) subtype, activated B-cell (ABC) subtype, and primary mediastinal DLBCL.^{175–177} The gene expression signature of the GCB subtype is characteristic of that of GCBs. In contrast, the gene expression signature associated with the ABC subtype is characterized by overexpression of a set of genes overexpressed in mitogen-activated B cells.¹⁷⁶

Patients with GCB subtype have a significantly better overall survival after chemotherapy than those with ABC subtype. The 5-year survival rate differed significantly between the GCB and ABC subtypes (62% vs. 26%, respectively) after CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone)-like chemotherapy,¹⁷⁶ a finding similar to that in an earlier report by Alizadeh et al.¹⁷⁵ Even among patients who were at low risk according to IPI, those with the ABC subtype had a distinctly worse survival than those with the GCB subtype. Moreover, ABC cell lines and primary ABC-DLBCL samples also showed increased activity of the NF- κ B signaling pathway, which could potentially block the apoptosis induced by chemotherapy and thus account for poor survival.

In addition to the GCB versus ABC distinction, Rosenwald et al.¹⁷⁸ identified 3 additional gene expression signatures, which included the MHC class II gene-expression signature, the proliferation signa-

ture, and the lymph node signature. High expression of lymph node signature is associated with improved survival in patients treated with CHOP or CHOP-like chemotherapy. The loss of MHC class II gene and protein expression and high expression of the proliferation signature have been shown to be predictive of poor survival in patients with DLBCL.¹⁷⁹

Lenz et al.^{180,181} recently re-examined the prognostic model in patients treated with CHOP-like chemotherapy combined with rituximab and reported a predictive model created from 3 gene-expression signatures (GCB, stromal-1, and stromal-2). In this analysis, patients with the GCB subtype still showed better survival than those with the ABC. High expression of stromal-1 signature was associated with favorable outcome and reflected extracellular matrix deposition and infiltration by histiocytic innate immune cells. In contrast, the stromal-2 signature was indicative of unfavorable prognosis and reflected angiogenesis and tumor blood vessel density. These results must be confirmed in another dataset or prospective trial. CALGB is conducting a prospective assessment of GEP in DLBCL in conjunction with a trial comparing R-CHOP with dose-adjusted EP-OCH-R (etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin, rituximab).

Several investigators have used the information generated by GEP studies to develop prediction models based on more commonly used techniques, such as immunohistochemistry and PCR-based methods.^{182–185}

Hans et al.¹⁸² used immunohistochemistry to identify the 2 subtypes of DLBCL. GCB subtype was identified by either of the following immunophenotypes: 1) CD10-positive, *BCL6* and *MUM1*-negative; or 2) CD10-negative, *BCL6*-positive, and *MUM1*-negative. The lack of both CD10 and *BCL6* or *MUM1* positivity indicated a non-GCB subtype, which in turn was associated with poor overall survival. The 5-year overall survival data (76% for the GCB group vs. 34% for the non-GCB group) were similar to those reported using GEP. Although the combined analysis of CD10, *BCL6*, and *MUM1*, according to the algorithm, may be a surrogate for the GEP classification of GCB and ABC subtypes, there is a misclassification rate of 14%. In an attempt to improve the accuracy of the Hans algorithm, Choi et al.¹⁸⁶ added 2 new antibodies that help distinguish between the ABC and GCB subtypes. The impact of

BCL6 in this algorithm is reduced and the misclassification rate has improved to 7% versus 14% for Hans's algorithm.

Lossos et al.¹⁸³ created a 6-gene predictive model using the real-time RT-PCR platform.¹⁸³ The expression of *FN1* along with *LMO2* and *BCL6* (both of which are highly expressed in the GCB subtype) correlated with prolonged survival. The other 3 genes (*BCL2*, *CCND2*, and *SCYA3*) are often highly expressed in the ABC subtype, and their high expression is associated with poorer outcome.

Studies based on immunohistochemistry have produced conflicting results, with the study by Fu et al.¹⁸⁷ reporting that the addition of rituximab to CHOP-like chemotherapy improved overall survival for both GCB and ABC subtypes, but the GCB subtype still had a more favorable 3-year overall survival rate than the ABC subtype (85% vs. 69%, respectively). In contrast, Nyman et al.¹⁸⁸ showed that GCB subtype defined by immunohistochemistry had a significantly better survival rates than the non-GCB subtype in patients treated with chemotherapy, whereas the GCB subtype lost its prognostic significance with the addition of rituximab to chemotherapy. A recent GEP study of 233 cases by Lenz et al.¹⁸⁹ indicates that the GCB subgroup continues to have better survival than the ABC subgroup, even with the addition of rituximab (86% vs. 68%, respectively).

Single Prognostic Markers: Many single markers have been proposed as prognostic indicators, with some useful for distinguishing between the GCB and ABC subtypes.¹⁷³ For example, *GCET2(HGAL)* and *LMO2* are very strongly associated with the GCB subtype, whereas *FOXP1*, when it is highly expressed is a good marker for the ABC subtype. Similarly, a high level of expression of *BCL2* helps identify cases with the ABC signature. *PKC-beta* and *cyclin D2* also indicate an ABC derivation. *BCL2*, *BCL6*, and *TP53* genes and their protein expression are probably the most intensively studied markers.

***BCL2*:** *BCL2* is an antiapoptotic protein that is up-regulated by the t(14;18) translocation, which juxtaposes the *BCL2* gene on chromosome 18 with the IgH locus on chromosome 14. *BCL2* protein is expressed in approximately 40% to 60% of patients with DLBCL. The *BCL2* gene rearrangement with t(14;18) has been detected in approximately 20% of patients with DLBCL. This translocation occurs in approximately one third of patients with GCB-DLBCL, but

not in those with ABC.^{190,191} This translocation also correlates with the expression of *BCL2* protein and mRNA in the GCB subtype. However, *BCL2* is also highly expressed in the ABC subtype in the absence of the t(14;18) translocation, indicating that the mechanism of *BCL2* upregulation in this subtype is independent of t(14;18).

High *BCL2* expression was shown to be more frequent in patients with stage III to IV disease, resulting in reduced disease-free and overall survival in patients treated with anthracycline-based chemotherapy alone.¹⁹² However, Iqbal et al.¹⁹³ recently reported that the prognostic significance of *BCL2* expression in patients treated with chemotherapy alone is subtype-dependent. In the GCB subtype, no difference in overall survival was seen between the *BCL2*-positive and -negative groups, whereas *BCL2* expression was associated with poor survival in the ABC subgroup. Hence, *BCL2* expression could be a marker for more aggressive disease in the ABC-DLBCL subtype but not the GCB-DLBCL.

Recent reports have shown that adding rituximab to CHOP modulates *BCL2*-associated resistance to chemotherapy.^{194,195} In the GELA study, complete response rates for R-CHOP and CHOP were 78% and 60%, respectively, for patients with *BCL2*-positive DLBCL.¹⁹⁴ The corresponding response rates were 76% and 73%, respectively, for patients with *BCL2*-negative disease. R-CHOP resulted in significantly better 2-year overall survival rates than CHOP (67% vs. 48%, respectively) in patients who were *BCL2*-positive, whereas the overall survival rates were not significantly different between the groups among patients who were *BCL2*-negative (72% for R-CHOP and 67% for CHOP). Taken together, these results suggest that the addition of rituximab offers a significant survival advantage for patients who are *BCL2*-positive, whereas its effect is limited in those who are *BCL2*-negative. However, in a re-analysis of the GELA data comparing R-CHOP and CHOP using a competing risk model, Mounier et al.¹⁹⁶ concluded that rituximab benefited both *BCL2*-positive and -negative patients. Given the conflicting data, selecting patients for inclusion of rituximab based on *BCL2* expression is not appropriate.

***BCL6*:** *BCL6* expression is associated with various chromosomal translocations involving chromosome 3q27. In an earlier report, Lossos et al.¹⁹⁷ identified high expression of *BCL6* gene assessed with quanti-

tative RT-PCR or immunohistochemistry as a favorable factor in patients with DLBCL. In a prospective correlative study, Winter et al.^{198,199} showed that adding rituximab to CHOP improved failure-free and overall survival in patients who were BCL6-negative, but had no effect on those who were BCL6-positive. This study also showed that BCL6 expression loses its prognostic significance with the addition of rituximab to CHOP, a finding that has now been confirmed by the LLBC (Figure 3).²⁰⁰ However, Malumbres et al.¹⁸⁵ showed that higher expression of BCL6 mRNA was associated with improved survival in patients treated with R-CHOP, suggesting that BCL6 mRNA versus BCL6 may have different predictive potential.

TP53: The TP53 tumor suppressor gene plays an important role in cell cycle checkpoint, apoptosis, and DNA damage response. Young et al.²⁰¹ recently reported that TP53 mutations in the DNA-binding loops were associated with poor survival in patients with DLBCL treated with anthracycline-based chemotherapy without rituximab, and that these mutations were also predictive of poor overall survival in the GCB subtype but not the non-GCB subtype. High levels of staining for p53 with absence of p21 staining have been associated with TP53 gene mutations, and have been used as an imperfect surrogate for mutated p53 in some studies.^{202,203} Larger studies are needed to confirm the adverse effect of TP53 mutations identified using sequencing in patients with DLBCL.

p21: p21 is a downstream effector protein of p53. In a prospective correlative study, Aurora et al.²⁰⁴ showed that p21 expression as determined by immunohistochemistry is predictive of a favorable outcome in older patients with DLBCL treated with R-CHOP but not CHOP. The addition of rituximab to CHOP improved 5-year failure-free survival for p21-positive (61% vs. 24% for CHOP) but not p21-negative cases (38% vs. 37% for CHOP). A similar trend was also observed with 5-year overall survival. Among patients treated with R-CHOP, failure-free and overall survival were better for patients who were BCL2-negative and p21-positive compared with those with BCL2-positive and p21-negative phenotype.¹⁹⁸

LMO2: Lossos et al.¹⁸³ identified the LMO2 gene to be a predictor of survival in patients with GCB-DLBCL subtype.¹⁸³ Natkunam et al.²⁰⁵ recently reported that the corresponding protein is expressed in germinal

center-derived lymphomas. In the subsequent study, LMO2 expression was identified as a prognostic factor in patients treated with anthracycline-based regimens with or without rituximab.²⁰⁶ LMO2 expression was significantly correlated with improved overall and progression-free survival; however, the difference in outcome between patients who were LMO2-positive and LMO2-negative was not significantly different among those with high-risk IPI. The combined protein expression of LMO2 and BCL6 correlated with improved outcome in patients treated with CHOP; however, LMO2 expression was the only predictive marker in patients treated with R-CHOP.²⁰⁶

Conclusions: Available evidence suggests that changes in the prognostic profile associated with the addition of rituximab to CHOP likely reflect its impact on survival pathways important to specific subsets of DLBCL, and thereby provide clues to the underlying heterogeneity of the disease.

- The addition of rituximab to CHOP chemotherapy provides greater benefit to patients who are BCL2-positive than those who are BCL2-negative.
- BCL6 expression loses its prognostic significance when rituximab is added to conventional CHOP chemotherapy.
- Patients who are BCL6-negative seem to benefit from rituximab, selectively. The lack of benefit for BCL6-positive cases must be confirmed.
- p21 emerges as a useful prognostic marker in the context of therapy that includes rituximab, and seems to identify a subset of patients who benefit from the addition of rituximab to CHOP.
- The presence of TP53 mutations, especially in the DNA binding loops, is associated with poor survival.
- LMO2 is a strong predictor of improved outcome, and the prognostic significance remains unaffected by the addition of rituximab.
- The prognostic significance of a particular biomarker may depend on the patient population treated and the treatment; therefore, the literature has conflicting reports about the prognostic significance of individual biomarkers.
- Combinations of molecular markers are likely to be more powerful predictors of clinical outcome than single markers.

In the future, biomarker-guided risk-adapted

Figure 3 (A, B) Failure-free survival (FFS) and (C, D) overall survival (OS) according to Bcl-6 expression for cases treated on R-CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone, and rituximab) and CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) induction arms. Analysis excludes patients who were randomized to experience molecular remission. From Winter JN, Weller EA, Horning SJ, et al. Prognostic significance of Bcl-6 protein expression in DLCL treated with CHOP or R-CHOP: a prospective correlative study. *Blood* 2006;107:4210; with permission.

therapy will be possible. Treatment will be tailored to the specific survival pathways unique to the particular disease subset.

Follicular Lymphoma

Follicular lymphoma is the most common indolent subtype of NHL, accounting for approximately 22% of all newly diagnosed cases. Approximately 90% of these have a $t(14;18)$ translocation, which juxtaposes the *BCL2* gene with the *IgH* locus that results in the deregulated expression of *BCL2*. No reliable molecular marker exists to predict the clinical course of follicular lymphoma at diagnosis.

Dave et al.²⁰⁷ identified 2 gene expression signatures to predict survival in patients with untreated follicular lymphoma. The immune response 1 (IR1)

included genes that are expressed in T cells and macrophages, whereas the immune response 2 (IR2) signature included genes preferentially expressed in macrophages and/or dendritic cells. Increased expression of IR1 signature was associated with a more favorable clinical course and prolonged survival. This study highlights the importance of host/tumor interactions in determining survival. Glas et al.²⁰⁸ developed a gene expression profile of 81 genes that can distinguish between indolent and aggressive forms of follicular lymphoma at diagnosis and relapse. Genes that are involved in the cell cycle control, DNA synthesis, and metabolism were up-regulated in the aggressive phase of the disease.

Transformation to DLBCL, which is generally associated with a poor clinical outcome, occurs at

a rate of approximately 2% to 3% per year for at least 15 to 18 years, with the risk for transformation decreasing thereafter.²⁰⁹ Various oncogenic mechanisms for the transformation of follicular lymphoma have been described using GEP.^{208,210–213} Lossos et al.²¹⁰ found that only a subset of transformed follicular lymphoma shows increased expression of cMYC and genes regulated by cMYC. Davies et al.²¹³ reported that transformed follicular lymphoma was predominantly of the GCB phenotype that evolves by 2 pathways; one is similar to antecedent follicular lymphoma and the other has a higher proliferation rate with activation of the cMYC pathway similar to the group described by Lossos et al.²¹⁰ This latter group is also characterized by the presence of several recurrent genetic abnormalities.

In another study, the transformed follicular lymphoma also exhibited a GCB-like profile.²¹¹ The authors identified a 3-gene predictor (*PLA₂*, *PDGFR α* , and *Rab-6*) for transformation, and also showed that inhibition of p38MAPK blocked the growth of t(14;18)-positive cell lines and of transplanted tumors in NOD-SCID mice, suggesting that pharmacologic targeting of p38MAPK may be an effective treatment strategy.

MCL

MCL is believed to have the worst characteristics of both indolent and aggressive NHLs. In general, it is believed to be incurable with conventional chemotherapy and to have a more aggressive clinical course than the indolent lymphomas. In most cases, MCL is characterized by the dysregulated expression of cyclin D1, a cell cycle regulatory protein, that results from t(11;14). Additional genetic alterations have been described in subsets of patients with MCL.²¹⁴ High tumor proliferation indexes (Ki-67), inactivation of *INK4a/ARF* locus, and *TP53* mutations have been associated with shorter survival.²¹⁴

Several GEP studies addressed the heterogeneity of clinical and biologic features in MCL.^{168,215} Rosenwald et al.²¹⁶ identified the proliferation signature as the strongest predictor of survival to date in patients with MCL using GEP of samples derived from 92 patients. The average expression of a signature composed of 20 proliferation signature genes was inversely correlated to survival, with a high statistical significance, and identified patient subsets that differed by more than 5 years in median survival. The authors also showed that it is possible to construct a

predictive model from only 4 proliferation signature genes. These findings suggest that therapeutic modulation of the cell cycle has the potential to prolong survival in patients with MCL. Several groups have shown that the Ki-67 expression according to immunohistochemistry provides a surrogate for the proliferation signature and predicts clinical outcome.^{217–219}

Conclusions

- Molecular markers and gene expression signatures that predict clinical outcomes in patients with NHL have been identified.
- Molecular markers must be re-evaluated in the context of each new treatment strategy, because changes in therapy are likely to alter the prognostic significance of established molecular markers.
- Standardization of methods and centralized consensus review are essential if molecular markers are to be used routinely for patient stratification.
- Molecular markers must be validated in large datasets, preferably in prospective uniformly treated patient cohorts.²²⁰
- GEP has been an important diagnostic tool for classifying DLBCL into at least 3 subtypes that are associated with different prognoses and in the development of gene expression signature models that predict clinical course and survival in patients with follicular lymphoma and MCL. However, this technique is not yet recommended for widespread clinical use. Robust signatures for uncommon groups of NHL are not yet available.
- Development of gene prediction models using routine laboratory techniques, such as quantitative RT-PCR, qNPA, or immunohistochemistry, will facilitate the clinical application of prognostic factors using formalin-fixed or paraffin-embedded tissue samples rather than fresh/frozen biopsy specimens.
- Based on available evidence, biomarker-guided risk adapted therapy is currently recommended only in the context of clinical trials.

MRD in NHL

PCR is used widely for the detection of MRD due to its high diagnostic sensitivity (1 malignant cell among 10^4 – 10^6 normal cells).²²¹ Rare event sensitivity ranges from 10^{-4} to 10^{-6} for the analysis of fusion-gene transcripts; 10^{-5} to 10^{-6} for the analysis of chromosomal translocation; and 10^{-3} to 10^{-4} for the analysis of *IgH* and/or *TCR* gene rearrangements.

Follicular Lymphoma

Detection of t(14;18) translocation: The t(14;18) translocation (resulting in the overexpression of BCL2) is a characteristic genetic marker in approximately 90% of patients with follicular lymphoma and is commonly used for the quantitative detection of MRD using PCR. However, this translocation is not detectable in the remaining 10% to 20% of patients with follicular lymphoma using other available methods, such as FISH, suggesting that other mechanisms are involved in the dysregulated expression of BCL2 in this subset of patients.³

The t(14;18) translocation occurs at multiple breakpoints. The breakpoints on chromosome 14 occur in the heavy chain joining region (J_H) or the heavy chain diversity segments (D_H). Most of the breakpoints on chromosome 18 are tightly clustered into 2 regions: major breakpoint cluster region (MBR), which occurs in approximately 60% of cases, and the minor breakpoint cluster region (mcr), which occurs in approximately 25% of cases.³ PCR assays using MBR/J_H or mcr/J_H primer sets can detect the translocation in 65% of cases.²²² Real-time PCR is more suitable than qualitative PCR for detecting MRD in bone marrow and peripheral blood and in stem cell harvests to assess the efficacy of in vivo purging before autologous stem cell transplant (ASCT).^{166,223} The sensitivity of real-time PCR ranges from 10⁻⁵ to 10⁻⁶. Long-distance PCR assay significantly enhances the detection rate of t(14;18).²²⁴ However, it requires high-quality DNA and is not routinely used in laboratories.

Recent studies have identified the presence of additional breakpoint clusters, referred to as 3' BCL2, 5' mcr, and an intermediate cluster region (icr). Weinberg et al.²²⁵ reported that the frequency of icr breakpoint is significantly higher compared with mcr (13% vs. 5%, respectively) in patients with follicular lymphoma. A higher detection rate for t(14;18) can be obtained through analyzing icr with standard PCR assays, as shown by Albinger-Hegy et al.²²⁶ The multiplex PCR assay can detect the translocation in 88% of patients with follicular lymphoma using primer sets capable of detecting translocations in MBR, mcr, and 2 other specific regions between MBR and mcr.²²⁷

The presence of a clonal rearrangement in non-malignant cells (e.g., BCL2/IgH rearrangement in "normal" individuals) may result in a false-positive

MRD if the sensitivity of PCR assay is extremely high.^{228,229} Residual t(14;18)-positive cells may not be clonogenic cells, and the optimal timing and frequency of monitoring MRD is not known.²³⁰

In an international multicenter, collaborative study, nested PCR was more sensitive than single-round PCR for detecting BCL2/IgH rearrangements, with a false-positive rate of 28%.²³¹ The results of a more recent collaborative study showed that the sensitivity for quantitative PCR had less variation with no false-positives.²³²

Quantitative PCR is potentially more useful in the clinical setting than conventional PCR. The standard or real-time PCR assays will only detect breakpoints within the 2 cluster regions (MBR and mcr). In clinical trials, using PCR assays with primer sets capable of detecting additional BCL2 breakpoint regions is essential for the reliable identification of MRD. In patients with follicular lymphoma in whom t(14;18) translocation is not present (10%–20%) or in whom the breakpoints of the rearrangement occur outside of regions detected by currently used PCR assays, rearrangements of variable, diversity, and joining segments (VDJ) of IgH gene are used for MRD detection.²³³

MRD After Chemotherapy: Molecular assessment of t(14;18)-positive cells in the peripheral blood and bone marrow has been studied over the past 2 decades in follicular lymphoma. In the pre-rituximab era, bone marrow was considered to be a better tissue source than peripheral blood for detecting MRD, because bone marrow is more likely to be positive even after the rapid clearance of t(14;18)-positive cells from peripheral blood.^{234,235} The choice of tissue source is of great significance for monitoring MRD, because the rate of clearance of residual cells varies among tissue compartments (peripheral blood, bone marrow, and lymph nodes). The clearance of residual tumor cells from the tested site must reflect the systemic disease burden for the reliable identification of MRD.

In the chemotherapy era, half or fewer patients with follicular lymphoma remained PCR-positive after a full course of treatment, suggesting that conventional therapy did not eradicate BCL2-positive cells.²³⁶ Some studies have shown correlation between MRD and clinical outcome in patients treated with chemotherapy.^{237,238} Molecular response as assessed using PCR in peripheral blood was identified

as one of the prognostic factors to predict failure-free survival. In patients with advanced stage follicular lymphoma, those who achieved a molecular response and sustained it during the first year of treatment with anthracycline-based chemotherapy had a significantly longer failure-free survival than those who did not (4-year failure-free survival: 76% and 38%, respectively).²³⁷

The achievement of molecular response also correlated with outcome in patients treated with CVP (cyclophosphamide, vincristine, prednisone) chemotherapy in conjunction with interferon alfa.²³⁸ At 2-year follow-up, continuous clinical remission was observed in 94% of patients with undetectable MRD (PCR-negative), whereas 50% of patients who reverted to PCR-positivity had relapsed disease.

However, other studies have shown persistence of t(14;18)-positive cells in patients with advanced follicular lymphoma experiencing long-term remissions after CVP chemotherapy.²³⁹ In another prospective study, first-line treatment with CVP chemotherapy with interferon resulted in rapid clearance of t(14;18)-positive cells from peripheral blood irrespective of the clinical response.²⁴⁰

MRD After B-Cell-Targeted Therapy: Rituximab alone or in combination with chemotherapy is associated with a rapid clearance of residual tumor cells. However, early data suggested that MRD assessment in the single-agent rituximab era was less predictive.^{241–245}

In the pivotal study of single-agent rituximab, t(14;18)-positive cells were rapidly cleared from the peripheral blood and bone marrow, but most patients still had evidence of residual nodal disease, suggesting the presence of a “compartmental effect” in patients receiving single-agent rituximab.²⁴¹ Ghielmini et al.²⁴⁴ showed that the clearance of circulating t(14;18)-positive cells was significantly associated with clinical response, whereas in another study, PCR-negativity in peripheral blood and/or bone marrow samples after rituximab did not correlate with clinical response.²⁴⁵

Czuczman et al.²⁴⁶ first reported that the addition of rituximab to CHOP chemotherapy induced durable clinical and molecular remissions in patients with untreated low-grade follicular lymphoma. Complete nodal remission was documented in all of the patients who became BCL2-negative in the bone marrow and peripheral blood after treatment

with CHOP plus rituximab.²⁴¹ At 9-year follow-up, 7 of 8 patients who were found to be BCL2-positive in peripheral blood and bone marrow converted to PCR-negative status after completion of therapy.²⁴⁷

The results of 3 randomized clinical trials showed the prognostic significance of achieving MRD and the higher rate of MRD achieved with chemotherapy plus rituximab.^{248–250}

Pott et al.²⁴⁸ showed that circulating lymphoma cells in peripheral blood at diagnosis and follow-up after treatment with CHOP or R-CHOP correlated with time to treatment failure (TTF). Patients with a lymphoma load greater than 0.01 had a significantly lower TTF than those with lymphoma load of 0.01 or less. R-CHOP removed residual lymphoma cells more efficiently (> 2 logs) than CHOP inducing a molecular remission in 76% of patients. Patients who achieved molecular remission after induction therapy had improved progression-free survival at 2 years (90% vs. 53% for patients who were MRD-positive). CHOP induced a reduction of approximately 2 logs, but 77% of patients remained MRD-positive.

In the trial conducted by the German study group, approximately 75% to 85% of patients treated with a full course of R-CHOP cleared the bone marrow and peripheral blood of t(14;18)-positive cells as assessed with real-time quantitative PCR. The log clearance or rate of reduction (≥ 2 log) of circulating tumor cells was more predictive of disease-free and progression-free survival.²⁵⁰

In the GITMO trial, Ladetto et al.²⁴⁹ compared rituximab-supplemented high-dose sequential chemotherapy (R-HDS) with ASCT and R-CHOP as first-line therapy in high-risk patients. MRD analysis using PCR showed that the patients in both groups who achieved molecular remission (PCR-negative) had a better PFS than those without molecular remission (Figure 4A). Molecular remission was achieved in 44% of patients receiving R-CHOP and 80% of patients in the R-HDS group. The progression free survival for the entire study population (without considering PCR status) was significantly higher in favor of R-HDS, because it is associated with increased number of molecular remissions. However, the outcome of patients achieving molecular remission was similar regardless of the treatment as was the outcome of patients not achieving molecular remission (Figures 4B and 4C).

Molecular remission after sequential admin-

istration of CHOP and rituximab was also associated with a better clinical outcome.²⁵¹ Patients who achieved clinical response after CHOP but remained PCR-positive were treated with rituximab. At 12 weeks follow-up, 59% converted to PCR-negative in peripheral blood and bone marrow, and 63% were PCR negative at 44 weeks follow-up. Freedom from recurrence was 57% in patients who were PCR-negative compared with only 20% in patients who lost molecular negativity. These results showed the predictive value of additional therapy with rituximab in patients who were PCR-positive after chemotherapy. **MRD After High-Dose Therapy and ASCT With or Without Rituximab:** Gribben et al.²⁵² first reported the prognostic significance of monitoring MRD after ASCT. The presence of BCL2-positive residual lymphoma cells identified patients who are at high risk for relapse. In a more recent report, Hirt et al.²⁵³ also showed that after ASCT, increasing numbers of circulating t(14;18)-positive cells is associated with relapse, and complete remission is associated with stable cell counts.

Freedman et al.^{254,255} reported the best results regarding the correlation of clinical outcome and PCR status of bone marrow in patients with follicular lymphoma in first remission after ASCT using in vitro purging with monoclonal antibodies. With more than 10 years of follow-up, ASCT results in follicular lymphoma continue to show the prognostic significance of achieving MRD after ASCT in both patients in first remission and the transplanted cells assessed ex vivo.^{256,257} At 12-year follow-up, the

results suggest that a subset of patients whose bone marrow was PCR-negative for t(14;18) have prolonged survival. Bone marrow involvement at harvest and PCR-detectable disease in the bone marrow after purging were identified as predictors of decreased progression-free survival.²⁵⁶

In vivo purging with rituximab during the process of stem cell harvest has been used in several studies to obtain t(14;18)-negative stem cell grafts.²⁵⁸ Recent studies have reported better progression-free survival and molecular remissions after in vivo purging with rituximab before ASCT;^{259,260} it produced 86% of harvests free of contamination compared with only 14% in the control group. All patients who were re-infused with negative stem cell grafts at transplantation experienced complete remission with significantly better 5-year progression-free survival (100%) compared with those reinfused with contaminated samples (41%).²⁵⁹

MCL

Detection of t(11;14) Translocation: The t(11;14) translocation is detectable in 70% to 100% of patients with MCL. Most of the breakpoints on chromosome 14 occur in the J_H region. Although the breakpoints are widely scattered on chromosome 11, 3 clusters have been identified so far. The most important one is referred to as major translocation cluster region (BCL1-MTC), which contains up to 50% of the translocations and has been a suitable target for detecting MRD.⁴

The translocations involving the MTC region are detectable by PCR in 30% to 40% of the cases.³ PCR

Figure 4 (A) Progression-free survival according to the presence of molecular remission (PCR-) or lack of molecular remission (PCR+). (B) Progression-free survival according to treatment arm in patients experiencing molecular remission. (C) Progression-free survival according to treatment arm in patients not experiencing molecular remission. From Ladetto M, De Marco F, Benedetti F, et al. Prospective, multicenter randomized GITMO/IIL trial comparing intensive (R-HDS) versus conventional (CHOP-R) chemoinmunotherapy in high-risk follicular lymphoma at diagnosis: the superior disease control of R-HDS does not translate into an overall survival advantage. *Blood* 2008;111:4009; with permission.

detection of t(11;14) is performed by nested PCR analysis using a consensus primer derived from the J_H regions and 2 primer sets for the MTC region.⁴ This can detect 1 malignant cell out of 10⁵ normal cells. Bottcher et al.²⁶¹ recently showed that qualitative consensus IGH-PCR is more sensitive than FCI for detecting MRD. Real-time quantitative PCR targeting the BCL1/J_H rearrangement was also more sensitive than the qualitative PCR assay for the detection of tumor burden in the BM.²⁶²

Alternatively, quantitative monitoring of cyclin D1 overexpression has been used for the detection of MRD in patients with MCL.²⁶³

MRD After B-Cell Targeted Therapy: Unlike follicular lymphoma, PCR-negative status has been more difficult to achieve in patients with MCL. The addition of rituximab to CHOP resulted in the clearance of residual disease from peripheral blood and bone marrow in only 36% of patients with newly diagnosed MCL, but the molecular remission rates after induction therapy did not translate into prolonged progression-free survival (16.5 months for those who did not experience molecular remission and 18.8 months for those who did).²⁶⁴ However, the results of a recent randomized trial showed that achievement of molecular remission after immunochemotherapy might predict treatment response in patients with MCL.²⁶⁵ Remission duration was significantly improved in patients who were MRD-negative in bone marrow compared with those who were MRD-positive (100% vs. 66%, respectively, at 24 months).

MRD After High-Dose Therapy and ASCT With or Without Rituximab: Pott et al.²⁶⁶ reported that MRD status after ASCT is a powerful indicator of treatment outcome in patients with advanced-stage MCL.²⁶⁶ The MRD-negative group had a median progression-free survival of 92 months compared with 21 months in the MRD-positive group. Cytarabine-based regimens and myeloablative radiochemotherapy significantly reduced MRD compared with CHOP-like regimens.

Recent studies incorporating high-dose chemotherapy, rituximab, and transplantation suggest that molecular remission after ASCT is associated with improved outcome in a significant portion of patients with MCL. Ladetto et al.²⁶⁷ evaluated the importance of monitoring of MRD in patients who had molecular relapse after R-HDS and ASCT. PCR-negative status was restored after 4 to 6 courses of rituximab in all patients who experienced molecular relapse after R-

HDS and ASCT. This was observed even in patients who had previously received several courses of rituximab, suggesting that the residual tumor cells are still sensitive to rituximab.

The results of 2 multicenter trials have shown the prognostic significance of rituximab-supplemented high-dose therapy with in vivo rituximab-purged ASCT.^{268,269} In vivo purging resulted in PCR-negative stem cell products in all patients with evaluable markers and was also associated with long-term progression-free survival and remission. These results must be confirmed in prospective trials.

Limitations of Monitoring MRD

Both clinical and laboratory criteria must be established for the successful implementation of MRD monitoring in routine clinical practice. Robust laboratory assays must be in place to allow the sensitive and specific detection of the biomarker. Development of robust laboratory-based assays facilitates the necessary clinical comparisons for validating a biomarker for MRD detection.

Timing of specimen sampling is crucial in the prognostic value of MRD detection, as is definition of the optimal reservoir for MRD evaluation dependent on the treatment modality, and location within the reservoir sampled being a particular consideration with specimens exhibiting results at the low end of the reporting range of the respective assay.²⁷⁰

Standardization of all phases of specimen testing is crucial. In the preanalytic phase, analyte and matrix stability, storage time, and temperature are practical considerations that can affect the overall performance of specimen testing. For RNA-based assays, the inherent instability of the analyte dictates particular care, with suggested use of RNA stabilization agents during specimen collection.²⁷¹

In the analytic phase, assay accuracy, precision, and reproducibility; reportable ranges; and analytic sensitivity and specificity are important criteria that must be standardized. European and international groups have pioneered the standardization of reagents and methods (flow cytometry immunophenotyping and quantitative PCR-based assays),²⁷²⁻²⁷⁵ rendering useful resources and guidelines for the implementation of MRD monitoring.

For the postanalytic phase, when data are interpreted, reference intervals/cutoff values are determined and reports generated, guidelines also must be implemented to ensure interlaboratory uniformity.

ty.^{271,273,276,277} Assurance of uniformity could be provided by the introduction of standard calibrators and strict proficiency testing programs.

Conclusions

Although MRD detection in NHL has been under investigation for many years, immense clinical, pathologic, and molecular heterogeneity that is inherently displayed in this disease and its subtypes have presented difficulties in implementing uniform standardized assays and have impeded the development of robust molecular markers.

Molecular markers for MRD detection in NHL currently being evaluated display variable efficacy in the informative detection of MRD, depending on the treatment modality. Assessment of MRD is technically feasible for most patients.

Incorporation of MRD assessment into clinical practice and interpretation of data needs standardization, quantification of MRD assessment, and uniform reporting at serial time-points from all laboratories. Typically, biomarkers are discovered in small data sets, validated in several retrospective trials, and then evaluated in large prospective trials. The correlation between MRD status and outcome must be established in well-designed, large, prospective, randomized clinical trials performed within the auspices of consortiums and/or collaborative study groups. Changing therapeutic approaches or agents must be accompanied by re-evaluation of the respective MRD biomarker.

MicroRNAs in Leukemias and Lymphomas

MicroRNAs (miRNAs) are short noncoding, single-stranded RNAs that play a role in gene regulation.^{278–280} miRNA genes are mostly transcribed by RNA polymerase II in the nucleus to form a large RNA precursor called *primary miRNA*, which is transformed to a mature 21–22-nucleotide miRNA through a multistep process in the nucleus and cytoplasm. miRNAs bind to the mRNA-containing complementary nucleotide sequence, resulting in the degradation of mRNA or inhibition of protein translation.

miRNAs have been shown to occur in the genome regions of cancer cells involved in deletion or amplification of chromosomes.²⁸¹ Tumor suppressor miRNAs are located in the deleted regions of the genome and are usually down-regulated in cancer cells and control oncogene expression. Oncogenic miRNAs are located in the amplified region of the genome and are usually up-regulated in cancer cells and enable

the upregulation of tumor suppressor genes. miRNAs have been used in diagnostics and prognostication for various hematologic malignancies.^{282,283}

Initial work showed that miRNA expression signatures can accurately discriminate ALL from AML,²⁸⁴ and among cytogenetic and molecular subsets of AML.^{285–287} Furthermore, it was demonstrated that miRNA signatures are associated with AML prognosis.²⁸⁸ For example, patients with high expression of miR-191 and miR-199a had significantly worse overall and event-free survival than AML patients with low expression. Similar findings were described in ALL.^{289,290}

Calin et al.²⁹¹ first showed that miRNA-15a and miRNA-16-1 are located in the region of chromosome 13q14 and are deleted in most patients with chronic lymphocytic leukemia (CLL), indicating that miRNAs are involved in the pathogenesis. miRNAs have also been shown to regulate the expression of BCL2 and TCL1 in patients with CLL. miRNA-15a and -16-1 negatively regulate BCL2 expression and promote apoptosis in leukemic cell lines.^{292,293} TCL expression is inversely correlated with that of miRNA-29b and -181b.²⁹⁴ miRNA signatures are also associated with prognosis and disease progression in patients with CLL.^{295,296} Calin et al.²⁹⁶ identified a signature composed of 13 miRNAs that differentiated patients with favorable and unfavorable prognosis based on the ZAP-70 expression and IgV_H mutational status. Of the 13 miRNAs, 9 were overexpressed in the group of patients with a short interval from diagnosis to the start of initial therapy.

miRNA-155 and its host gene *BIC* have been reported to accumulate in human B-cell lymphomas.^{297–299} Higher copy numbers of miRNA-155 and elevated expression of *BIC* RNA have been found in lymphoma cells compared with normal circulating B cells.²⁹⁷ miRNAs could also be useful as molecular markers for the differentiation of DLBCL subtypes.^{297,300,301} ABC phenotype is characterized by the higher expression of miRNA-155.^{297,301} Lawrie et al.³⁰⁰ also identified 2 additional miRNAs (-21 and -221) that were more highly expressed in ABC-type than GCB-type. This study also showed that miRNAs can be measured reliably from routine paraffin-embedded biopsies of archived samples.

Conclusions

- miRNA technology has the potential not only to improve the ability to predict outcome and

differentiate clinically distinct subtypes but may also identify new therapeutic targets.

- Quantification and standardization of expression analysis is essential to establish a library of miRNA signatures that can be used in clinical trials for diagnosing and treating cancer.

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