

Chronic Myeloid Leukemia

Laboratory Monitoring of Minimal Residual Disease

BY RICHARD D. PRESS, MD, PHD

Chronic myeloid leukemia (CML) is a slowly progressing cancer that affects each of the body's white blood cell subtypes. Of the 6,000 patients diagnosed with CML every year, 90%–95% of cases are associated with a specific chromosomal aberration known as the Philadelphia chromosome (Ph), which can be detected cytogenetically as a reciprocal translocation between the long arms of chromosomes 9 and 22. Until recently, standard treatment options for CML included allogeneic stem cell transplantation, interferon (IFN)-alpha containing chemotherapeutic regimens, and hydroxyurea. Although allogeneic stem cell transplantation can cure approximately 65% of patients, only about one-third of patients are eligible for this high morbidity procedure. Interferon-based therapies, with or without cytarabine (Ara-C), were the gold standard chemotherapy for the majority of these patients. Now, however, a new molecularly targeted drug, Gleevec (imatinib mesylate, formerly STI571), is radically changing treatment regimens and improving outcomes for CML patients.

The laboratory plays a major role in monitoring a patient's response to anti-leukemic therapy. The best surrogate laboratory marker for survival is the degree of cytogenetic remission, as measured by the percentage of metaphase cells with a visible Ph chromosome. But with the advent of the more successful imatinib therapy, increasing numbers of laboratories are employing the polymerase chain reaction (PCR) to more sensitively monitor for minimal residual disease (MRD).

The Molecular Consequences of the Philadelphia Translocation

The hallmark of the Ph translocation is a physical juxtapositioning of sequences from the chromosome 22 bcr (breakpoint

capability, transformation being the likely result of bcr-abl activation of kinase-dependent signaling pathways deregulating the stem cell's growth, differentiation, apoptosis, and adhesion properties. As definitive proof of the leukemogenic activity of this chimeric protein, scientists have shown that direct bcr-abl overexpression in mice causes an *in vivo* CML-like leukemia.

A New Gold Standard for Treatment: Imatinib

Of those CML patients in chronic phase who have been treated with interferon, only 30% experience a major cytogenetic response, which is defined as less than 35% of cells with a detectable Ph+ chromosome. As few as 10% of patients treated with interferon have a complete cytogenetic response or no

used to monitor the pre- and post-therapy disease burden. The former goal has now been largely realized with the discovery, validation, and rapid approval by the Food and Drug Administration (FDA) of the small molecule tyrosine kinase inhibitor imatinib as an effective therapy for Ph+ leukemias. Perhaps the first example of a targeted molecular therapy for cancer, researchers designed the drug to specifically occupy the ATP-binding "kinase pocket" of the bcr-abl protein. Upon binding the protein, imatinib competitively inhibits bcr-abl's pathogenic kinase activity. Because it targets only those cells with the Ph chromosome, non-tumor cells are largely spared from the drug's inhibitory action. As a result, patients on imatinib therapy experience minimal toxicity.

Growing evidence suggests that imatinib is a highly effective single-agent therapy for CML, inducing a complete hematologic response in 95% of chronic phase patients. Remarkably, the drug has produced a complete cytogenetic response with no detectable Ph chromosomes in 76% of early chronic phase patients and 41% of late chronic phase patients. Imatinib also shows substantial activity in the later disease stages of CML, including accelerated-phase, blast-crisis, and Ph+ ALL, but these responses are often not durable. Because of these extraordinary efficacy results, imatinib is now widely considered the gold standard chemotherapy for CML when curative stem cell transplantation is not an option.

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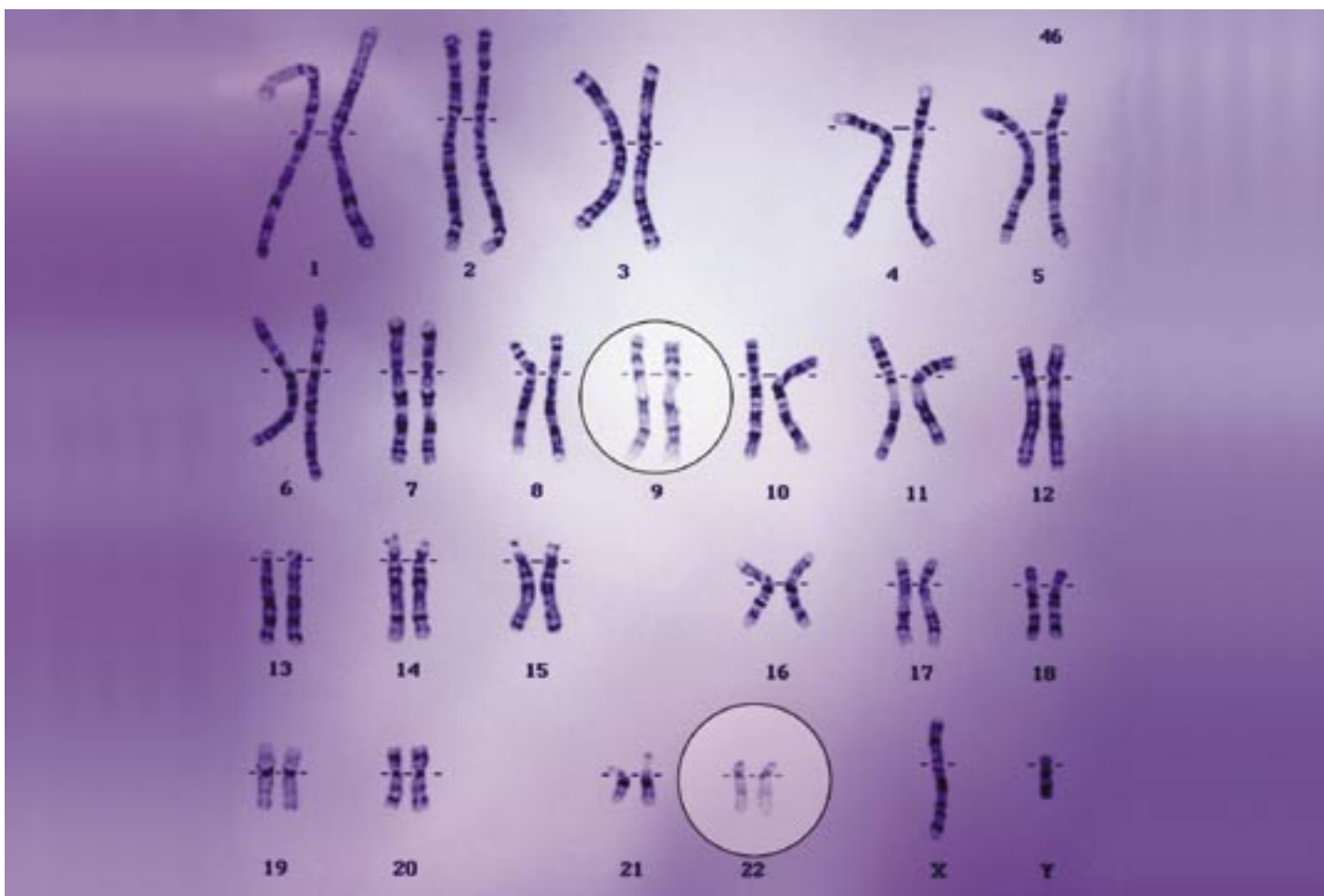
Responses to anti-leukemic therapy in CML, as assessed by surrogate laboratory markers of MRD, are a primary prognostic indicator of disease status. These responses are typically expressed at three levels by laboratory assays used to measure the response: hematologic response, defined as a normalization of peripheral blood cell counts; cytogenetic response, defined as the proportion of residual Ph+ metaphase cells after routine cytogenetic evaluation; and molecular response, defined as the presence or amount of residual bcr-abl RNA as measured by reverse transcriptase-polymerase chain reaction (RT-PCR). The table on page 11 presents a comparison of these assays.

Cytogenetics. The current gold standard method for predicting clinical outcomes in CML remains conventional cytogenetic

cluster region) gene adjacent to sequences from the chromosome 9 c-abl gene that encodes a non-receptor tyrosine kinase. The resulting bcr-abl fusion gene is transcribed and translated into a 210 kDa (p210) or 185 kDa (p185) bcr-abl fusion product with significantly enhanced (deregulated) tyrosine kinase activity. Several studies have shown that the kinase activity of bcr-abl is absolutely required for its transforming

detectable Ph+ metaphase chromosomes. Clearly, alternative treatment options were required to improve patient outcomes.

The direct cause-and-effect relationship between bcr-abl and CML created an ideal model system to test whether molecularly targeted inhibition of a tumor-specific gene product could improve treatment of this disease, and also whether the persistent presence of this tumor marker could be



detection of the Ph chromosome in metaphase spreads after in vitro bone marrow culture. This gold standard designation stems from the observation that metaphase cytogenetic responses have been shown to directly correlate with improved CML survival times.

However, there are many practical disadvantages of monitoring CML therapy by bone marrow cytogenetics, including the requirement for proliferating cells, sampling errors due to the small number (usually 20) of metaphase chromosomes typically analyzed, the slow turnaround times necessitated by the long-term culture requirements, the need for invasive bone marrow aspirations, and the relatively poor sensitivity for detecting low-level MRD. A significant advantage of cytogenetic monitoring, however, is its ability to detect secondary chromosomal aberrations distinct from Ph (clonal evolution), many of which have independent poor prognostic significance.

Fluorescence in situ hybridization (FISH). Most laboratories also assess CML MRD by in situ hybridization of fluor-labeled, large bcr (chromosome 22) and abl (chromosome 9) DNA probes to interphase or metaphase nuclei. In this method, a cytogeneticist microscopically examines individual cell nuclei, looking for physically overlying or juxtaposed mixed-color fluorophore signals that indicate physical joining of chromosomes 9 and 22. As these hybridizations can be performed on interphase cells, the requirement for dividing target cells is obviated. Moreover, larger numbers of cells—typically 200—can be examined, which increases the assay sensitivity over that of metaphase cytogenetics.

A significant disadvantage of some FISH methods is the relatively high background of false-positive signals that can result from the random chance, two-dimensional physical overlapping of chromosomes 9 and 22 in the three-dimensional nucleus. This juxtaposition artifact can affect 2%–10% of analyzed nuclei for methods in which the FISH probes cover only one side of the translocation breakpoint.

The FISH assay performed in many laboratories overcomes this problem by utilizing a dual-fusion probe set consisting of a green-labeled abl probe for chromosome 9 and a red-labeled bcr probe for chromosome 22, both of which span the typical Ph translocation breakpoint. In this assay, the probe recognizes not one, but both translocation partner chromosomes. When a reciprocal translocation (9;22) is present, wavelength interference between the closely juxtaposed red and green fluorophore probes produces

a yellow “fusion” signal on both the derivative 9 and the derivative 22 chromosome. Consequently, a cell that contains the Ph chromosome will have two yellow fusion signals, one red signal (the normal 22), and one green signal (the normal 9). This type of dual-fusion probe set essentially eliminates the possibility of random juxtaposition artifacts and further reduces the sensitivity limit for the detection of residual leukemia to a lower limit defined by the number of cells examined, typically 1 in 200–300.

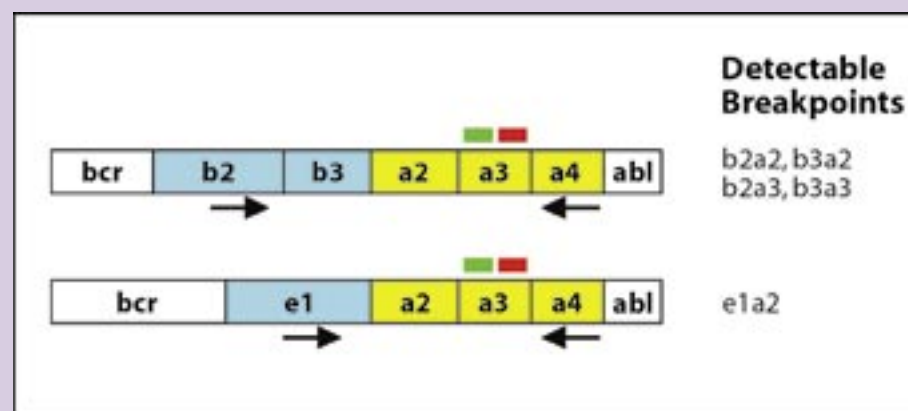
Despite these improvements, the interpretation of FISH data remains highly subjective and prone to occasional false negative results. These problems arise from three possible sources: technical considerations in performing the assay; the frequent large chromosomal deletions that often accompany CML disease progression; and the presence of Ph+ cells that do not actively express the bcr-abl kinase.

RT-PCR. The tumor cell-specific bcr-abl fusion gene has long been a prime target for the ultra-sensitive PCR-based detection of CML MRD. Because the breakpoint cluster regions extend across a long stretch of genomic DNA, designing a DNA-based PCR primer strategy is impractical. However, the splicing process creates one of two well-conserved bcr-abl mRNAs that encodes the p210 or p185 kinase, each of which can be detected by RT-PCR. Based on the location of the specific breakpoints, scientists have designed RT-PCR methods using only two PCR primer pairs that cover the major and minor breakpoint regions (see box above). These PCR assays amplify extremely small input amounts of bcr-abl RNA, producing a lower limit of detection that is often in the range of one Ph+ tumor cell in a background of 1×10^5 to 1×10^6 normal cells and a sensitivity far exceeding that of metaphase cytogenetics or FISH.

The first generation bcr-abl RT-PCR assays in clinical diagnostic labs were qualitative assays used for both primary diagnosis and post-therapy detection of MRD. Although diagnosis and disease classification for prognosis remain a valuable use for these qualitative assays, optimal post-therapy monitoring of disease status is probably better accomplished using quantitative PCR-based assays. Qualitative PCR-based assays have limited utility for disease monitoring because they remain consistently “positive” in the vast majority of imatinib- or interferon-treated patients, long after they achieve complete cytogenetic remission.

The limited value of qualitative PCR for CML MRD detection has led to the devel-

Schematic Representation of a Fluorescent Real-time Quantitative RT-PCR Assay for Bcr-abl



The assay performed in our laboratory uses two forward bcr PCR primers (corresponding to exon b2 within the major breakpoint cluster region and upstream exon e1 within the minor breakpoint cluster region) and a single common reverse primer (abl exon a4) to amplify the p210 and/or p185 cDNAs. The amplified products are quantitatively detected in real-time by fluorescence resonance energy transfer (FRET) using a pair of fluorophore-labeled hybridization probes specific to exon a3 (green and red bars). Instead of FRET-based hybridization probes, another commonly-used assay platform uses hydrolysis (Taqman) probes carrying both quencher and reporter dyes. The various exons of bcr (b) and abl (a) are depicted as boxes.

opment of sensitive, quantitative bcr-abl RT-PCR assays. These assays are quickly becoming the standard of care for CML patients who achieve a complete cytogenetic response after imatinib therapy—76% of newly diagnosed CML patients and 41% of those in late chronic phase (see box, p. 12). In most molecular diagnostic labs, these assays are performed on fluorescent PCR instruments that accurately quantify even low abundance transcripts by monitoring cycle-to-cycle, fluorophore-labeled PCR product accumulation kinetics in real-time. Because these instruments quantify template levels by determining the cycle number at which the exponential log phase of the PCR reaction begins, they are inherently more precise than first-generation competitive PCR quantifications in which only the end point of the PCR reaction is monitored.

Other significant advantages of the real-time PCR method include its relative ease of use compared to competitive PCR, its comparable results in blood and bone marrow samples, the single-tube amplification and detection format that minimizes handling and contamination errors, and its semi-automated nature. In addition to controlling for the possible degradation (partial or complete) of labile RNA samples,

most laboratories quantifying bcr-abl RNA by real-time PCR choose to simultaneously quantify a second ubiquitously expressed “housekeeping” transcript from the same RNA sample. The final result is then often reported as a relative ratio of bcr-abl RNA to a housekeeping mRNA.

While this type of assay developed in-house can typically detect residual CML cells down to a dilution of one tumor cell in 100,000—or even one million—background cells with excellent analytical precision (CV ~15%–40%), unfortunately, the extreme lab-to-lab variability in methodology precludes the practical comparison of results generated in different laboratories. As is the case for most molecular diagnostic assays that are not yet approved by the FDA, a significant obstacle to more widespread clinical utilization of these tests remains the absence of recognized standards for assay methodology, analytical sensitivity, reporting, and proficiency testing.

The Pharmacogenetic Paradigm: Smart Drugs

The imatinib success story has quickly spawned a popular new “pharmacogenetic paradigm” for developing molecularly targeted “smart” drugs in the war against cancer. As the demand for these therapies grows, laboratorians will be asked to design, optimize, and validate assays to serially monitor the disease status of patients being treated with the drugs. Not only will specific laboratory methods need to be developed for determining the initial activity of such drugs, but additional methods will be required to monitor the drug’s ability to keep residual disease in check.

The best analogy for the laboratory’s critical role in monitoring molecularly targeted cancer therapies is laboratory monitoring of patients with human immunodeficiency virus (HIV) disease. Like patients undergoing HIV therapy with virus-specific protease and reverse transcriptase inhibitors, leukemia patients receiving imatinib require serial laboratory monitoring by quantitative, ultra-sensitive diagnostic assays directed at the same disease-specific molecular target as the treatment. The goal in each disease is

Lab Methods for Detecting Minimal Residual Disease

Method	Target	Analytical Sensitivity	Pros	Cons
Morphology	Blood / bone marrow	Depends on number of cells examined	Detects other relevant pathologies	Subjective; difficult to quantitate
Cytogenetics	Ph chromosome	1:20	Detects other prognostic chromosome changes	Slow; bone marrow only; metaphases required
FISH	Bcr-abl fusion gene	1:200–1:500	Can use interphase cells and blood	Juxtaposition artifacts; non-expressing Ph+ cells; subjective
RT-PCR	Bcr-Abl RNA	1:100,000–1:1,000,000	Ultra-sensitive method; blood or bone marrow	Amplicon contamination; assay standardization; false negatives for “unusual” breakpoints; labile RNA

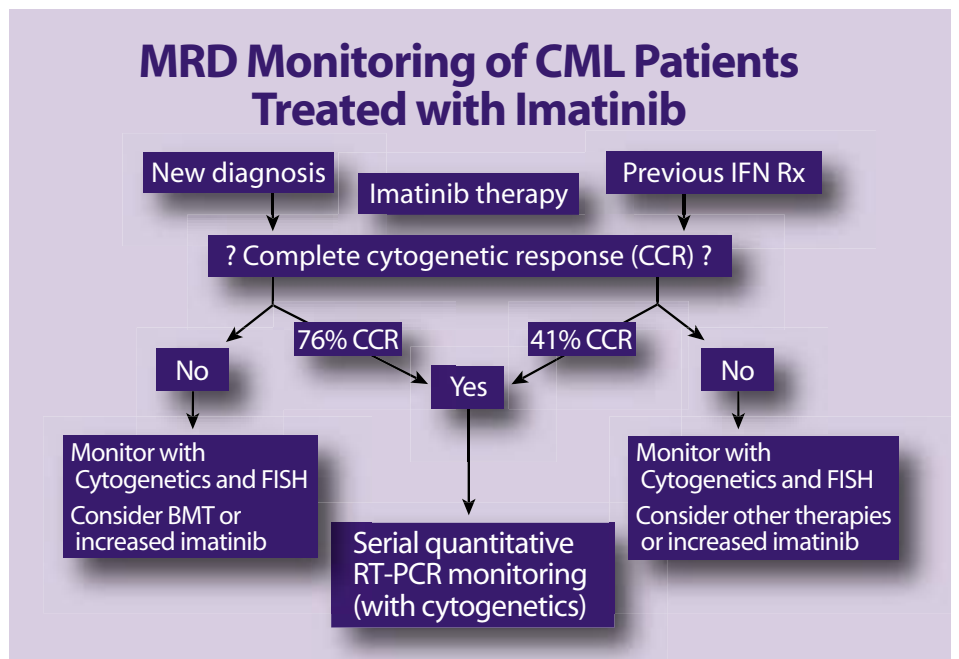
identical: the early detection of pre-clinical MRD so that clinicians can initiate early alternate therapies for the prevention of frank clinical relapse.

A Growing Need for Monitoring MRD

The recent widespread acceptance of imatinib as the gold standard chemotherapy for CML has created the need for better laboratory methods to monitor its anti-leukemic efficacy. Although quantitative PCR-based methods are clearly the most sensitive methods for detecting MRD, the use of these ultra-sensitive assays has not yet been shown to directly predict a long-term clinical outcome such as survival.

An indirect proof of the potential clinical utility of quantitative PCR-based assays comes from a recent finding published by Merx and colleagues (see ref.). The researchers measured bcr-abl RNA levels in patients by quantitative RT-PCR after two months

of imatinib therapy and found them to be a significant prognostic marker for the later achievement of a predictive cytogenetic response, either major or complete. In a recent *New England Journal of Medicine* paper, Hughes and colleagues (see ref.) published a more direct proof of the importance of bcr-abl RNA levels to clinical outcomes. The researchers found that reduced bcr-abl RNA levels after 12 months of imatinib therapy was a significant predictor of clinical disease progression in chronic phase patients with a complete cytogenetic response. Although further follow-up of patients on imatinib therapy will obviously be needed before definitive conclusions about survival can be reached, the amazing success of this drug in substantially reducing the burden of Ph+ cells strongly suggests that the new goal for defining treatment success will require quantitative, serial RT-PCR monitoring of bcr-abl RNA.



SUGGESTED READING

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