Laboratory Tools for Diagnosis and Monitoring Response in Patients with Chronic Myeloid Leukemia

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ABSTRACT: Chronic myeloid leukemia (CML) is a clonal hematological disease that represents 15–20% of all adult leukemia cases. The study and treatment of CML has contributed pivotal advances to translational medicine and cancer therapy. The discovery that a single chromosomal abnormality, the Philadelphia (Ph) chromosome, is responsible for the etiology of this disease was a milestone for treating and understanding CML. Subsequently, CML became the first disease for which allogeneic bone marrow transplantation is the treatment of choice. Currently, CML is one of the few diseases where treatment targeted against the chromosomal abnormality is the sole frontline therapy for newly diagnosed patients. The use of directed therapy for CML challenged disease monitoring during treatment and led to the development of definitions that document response and predict relapse sooner than the former routine methods. These methods relied on classical cytogenetics through molecular cytogenetics (FISH) and, finally, on molecular monitoring assays. This review discusses the laboratory tools used for diagnosing CML, for monitoring during treatment, and for assessing remission or relapse. The advantages and disadvantages of each test, the common definition of response levels, and the efforts to standardize molecular monitoring for CML patient management are discussed.

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Chronic myeloid leukemia is characterized by the Philadelphia chromosome, which originates in a balanced reciprocal translocation between the *ABL* gene on chromosome 9 and the *BCR* gene on chromosome 22 [t(9;22)(q34;q11)] [1]. This aberration leads to the formation of a new *BCR-ABL1* gene that encodes a chimeric oncoprotein with constitutive kinase domain activity [2]. The literature indicates that the Ph chromosome is responsible for the etiology of CML in 95% of all patients. This fact makes CML unique and one of the most investigated hematologic diseases.

The evolution of CML involves three phases defined by clinical characteristics and laboratory findings [3]. It typically begins in the chronic phase (characteristic in 85% of newly diagnosed patients). Over the course of several years it progresses to the accelerated phase and ultimately to the blast crisis phase. The BC phase, which is the terminal phase, exhibits clinical behavior similar to that in acute leukemia. The mechanism responsible for progression from CP to AP and then to the BC phase is complex and not well understood [2]. It can be partly explained by the formation of new chromosomal abnormalities (in addition to the Ph chromosome), up-regulation of genes responsible for the arrest of differentiation, genomic instability, and inactivation of tumor suppressor genes [2].

TREATMENT: PAST AND PRESENT

For many years, the treatment strategy for CML patients was based on chemotherapeutic agents such as busulfan and hydroxyurea. These agents provided clinical maintenance but failed to eliminate the malignant clones [4]. In the mid-1970s, an important breakthrough was made by the Seattle group, which reported the disappearance of the Ph-positive clone in patients treated with allogeneic stem cell transplantation. Another breakthrough occurred in the early 1980s with the introduction of interferon-alpha to the clinical treatment, which led to complete cytogenetic response and long-term survival, but not in all patients [5]. In subsequent years allogeneic SCT and INFα therapy became the treatments of choice offered to CML patients, based on the availability of a

Ph = Philadelphia
CML = chronic myeloid leukemia
BC = blast crisis
CP = chronic phase
AP = accelerated phase
SCT = stem cell transplantation
INFα = interferon-alpha

Table 1. Response definition in CML [6,15]

Level of response	Definition	Laboratory method	Time point of optimal response*
Complete hematological response	Normal complete blood count and differential	Blood count	3 mos
Minimal cytogenetic response (minCyR)	66–95% Ph+ metaphases	Karyotyping	
Minor cytogenetic response (mCyR)	36–65% Ph+ metaphases	Karyotyping	3 mos
Partial cytogenetic response (PCyR)	1–35% Ph+ metaphases	Karyotyping	6 mos**
Complete cytogenetic response (CCyR)	0% Ph+ metaphases	Karyotyping	12 mos
Major molecular response (MMoR)	*BCR-ABL1*/housekeeping gene ≤ 0.1%#	Quantitative real-time PCR	18 mos
Complete molecular response (CMoR)	Negativity of *BCR-ABL1* mRNA##	Quantitative real-time PCR	

* From imatinib therapy initiation
** ≥ PCyR
# According to international scale
In two consecutive blood samples

human leukocyte antigen-matched donor, and the patient's condition, age, co-morbidities and disease status [6].

Recognition that the *BCR-ABL1* chimeric protein is a pivotal contributor to the disease pathogenesis and progression led, in the late 1990s, to the synthesis of a compound (now known as imatinib) that inhibited ABL and other tyrosine kinases and revolutionized CML therapy [7,8]. Imatinib was found to specifically inhibit the tyrosine kinase enzyme and prevent its activity [7,8]. It is composed of two phenylamino-pyrimidine derivates that bind to the TK active sites, specifically to the TK domain of ABL1, c-kit, and PDGFR (platelet-derived growth factor receptor), and inhibit their constitutional activity [9]. A new generation of TK inhibitors, nilotinib (Tasigna®, Novartis AG, Basel, Switzerland) and dasatinib (Sprycel®, Bristol-Myers Squibb, Princeton, NJ, USA) are now available and are used if imatinib therapy fails [10,11]. In CML patients, TKI bind to the highly conserved Asp-Phe-Gly residues (DFG) in the catalytic domain, displacing ATP and preventing molecular phosphorylation of signaling molecules. While imatinib and nilotinib bind to the inactive "DFG-out" conformation, dasatinib binds to the active "DFG-in" conformation [12].

Advances in the therapy of myeloid leukemia (using tyrosine kinase inhibitor) and better understanding of the mechanism have prolonged patient survival

Imatinib has replaced allogeneic stem cell therapy and INFα as the frontline therapy for CML. Most CML patients do well with this standard treatment but some require dose optimization of imatinib or alternative approaches including second-generation TKI, or combination therapy with INFα or chemotherapy and/or allogeneic SCT [reviewed in 13].

TK = tyrosine kinase
TKI = TK inhibitors

RESPONSE DEFINITION

Historically, before the 1980s, hematologic remission (normalization of the peripheral blood cell count and differentiation, as well as spleen size) was the only therapeutic response achieved in CML patients. Later, INFα treatment led to partial cytogenetic responses (1–34% Philadelphia positive) or a complete cytogenetic response (no Philadelphia-positive cells in the metaphase bone marrow cells) in a subset of patients and correlated with significantly improved survival. Kantarjian et al. [14] reported that according to the data of MD Anderson, 78% of patients who achieved a CCyR with INFα therapy were alive after 10 years. These studies highlight the importance of monitoring CML patients' response during treatment [Table 1]. The association between cytogenetic response and improved survival made the cytogenetic response the gold standard of CML therapy. Together with the introduction of TKI as the frontline therapy, new studies precisely defined the conditions for the optimal, suboptimal, or no response to treatment. These are based on the total peripheral blood cell count normalization and the achievement of CCyR by conventional cytogenetics, relative to the time from diagnosis (therapy initiation) [6,16]. Nevertheless, more sensitive monitoring assays, such as the polymerase chain reaction, show that even in the state of CCyR, more than 2.5 x 10^7 leukemic cells might still be present in the circulation. This finding led to the definition of a third response level, the molecular response, based on molecular assays that detect residual leukemic cells. Currently, the European LeukemiaNet and National Cancer Network guidelines [6] require molecular response monitoring every 3 months for the follow-up of CML patients and for all clinical trials of new TKI agents. Moreover, major molecular response at 12 months was the primary endpoint of the recent ENESTnd study that compared imatinib 400 mg once a day to nilotinib 300 or 400 mg twice a day [17].

LABORATORY ASSAYS FOR DIAGNOSIS AND DURING THERAPY

Diagnostic assays for new CML patients are based on the standard tests of cytogenetics, fluorescence in situ hybridization, and PCR [18]. All three kinds specifically detect

CCyr = complete cytogenetic response
PCR = polymerase chain reaction

Table 2. Laboratory methods in use for CML patient management [18]

Method	Target	Time to use	Advantages	Disadvantages
Karyotyping	Metaphase chromosomes	At diagnosis and in case of advanced disease	Available in most laboratories	Low sensitivity (1–5%) BM aspiration only
Qualitative PCR for *BCR-ABL1*	RNA sequence of BCR-ABL	Diagnosis	Monitoring the specific breakpoint of *BCR-ABL1*	Not sensitive for *BCR-ABL1* monitoring during treatment
FISH	DNA-specific markers	Every 3 mos *	Rapid (1–2 days) More sensitive than metaphase karyotyping (0.1–3%)	Does not detect other clonal events
Quantitative PCR for *BCR-ABL1*	RNA breakpoint of specific BCR-ABL transcript	Every 3 mos **	Very sensitive (0.001–0.0001%)	Requires standardization Is labor-intensive
Mutation screening of ABL1 kinase domain	RNA sequencing of ABL kinase domain	Suspected progression or resistance	In case of mutation: overcome resistance with more potent TKI therapy	Depends on the specific assay for mutation detection #

*Or when metaphase cytogenetics are not successful
**Important especially when complete cytogenetic response
#See Table 3

the Philadelphia chromosome and the *BCR-ABL1* fusion abnormality. Since TKI therapy leads to cytogenetic and molecular remission, resulting in a prolonged chronic phase and improved outcome, assays for *BCR-ABL1* monitoring have become important not only for diagnosis but during therapy as well [19]. Appropriate monitoring also permits early detection of progress to an advanced-phase disease. In order to optimize monitoring and ensure early detection of disease progression the advantages and disadvantages of each method [15] must be considered [Table 2].

CYTOGENETICS

Cytogenetics is the gold standard for detecting the Ph chromosome and for monitoring cytogenetic response. It is also important for detecting additional chromosomal abnormalities, especially in the case of clonal evolution in the Ph-negative clones (during therapy), which may in rare cases progress to myelodysplastic syndrome) or even to acute myeloid leukemia [20]. The karyotyping results report the number of Ph-positive metaphases out of at least 20 metaphases [6]. Among the disadvantages of conventional cytogenetics is the low sensitivity, which is limited to 1–5% of Ph-positive cells in the sampled culture. Other disadvantages are the time lag due to the need to obtain growing cells and the need for bone marrow aspiration as a source for dividing cells for metaphase analysis. Still, cytogenetics is the gold standard technique and the most standardized assay. It is used in most medical centers and in clinical trials, including the IRIS study for definition of response during TKI treatment [6].

Proper choice of laboratory tests and obtaining reliable results are essential for accurately determining the patient's status and response to therapy

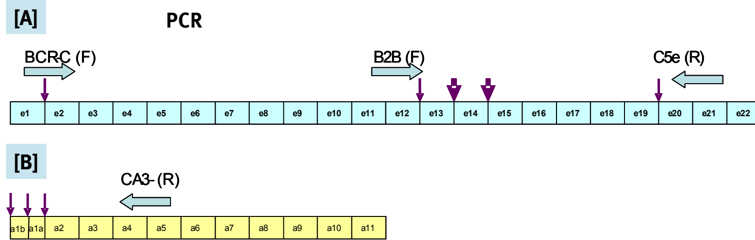
FISH

FISH, as a specific assay, is widely used in hematological malignancies. In contrast to conventional cytogenetics, it rapidly identifies specific genomic abnormalities needed for clinical decision making. FISH is used to detect *BCR-ABL1* for diagnosis, especially when cytogenetics is negative or when no metaphase cells can be obtained. In addition, FISH can identify deletions flanking the *BCR-ABL1* breakpoint, which are important because early studies suggest that these deletions may affect the duration of imatinib response [21]. However, as the importance of these deletions has not been confirmed, FISH is not a mandatory assay for diagnosis if conventional cytogenetics confirms the presence of the Ph chromosome [22].

FISH is frequently used to monitor the response to treatment, especially when the therapeutic goal is CCyR. The reproducible results depend on the use of good quality commercial probes with a low false positive rate that brings the sensitivity of FISH assays to 1%–6% [23]. Some centers use FISH instead of cytogenetics, but the correlation between cytogenetics and FISH has not yet been established [16]. Moreover, the ability of cytogenetics to monitor clonal evolution in advanced-phase disease cannot be matched by FISH [20]. Currently, many medical centers use FISH for routine monitoring of *BCR-ABL1* during TKI treatment, usually in combination with conventional cytogenetics and particularly when quantitative PCR monitoring is not available.

FISH = fluorescence in situ hybridization

Figure 1. Transcripts of *BCR-ABL1* recognized by multiplex RT-PCR [according to ref 24] **[A]** Primers position used for multiplex PCR detecting the *BCR-ABL1* variants. The wide arrows indicate the most frequent positions of the breakpoints in the *BCR* gene and the thin arrows represent the less frequent breakpoints in either the *BCR* or the *ABL1* gene **[B]** The table summarizes the *BCR-ABL1* fusion subtypes that can be detected with this assay



Fusion subtype	Primers	PCR product size
BCR control (normal)	B2B+C5e-	801 bp
e13a2 (b2a2)	B2B+CA3-	310 bp
e13a3 (b2a3)	B2B+CA3-	136 bp
e14a2 (b3a2)	B2B+CA3-	385 bp
e14a3 (b3a3)	B2B+CA3-	211 bp
e1a2	BCR-C+CA3-	211 bp
e1a3	BCR-C+CA3-	307 bp
e19a2 *	B2B+CA3-	925 bp
e19a3	B2B+CA3-	751 bp

*coding for P230 protein

The PCR-based assay for *BCR-ABL1* transcripts for newly diagnosed patients defines the specific transcript resulting from the breakpoints in the *BCR* and *ABL* genes. The breaks in chromosome 22 occur within a 5.8 kb segment (named breakpoint cluster region, BCR), specifically in *BCR* exons 12–16 (b1-b5), and the reciprocal break occurs in chromosome 9 before exon 2 of the *ABL* gene [2]. The different breakpoint sites result in a variety of transcripts [Figure 1], but in the majority of Ph-positive CML patients (99%) two types of *BCR-ABL1* transcripts have been identified by reverse transcription PCR, the b2a2 and the b3a2 (breakpoint in exon 2 or exon 3 of *BCR* respectively), which are referred to as the major transcripts. The major transcripts encode for forming the p210-kDa protein [2]. The 9;22 translocation is found also in acute lymphocytic leukemia (6% of childhood ALL and 17–25% of adult ALL), with a chromosomal breakpoint in the first intron of *BCR*, while the *ABL* sequence remains the same. This breakpoint results in a 7 kb chimeric mRNA transcript, called e1a2, translated into a p190-kDa protein with the same constitutive tyrosine kinase activity as the p210 [2]. The e1a2 transcripts are present in fewer than 1% of CML patients. Some less frequent *BCR-ABL1* transcripts are found because of other rare breakpoint regions in the *BCR* gene. One of these rare breakpoints is the e19a2 transcript [Figure 1] that codes for the p230-kDa protein first identified in chronic neutrophilic leukemia [25]. Karyotyping does not distinguish between the transcripts, which can be identified by qualitative multiplex RT-PCR [24]. This assay is performed even when no chromosomal translocations or Ph chromosome are observed by cytogenetics. In this assay, RNA is isolated from the patient bone marrow or peripheral blood, reverse transcribed into cDNA, and subjected to PCR amplification using primers specific for the different regions of the *BCR* and *ABL* exons. The primer set is designed to detect the different fusion products as well as a segment of the normal *BCR* gene that is not involved in the translocation and constitutes the control for sample cDNA quality. PCR products are analyzed by electrophoresis and ultraviolet transillumination of ethidium bromide-stained gels that depict PCR products of different lengths for each kind of transcript detected [24]. However, the limit of detection for this assay is at least 1 in 100,000 cells, which is suitable for diagnosis but is not sensitive enough for monitoring molecular response during treatment. Moreover, qualitative RT-PCR cannot be used for quantification of the *BCR-ABL1* transcript level in the peripheral blood, which is of particular significance for patients in CCyR under TKI therapy. Thus, it cannot predict relapse and is not suited for follow-up of response to therapy.

Developing an international standardization of laboratory assays will help medical centers evaluate successful TKI treatments for CML patients

Patients receiving TKI are usually monitored by real-time quantitative PCR. This test is designed to quantify the specific *BCR-ABL1* transcript originally identified at diagnosis. There are a number of accepted real-time PCR techniques [26]; the most commonly used and recommended by the Europe Against Cancer protocol is based on TaqMan chemistry [26]. The assay template is cDNA isolated from peripheral blood or bone marrow followed by two separate PCR reactions: one with primers specific for the *BCR-ABL1* breakpoint identified at diagnosis and the second with primers for the detection of a control gene (internal standard) such as *BCR*, *ABL*, or the *B2M* genes [27]. The specificity of the TaqMan assay [summarized in ref. 26] is the use of probes (short DNA fragments) designed to hybridize to a region within the amplified region of each *BCR-ABL1* sequence and the normalized gene sequence. This probe carries a fluorescent reporter and a fluorescent quenching dye. If there is no amplification (no *BCR-ABL1* transcripts in the sample), the close proximity of the quencher suppresses the fluorescence of the reporter, but in the

BCR = breakpoint cluster region
ALL = acute lymphocytic leukemia

RT = reverse transcription

presence of *BCR-ABL1* in the sample the exonuclease activity of the Taq polymerase leads to lysis of the probe, which results in the emission of a fluorescent signal. A detector monitors this fluorescent signal in real-time and the intensity of the signal is defined by the PCR cycle at which the fluorescence exceeds a threshold limit, the cycle threshold. Actually, Ct corresponds to the amount of target transcripts in the sample; the more templates present at the beginning the lower the Ct. Translation of the arbitrary Ct value into an absolute number is done by constructing two standard curves (one for the fusion transcript and the other for the control housekeeping gene) of known transcript quantity. The patient Ct is then plotted against the standard curve to obtain enumeration of the number of target molecules in each sample. Quantification of *BCR-ABL1* transcripts has proven to be the most sensitive ($1:10^{-5}$) method available and is now the seminal test for evaluating treatment success in CML patients [6]. However, even if the desired response rate, CMoR [Table 1], is achieved according to RQ-PCR monitoring, a recent publication reported persistent leukemia by DNA PCR after imatinib was discontinued (sensitivity $1:10^{-6.2}$) [28].

THE INTERNATIONAL SCALE OF *BCR-ABL1* MOLECULAR MONITORING

The definition of molecular response is based on calculation of the log reduction in the *BCR-ABL1* transcripts identified by RQ-PCR. In patients treated with imatinib, a 3 log reduction (at least) from a standardized baseline value is associated with improved probability of long-term response and improved progression-free survival. This desired value is considered the major molecular response [29]. Patients who achieved CCyR and MMR after 18 months of imatinib therapy have an estimated 100% rate of progression-free survival for 5 years [29]. However, wide variation in the RQ-PCR methods and absence of accepted standards make comparisons between laboratories very difficult. Moreover, MMR terminology is confusing. Comparing equipment and protocols among a large number of laboratories is not a realistic approach, but in 2005 an expert panel suggested improving the analytical step of the RQ-PCR assay [16]. This expert panel defined an international scale for *BCR-ABL1* measurements in an effort to coordinate molecular monitoring. A standard baseline value was determined by processing 30 samples from newly diagnosed CML patients before treatment in three central laboratories of the International Randomized Study of Interferon versus STI571 (IRIS) trials [30]. This baseline value was set at 100% and achievement of MMR was considered as 0.1% (or below) of the *BCR-ABL1* transcript level [29]. Central reference laboratories are now attempting coordination of the *BCR-ABL1* monitoring process by exchanging *BCR-ABL1*-positive samples among themselves and local laboratories that wish to participate in the process. (The Molecular Hematology Laboratory at the Sheba Medical Center, Tel Hashomer serves as the reference laboratory for Israel.) The RQ-PCR results from the reference laboratory and the participating laboratory are compared and a conversion factor based on the international scale is calculated for the local laboratory. This conversion factor applies to less than 10% of *BCR-ABL1* values by RQ-PCR [summarized in ref 31] and its stability has to be validated frequently [32].

Ct = cycle threshold
RQ-PCR = real-time quantitative polymerase chain reaction
MMR = major molecular response

Table 3. Methods for mutation detection

Technique	Detection of known or new mutations*	Sensitivity**	[Reference]
Direct sequencing (following nested PCR)	New	10–25%	[16]
DHPLC (denaturing high performance liquid chromatography)	Known	5–10%	[36]
Fluorescent-based ASO-PCR (allele-specific oligonucleotide)	Known	0.1–1%	[37]
Sequencing of single clones	New	10%	[33]
MALDI-TOF-MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry)	Known	3%	[38]

*For previously detected (known) or new mutations
**The percentage of mutated clones from the total cells analyzed

ABL1 KINASE MUTATIONS: PART OF THE RELAPSE MECHANISM

TKI have revolutionized CML therapy in particular and cancer therapy in general. However, some patients still progress to advanced-phase disease [19] and others respond to imatinib and then relapse. These patients have shown reactivation of the *BCR-ABL1* tyrosine kinase, which may be rationalized by one of two possible mechanisms: a) imatinib fails to reach the "target" due to drug efflux or its activity is inhibited by unknown proteins; or b) the target is insensitive to imatinib due to mutations in the *BCR-ABL1* kinase domain [7]. Early studies reported mutations as the cause for imatinib failure in 40–90% of patients [33]. The rest showed resistance due to undefined molecular events caused by genomic amplification of *BCR-ABL1* or by clonal evolution events [2]. Identification of mutations in the ABL1 kinase domain that caused resistance to imatinib led to the development of new TKI to restore the sensitivity to treatment [34,35]. Most known imatinib-resistant mutations are sensi-

tive to the second-generation TKI, i.e., dasatinib or nilotinib [2]. Therefore, tools for monitoring treatment resistance must be part of the basic routine of molecular assays.

MAPPING THE MUTATIONS ON THE *ABL1* KINASE DOMAIN

More than 100 different point mutations located between amino acid residues 244 and 500 have been reported in the literature. They are found in CML patients developing resistance to imatinib as well as in in vitro mutagenesis assays [33]. The various mutations cause different strengths of resistance, affect the specific therapeutic response, and dictate the selection of the TKI for optimal response.

The domain of *BCR-ABL1* kinase consists of four major regions: a) P-loop, b) ATP-binding site, c) catalytic domain, and d) A- (activation) loop [2]. The two major categories of mutations have been defined as those at positions where direct contact with imatinib occurs and those that affect the conformation of *BCR-ABL1*, thus preventing imatinib binding. The first category comprises the most resistant mutation, namely T315I, located in the ATP-binding site that prevents the formation of hydrogen bonds with imatinib, and the F359V mutation located in the catalytic domain. The second category comprises mutations in the P-loop (M244V, G250E, Q252H, Y253F/H, E255K/V) that decrease the flexibility of *BCR-ABL1* and change the conformational status required for imatinib binding. Mutations in the A-loop (H396R/P) also affect the conformation of *BCR-ABL1*. Specifically, they prevent the kinase from adopting the inactive conformation to which imatinib binds [33].

ANALYSIS OF MUTATION USING MOLECULAR TECHNIQUES: ADVANTAGES AND DISADVANTAGES

Various techniques are available for detection of the *ABL* kinase mutation [Table 3]. There is no consensus and no recommendation regarding the best routine assay, but efforts to harmonize the testing for mutations of *ABL* kinase are currently in progress [39]. Mutations can be reliably detected by nested-PCR amplification of the translocated *ABL* kinase domain, followed by direct sequencing of the entire amplified kinase domain. This is the most widespread method for routine monitoring and is suitable for detecting known and unknown mutations. The sensitivity of the assay enables detection of mutations in samples containing at least 15%–20% of mutated clones. A lower percentage of mutated clones may cause false negative results. Subcloning and sequencing, which are based on selection and expansion of specific clones, followed by direct sequencing, can detect a lower level of mutated clones compared to regular direct sequencing. However, this technique is cumbersome and is not applicable for routine monitoring. Assays based on allele-specific PCR (approximately 0.1% sensitivity), DHPLC (denaturing high performance liquid chromatography), or MALDI-TOF-MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) [38] are more sensitive but are suitable primarily for finding mutations already detected by sequencing and not for screening the entire kinase domain for unknown mutations.

Mutations are found mostly in accelerated phase or blast crisis patients and rarely in chronic-phase patients [40]. In some cases, mutations found in patients with low level disease burden may be transient or unstable. Moreover, early publications demonstrated that the most resistant T315I mutation, found at low levels in pretreated patients, was not selected during therapy [37] and mutations found in patients with stable CCyR have little prognostic significance. In practice, screening for mutations is justified when an increase in the *BCR-ABL1* transcripts is measured by RQ-PCR (especially when passing the MMR level) or in any advanced-phase disease such as in CP patients who do not achieve the appropriate cytogenetic response [6].

CONCLUSIONS

TKI has led to impressive progress in the understanding and treatment of CML. Determining the stage of the disease and the patient's response to treatment depends heavily on generating clear and meaningful laboratory tests. The ongoing concentrated widespread effort by medical centers to coordinate laboratory essays will facilitate development of reliable reproducible tests and achieve standardization according to the international scale.

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Trust in Allah, but tie your camel

Moslem proverb

Some scientists work so hard there is no time left for serious thinking

Francis Crick (1916-2004), English molecular biologist, biophysicist and neuroscientist, most noted for having co-discovered the structure of the DNA molecule in 1953 together with James D. Watson