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REVIEW ARTICLE

Laboratory Monitoring of Chronic Myeloid Leukemia in Patients on Tyrosine Kinase Inhibitors

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Abstract Chronic Myeloid Leukemia (CML) is a myeloproliferative neoplasm characterized by translocation of genetic material from chromosome 9 to chromosome 22 to form a fusion gene (BCR-ABL1) that is responsible for abnormal tyrosine kinase activity and alteration of various downstream signaling pathways. In addition to morphological diagnosis of CML phase, it is essential to detect BCR-ABL1 fusion by either metaphase cytogenetics or reverse transcriptase polymerase chain reaction that also determines type of mRNA transcript. Once treatment begins, monitoring the response to Tyrosine Kinase Inhibitor (TKI) using standardized techniques and guidelines is important to check for failure of response and thus, plan timely intervention by increasing the dose of TKI or opting for second line TKIs. The goal is to stop evolution of CML to accelerated phase or blast crisis that has poor response to treatment. Also, it is desirable to achieve good outcomes and even treatment free remission in patients of CML on TKI. Thus, molecular monitoring by reverse transcriptase quantitative PCR (RT-qPCR) is done at regular intervals. There are international recommendations and quality control measures to standardize the reporting of fusion gene transcript levels by quantitative PCR (RT-qPCR) in CML to achieve and maintain sensitivity in molecular detection of CML disease burden. Various state-of-the-art molecular techniques have emerged to accurately determine the number of fusion-gene transcript levels. This review highlights various methodologies and their practical implications in management of CML patients on TKI.

keywords Chronic Myeloid Leukemia · Monitoring · Tyrosine kinase inhibitors · Reverse transcriptase quantitative polymerase chain reaction

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Introduction

Chronic Myeloid Leukemia (CML) is characterized by recurring cytogenetic abnormality (shortened long arm of chromosome 22, i.e. Philadelphia chromosome-Ph) [1]. It involves reciprocal translocation of genes from chromosome 9 to chromosome 22 to form a fusion gene (FG) BCR-ABL1 [2]. It results in the formation of an abnormal fusion protein that is responsible for the pathogenesis of disease [3]. The abnormal kinase activity of the oncogene can be targeted using the first line tyrosine kinase inhibitors (TKI) such imatinib, dasatinib and nilotinib [4]. In CML patients, monitoring the response to TKI (using standardized techniques and guidelines) is essential to achieve good outcome and even treatment free remission [5]. At the time of diagnosis, it is essential to document morphological diagnosis, phase of the disease, detection of BCR-ABL1 fusion and also the type of fusion transcript [6].

Different Fusion Transcripts in Chronic Myeloid Leukemia (CML)

There are three types of fusion transcripts in CML depending upon the break points in BCR and ABL genes, detected by RT-PCR at the time of diagnosis (Table 1). *Major BCR fusion transcript*-M-bcr (p210 transcript) is most commonly exon 13 or 14 (e13 or e14; previously

Table 1 Types of BCR breakpoints in CML, their mRNA transcripts and different disease phenotypes

Disease phenotype	Breakpoints	Hybrid mRNA transcript	Protein product
CML	M-bcr	e13a2 and e14a2	p210
	m-bcr	e1a2	p190
CML with neutrophilia/thrombocytosis	μ-bcr	e19a2	p230
CML with monocytosis	m-bcr	e1a2	p190

known as b2 or b3) and the breakpoint in ABL1 gene is commonly upstream (a2), and rarely downstream (a3) of exon 2 [7]. The most common fusion mRNA products are e13a2 or e14a2. *Minor BCR fusion transcript*-m-bcr (p190 transcript)-exon 1 and the breakpoint in ABL1 gene is commonly upstream a2 of exon 2 resulting in e1a2 fusion transcript which is seen less frequently in CML but in > 75% cases of acute lymphoblastic leukemia with BCR-ABL1 (Ph+ ALL). Other fusion transcripts include *Micro BCR fusion transcript*-μ-bcr (p230 transcript) rarely seen in CML and involves BCR exons 19 and ABL exon 2 (e19a2) [8].

Qualitative analysis of BCR-ABL1 transcript (RT-PCR) should be followed by quantitative analysis (RT-qPCR) before the start of therapy in order to determine the baseline value of BCR-ABL1 transcripts according to National Cancer Comprehensive Network (NCCN) guidelines [9]. This should be useful in determining the treatment response in the first few months. However, some set ups tend to do away with baseline RT-qPCR testing. This is because serial BCR-ABL1 monitoring of CML patients on treatment offers a satisfactory 'trend over time' pattern for clinical assessment of response [10].

Methods of Monitoring Tyrosine Kinase Inhibitor (TKI) Response

After the commencement of TKI, monitoring of response is done at regular intervals by conventional techniques including peripheral blood (PB) counts and cytogenetic analysis of bone marrow (BM) metaphases. However, once the leukemic burden is reduced to levels that cannot be detected by cytogenetics, more sensitive molecular methods like RT-qPCR are needed [11].

The criteria for response asessment are detailed below:

1. *Hematological response* includes attainment of total leucocyte count $< 10 \times 10^9$/L, absence of immature myeloid cells and basophils <5% in the differential leucocyte count, platelet count $< 450 \times 10^9$/L and non-palpable spleen [12].
2. *Cytogenetic response* is assessed by conventional metaphase cytogenetics (CTG) in a bone marrow sample [13]. At least 20 metaphase spread should be analysed. Depending upon number of metaphases showing Ph chromosome, responses are as below:

Complete cytogenetic response (CCyR)	Ph+ 0%
Partial CyR	Ph+ 1–35%
Minor CyR	Ph+ 36–65%
Minimal CyR	Ph+ 66–95%
No CyR	Ph+ >95%

Cytogenetics is also helpful in monitoring CML cases with atypical BCR-ABL1 fusion transcript where molecular methods are not available [5]. Fluorescent In Situ Hybridization (FISH) is done if BM cannot be obtained or CTG cannot be analysed or in case of cryptic translocations. It can be done on peripheral blood and at least 200 interphase nuclei should be analysed before labelled undetectable [14].

3. *Molecular response* is assessed by RT-qPCR and it increases the sensitivity of detection of leukemic burden [12, 15–17]. This is done by the detection of number of copies of the fusion transcript mRNA in patient sample as compared to transcript load of control genes. It can be expressed as either $\log_{10}$ reduction below the standardized (specified pooled patient) baseline (as in IRIS study- International Randomized Interferon vs. STI571) or as a ratio of BCR-ABL1 copies to control gene expressed as percentage (as in European studies). Major Molecular Response (MMR) is defined as ≥ 3 $\log_{10}$ reduction in BCR-ABL1 copies from the baseline on TKI treatment or < 0.1% BCR-ABL1 copies on the International Scale (IS). Deep molecular response is ≥ 4 $\log_{10}$ reduction in BCR-ABL1 copies (MR^4 or more) from baseline. Early molecular response (EMR) is achievement of BCR-ABL1 ratio $\leq$ 10% at 3 months of commencing TKI [18, 19]. It may be more important than MR at 6 months to predict progression free survival or overall survival [6]. Also, the response monitoring is basically a trend rather than single time point evaluation [6, 20]. EMR is also valid for 2nd generation TKIs used as frontline therapy.

Treatment free remission (TFR) is defined as the

ability to maintain molecular response after stopping therapy. As deeper MR is an achievable target now-a-days, the scope of stopping TKI treatment has surfaced and is still debatable. If deep molecular response is achieved for > 3 years, substantial number of such patients will eventually discontinue therapy without molecular relapse. Jabbour et al. have recently summarized parameters required for consideration to stop TKIs. They include quantifiable RT-PCR transcripts, chronic phase, low-intermediate SOKAL score with optimal response to first line TKI, deep molecular response (> MR^4), and easy availability for monitoring [21].

Time Points for Performing BCR-ABL1 RT-qPCR Testing

It should be done every 3 months till major molecular response (MMR) is reached [6, 13]. After achieving MMR, the frequency may be increased to every 6 months. In stoppage trials for treatment free remission (TFR), monitoring is advised monthly for the first 12 months followed by less frequent monitoring (once every 3 months indefinitely) or as per trial requirements [22].

Definition of Optimal Response to First Line TKI in CML At various Time Points

There are two major recommendations that define treatment milestones: European Leukemia Network (ELN) [18] (Table 2) and the National Comprehensive Cancer Network (NCCN) [19]. These milestones help the clinician to evaluate optimal response and to communicate the results to the patients for their active involvement [10]. The 3-month monitoring results hold different clinical implication amongst the ELN recommendations and the NCCN guidelines. The ELN considers 3-month BCR-ABL1 transcript level > 10% a warning, irrespective of the TKI. However, the NCCN advocates an increase in dose or alternate TKI if the primary treatment is imatinib, whereas treatment continuation at the same dose or change to an alternate TKI is possible if the primary treatment is dasatinib or nilotinib.

According to ELN, optimal response indicates the current treatment to be continued as it predicts excellent outcome [16]. The treatment failure suggests the change in treatment as patient is at significant risk of disease progression and death. The TKI failure can be primary (optimal response never achieved) or secondary (loss of response). In case of failure of response, drug non-compliance and drug interaction should be ruled out. Along with that, BCR-ABL1 kinase domain mutation analysis by Sanger sequencing and cytogenetic analysis for additional mutations in Ph+ cells should be carried out [9, 23, 24]. ELN has an option of wait and watch policy at timepoints where BCR-ABL1 above certain level may not qualify for optimal response. Warning on treatment indicate that the current TKI may not be the best choice but there is no solid data that changing treatment will improve outcome. Hence, patients are more closely monitored for disease progression. However, guideline by NCCN has no grey zone. It advocates change of treatment when the desired milestone is not achieved.

There are some established and provisional factors present at baseline that influence response to TKI [5]. These patients require careful monitoring and withhold future possibility of using investigational therapies. The established factors are additional chromosomal anomaly (ACA) in Ph+ cells [+ 8, + Ph, i(17)(q10), ider(22)(q10), + 19] and high risk patient with high SOKAL, EUTOS or EUROS score [5, 25]. Also, atypical transcripts e.g. b2a3, b3a3, e6a2, e1a3, transcript levels, gene expression profile, polymorphism of genes involved in metabolism of TKI or transport of the drug, low level BCR-ABL1 mutation are provisional factors to be considered at baseline.

Role of International Scale (IS) in Molecular Monitoring of CML Patients on TKI

There are various issues in BCR-ABL1 analysis by RT-qPCR across labs due to differences in protocols, primers, plasmid standard concentrations, interpretation of Cycle threshold (C_t) values and reference/control genes. The

Table 2 The optimal response as defined by ELN 2013 at various timepoints on first line TKI (imatinib, nilotinib or dasatinib)[17]

Time	Optimal	Warning	Failure
Baseline	NA	High risk or CCA/Ph+, major route	NA
3 months	BCR-ABL1 ≤ 10% or Ph+ ≤ 35%	BCR-ABL1 > 10% or Ph+ 36–95%	Non-CHR or Ph > 95%
6 months	BCR-ABL1 ≤ 1% or Ph+ 0 (CCyR)	BCR-ABL1 1–10% or Ph+ 1–35%	BCR-ABL1 > 10% or Ph+ ≥ 35%
12 months	BCR-ABL1 ≤ 0.1% (MMR)	BCR-ABL1 0.1–1%	BCR-ABL1 > 1% or Ph+ ≥1%

NA not applicable, *CCA* clonal chromosomal abnormalities, *CcyR* complete cytogenetic response, *MMR* major molecular response

reporting is done on international scale (IS) so as to bring patients from all over the world at a single platform where treatment response can be compared to standard baseline, thus eliminating variability in results due to difference in analytical systems [26, 27].

Standard baseline is defined as the average copy number of BCR-ABL1 transcripts in baseline group of the 30 untreated CML-chronic phase patients enrolled in IRIS trial (The International Randomized Study of Interferon vs. STI571) which is accepted as an arbitrary value of 100% [15]. Quantitative results of patients are normalized against a reference gene and subsequently the results are converted to IS using laboratory conversion factor (CF). The values on IS are expressed as percentage. It is independent of patient's initial BCR-ABL1 expression and allows comparison of RT-qPCR values amongst different laboratories.

Conversion Factor

To express the results on the IS scale, each testing laboratory should obtain a laboratory-specific conversion factor by sample exchange with an established reference laboratory or by using kits and reagents that have been calibrated to the WHO International Genetic Reference Panel for quantitation of BCR-ABL1 mRNA. Secondary standards can also be obtained from the Institute for Reference Materials and Measurements (Belgium). Formula for conversion to IS for any given result in a lab (L), is as follows:

$$\text{BCR-ABL1}^{\text{L}} \times \text{CF} = \text{BCR-ABL1}^{\text{IS}}$$

For example, a lab calculated MMR equivalent (Eq) of MMR sample from IRIS trial. Its MMR Eq was 0.08% and IRIS MMR 0.1% so CF would be 0.1/0.08 = 1.25'.

Role of Control Gene to Assess the Level of Molecular Response

Quantification of mRNA of target gene by RT-q-PCR can be affected by quality of RNA isolated, time from obtaining sample to RNA isolation, cDNA yield and enzyme inhibitors in the sample. In order to normalize the steps of mRNA quantification, expression of control gene is important [28]. This helps to check the dynamic range of assay. Different control genes used by various laboratories are—ABL1, GUSB, BCR, β_2M, G6PD genes [29]. An ideal control gene should have similar expression levels in all types of blood cells, normal and leukemic cells. Also, its expression level and stability should be similar to BCR-ABL1. Greater the copy number of control genes an assay can measure, more sensitive it becomes.

For defining MR^3 ELN recommends at least 10,000 copy number for control gene that should be amplified for the same volume of sample for BCR-ABLI transcript. Samples with an ABL1 control for $< 10{,}000$ should be discarded. More than 3 $\log_{10}$ reduction (≥ 4 $\log_{10}$) in BCR-ABL1 copies from baseline is defined as deep molecular response. Deep MR can be MR^4, $MR^{4.5}$, MR^5 are defined below [30].

$MR^4 = \geq 4$ $\log_{10}$ reduction from IRIS baseline—either

- Detectable disease $\leq 0.01\%$ $\text{BCR-ABL1}^{\text{IS}}$
- Or undetectable disease in cDNA with 10,000–31,999 ABL1 transcripts or 24,000–76,999 GUSB transcripts.

$MR^{4.5} = \geq 4.5$ $\log_{10}$ reduction from IRIS baseline—either

- Detectable disease $\leq 0.0032\%$ $\text{BCR-ABL1}^{\text{IS}}$.
- Or undetectable disease in cDNA with 32,000–99,999 ABL1 transcripts or 77,000–2,39,999 GUSB transcripts.

$MR^5 = \geq 5$ $\log_{10}$ reduction from IRIS baseline—either

- Detectable disease $\leq 0.001\%$ $\text{BCR-ABL1}^{\text{IS}}$.
- Or undetectable disease in cDNA with $\geq 100{,}000$ ABL1 transcripts or $\geq 240{,}000$ GUSB transcripts.

Quality Control Measures in RT-qPCR for BCR-ABL1 Transcripts

Pre-analytical variables: At least 10–20 mL of EDTA anticoagulated PB should be taken. The sample should reach the laboratory preferably within 24 hours and definitely within 48 hours to prevent RNA degradation because these are RNA based tests. A minimum nucleated cell count of $1–2 \times 10^7$ should be present to ensure adequate RNA quantity [15]. Sensitivity of qPCR depends on sample quality and test or assay performance [30].

All efforts should be taken to minimize contamination. The RNA extraction should be done using in-house standardized protocol or protocol provided by kit insert. To standardize the steps of RNA extraction, 'armored' RNA plasmid control may be used. RNA is isolated from peripheral blood leucocytes and the assessment of purity and quantity of RNA is measured using spectrophotometric method like Nano-drop. Also, running RNA sample on agarose gel, checks for any degradation of the RNA sample.

Analytical variables: The cDNA samples should be run in duplicates or triplicates. The ELN recommends that sample should have at least 10,000 ABL1 or at least 24,000 GUSB copies to pass minimum quality standard.

Assay performance: In absolute quantification assays, concentrations of standard samples of known cDNA concentrations are plotted against their Ct values to obtain a standard curve (Fig. 1). Log concentration of unknown samples can be extrapolated from their Ct values

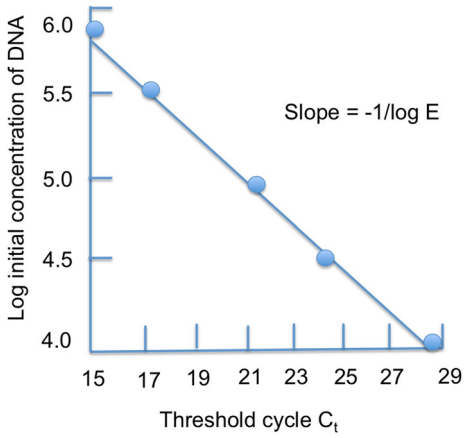

Fig. 1 A standard curve of C_t value vs. logarithmic of initial DNA concentration. A calculation for estimating the efficiency (E) of a real-time PCR assay is $E = (10^{-1/slope} - 1) \times 100$ (adapted from Sandy B. primrose. Principles of gene manipulation, 7th edition, page 31)

on the curve. To increase linearity of assay, correlation coefficient (R^2) of standard curve should be > 0.980 and the slope of standard curve should be -3.20 to -3.60, as close to 3.32. The identical replicates should have a Cycle threshold (C_t) standard deviation < 0.3 so that any drift of Ct values for plasmids and high and low quantification controls can be recorded [15, 31].

For quality practice to enhance precision in qPCR and to define MR in a reproducible manner, MIQE guidelines (Minimum Information for Publication and Quantitative RT-PCR experiments-2009) are followed [32].

In Europe against Cancer program (EAC) for minimal residual disease (MRD) in CML, a total of 26 European university laboratories from 10 countries have collaborated to establish a standardized protocol for TaqMan based RT-q-PCR analysis of the main leukemia-associated fusion genes for molecular determination of MRD levels [33].

Reporting of Molecular Response (MR)

BCR-ABL1 ratios are expressed as percentage (%): BCR-ABL1 molecules divided by the total number of ABL1 molecules and multiplied by 100; for example: BCR-ABL1: 5000 molecules, ABL1: 20,000 molecules; Ratio = 25.0%

According to ELN guidelines, reporting should include the control gene copy numbers [31]. If control gene copy number is adequate then the report is reliable and sensitive. The sample result must be discarded if ABL copy number is $< 10{,}000$ or GUSB copy number is $< 24{,}000$ regardless of BCR-ABL1 copy number [31, 34]. In case of satisfactory control gene numbers amplified, the report should indicate the level of MR defined. In case of inconsistent MR results at previous time points, BCR-ABL1 transcript level fluctuations in the absence of MMR loss, and borderline results, re-sampling and reconfirmation of results should be advised before any clinical decision is taken.

Mutation analysis should be recommended in case BCR-ABL1 transcript levels indicate warning (or failure). Bauer et al. suggested that inclusion of a chart recorded by the patient himself that monitors RT-qPCR results at various time points, will not only increase adherence to therapy but also help the clinician to track the decrease or increase in disease burden. This '*trend over time*' curve helps overlook any random single point unsatisfactory result [10].

Future Directions in Technologies for BCR-ABL1 Monitoring in Patients on TKI

There are emerging technologies for MR assessment that includes digital drop PCR (ddPCR) and automated technology that incorporates relative quantification of BCR-ABL1 fusion transcripts.

The ddPCR Technique

It is based on the separation of a standard PCR reaction into many thousand single nanoliter droplets, each of which is tested independently for the presence of the target with absolute number of copies, i.e. none, one or more copies of the target DNA molecule. As the assay is dependent on end-point detection of the amplification product, the effect of PCR efficiency is reduced, minimizing the requirement for internal standardization and calibration curves. It monitors very low BCR-ABL1 levels in CML patients with a high degree of reliability and sensitivity (1–2 log improvement in sensitivity) [35]. The advantage is that there is no need of standard curve analysis and analysis is independent of C_t. However, the dilemma is over cut off levels. Due to high sensitivity it can detect even a single copy of BCR-ABL1 in a given sample. The significance of finding BCR-ABL1 copies in sample that are in MR by absolute quantification is clinically debatable.

Automated and/or Point-of-Care Testing Systems

GeneXpert technology [36] is the latest technology useful in routine practice due to advantage of ease of operation. It is a quantitative test that can be done only for BCR-ABL1 major breakpoint (p210) transcripts. It automates the entire test process including RNA isolation, reverse transcription, and fully nested real-time PCR of BCR-ABL1 target gene and ABL reference gene in one fully automated cartridge. Moreover, standard plasmids for BCR-ABL1 and control genes are used to draw standard curves at the initial calibration, they aren't required thereafter with each sample lot run. It is recommended to use more than one control genes for normalization of target BCR-ABLI to increase accuracy

of results. Its performance was compared prospectively with the manual RT-qPCR based reference method on peripheral blood samples from 129 patients [37]. The overall and MMR concordance were 85.7 and 94% respectively. However, GeneXpert is used only for the monitoring and not for diagnosis. Also, transcripts other than p210 cannot be monitored by the system.

Conclusion

Real time PCR is essential for monitoring response to TKI in CML patients. Proper monitoring helps in preventing the evolution of CML to accelerated phase or blast crisis and also to plan timely intervention by increasing the dose of TKI or opting for second line TKIs. Long term monitoring along with 'trend over time' for deeper molecular response helps in monitoring compliance of patient, occurrence of additional chromosomal anomalies in Ph+ cells or BCR-ABL1 kinase domain mutation and to minimize drug toxicity. Achieving deep and stable response is necessary before stopping TKI therapy can be considered. Therefore, standardized molecular monitoring (on IS) of CML patients as per ELN recommendations or NCCN guidelines recommendations is vital. This should be done by undertaking adequate laboratory quality control measures for cytogenetics and molecular reporting along with continuous education and update of information of both the diagnosticians and the clinicians.

Though, newer methodologies for BCR-ABL1 monitoring to enhance automation and minimize errors due to manual intervention have been developed, these are still used in research centers and these techniques haven't yet been incorporated in routine patient care. Multi-center studies to establish their utility and ability to offer standardized patient testing should be undertaken.

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Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

Research Involving Human and/or Animals This article does not contain any studies with human participants or animals performed by any of the authors.

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