Article published online: 2023-11-27

Disease Response Assessment Modalities in Chronic Myeloid Leukemia: Past, Present, and Future

Deepak Kumar Mishra[1] Indranil Dey[2] Rakesh Demde[2] Sushant Vinarkar[3] Mayur Parihar[4]

[1] Department of Laboratory Sciences, Haematology and Molecular Pathology, TATA Medical Centre, Kolkata, West Bengal, India
[2] Department of Molecular Pathology, TATA Medical Centre, Kolkata, West Bengal, India
[3] Department of Haematology and Molecular Pathology, TATA Medical Centre, Kolkata, West Bengal, India
[4] Department of Haematology, Cytogenetics and Molecular Pathology, TATA Medical Centre, Kolkata, West Bengal, India

Address for correspondence Deepak K. Mishra, MD, PDF Haematology, Department of Laboratory Sciences, Haematology and Molecular Pathology, TATA Medical Centre, Kolkata 700160, West Bengal, India (e-mail: deepak.mishra@tmckolkata.com).

Ind J Med Paediatr Oncol 2023;44:592–601.

Abstract

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm caused by the *BCR::ABL1* fusion gene, which results from a reciprocal translocation between chromosome 9 and 22 t(9;22)(q34;q11). The use of tyrosine kinase inhibitor (TKI) against the chimeric *BCR::ABL1* fusion protein has led to a paradigm shift in CML patient outcomes. Despite generational advancements in TKI, a fraction of patients harbor residual disease or exhibit resistance to TKI. The importance of disease monitoring and detection of resistance mechanisms has gained prominence with increasing knowledge about disease evolution. In the past, cytogenetic techniques such as karyotyping and fluorescence in situ hybridization were widely utilized for monitoring disease and prognostication. These techniques had various challenges related to limited sensitivity in minimal residual disease (MRD) monitoring; however, their importance still holds in the detection of additional chromosomal aberrations and in cases with cryptic insertions, variants, and masked Philadelphia chromosome. Molecular genetics has evolved significantly from the past to the present times for MRD monitoring in CML patients. Qualitative reverse transcription polymerase chain reaction (RQ-PCR) can be performed at diagnosis to detect the *BCR::ABL1* transcript, while quantitative RQ-PCR is the most widely used and well-standardized MRD monitoring method. The DNA-based assays demonstrated high sensitivity and specificity, with many efforts directed toward making the laborious step of *BCR::ABL1* breakpoint characterization less tedious to increase the utility of DNA-based MRD approach in the future. Flow cytometric–based approaches for the detection of the *BCR::ABL1* fusion protein have been under trial with a scope of becoming a more robust and convenient methodology for monitoring in the future. Upcoming techniques such as digital PCR and ultra-deep sequencing next-generation sequencing (UDS-NGS) have shown promising results in residual disease monitoring and detection of resistance mutations. Novel MRD monitoring systems that are independent of *BCR::ABL1* fusion such as the detection of CD26+ leukemic stem

Keywords
- hematology
- medical oncology
- pathology

DOI https://doi.org/10.1055/s-0043-1771186.
ISSN 0971-5851.

Thieme Medical and Scientific Publishers Pvt. Ltd., A-12, 2nd Floor, Sector 2, Noida-201301 UP, India

cells and microRNA mutations are the future of residual disease monitoring, which can go up to the level of a single cell. In this review, we tried to discuss the evolution of most of the above-mentioned techniques encompassing the pros, cons, utility, and challenges for MRD monitoring and detection of TKI resistance mutations.

Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm that accounts for approximately 10 to 15% of all newly diagnosed cases of leukemia. In India, the incidence of CML is around 2/100,000 in men and around 1.5/100,000 among women, with a median age varying between 30 and 45 years.[1,2]

This was one of the initial hematological neoplasms that could be linked to a specific cytogenetic abnormality known as Philadelphia (Ph) chromosome. The Ph chromosome involves reciprocal balanced translocation involving Abelson murine leukemia (*ABL1*) gene on chromosome 9 and the breakpoint cluster region (*BCR*) gene on chromosome 22 and forms a chimeric protein that led to the pathogenic events of leukemogenesis. This phenomenon later became instrumental in the tyrosine kinase inhibitor (TKI) discovery that greatly changed the therapeutic landscape of CML. The use of TKI therapy altered the natural history of CML, so much so that it improved the overall survival rate of 10 years from approximately 20% to almost 90%.[3]

Despite the promising outcome result of TKI therapy, there are few number of cases (~5%) that still have a progression of disease.[4] In earlier days, prolonged treatment by TKI throughout the lifetime of patient was the only belief for complete cure. More recently, however, the concept of "treatment-free remission" (TFR) has come into prominence.[5] Current practices are more focused on avoiding resistance and increasing the TFR rate for patients.

Various technologies for minimal residual disease (MRD) monitoring and mutation testing have evolved with time, each having their own inherent advantages and drawbacks. It is therefore requisite to discuss the past, currently available, and novel technologies that may have far-reaching effect upon the theragnostic landscape of CML. Here, we endeavor to critically review various research studies that have been performed toward this end.

Cytogenetic Disease Monitoring

The Ph chromosome is pathognomonic of CML; however, additional chromosomal aberrations were also noticed in 3 to 5% of CML cases at diagnosis.[6–9] These abnormalities will lead to decreased survival rate and an early conversion of chronic phase to accelerated/blast phase. Among these, the most frequently seen abnormalities are presence of additional Ph (~35%), trisomy 8 (~35%), i(17q) (~20%), trisomy 19 (~20%), trisomy 21 (~10%), and loss of the Y chromosome (~10% in males).[8,10–12] Conventional karyotyping should be performed upfront to detect these clonal aberrations to predict the outcome in CML cases; however, this technique is not adequately sensitive as a standalone modality for treatment response monitoring.

In present times, highly sensitive fluorescence in situ hybridization (FISH) is a routinely used cytogenetic technique, which can be wielded on both metaphase and interphase cells. In CML, one red, one green, and two yellow (fusion) signals of *BCR::ABL1* are commonly observed pattern on FISH. A dual-color, dual-probe fusion FISH probes can detect additional abnormalities and also the cryptically inserted Ph with a 1% cutoff, which can become very useful in identification and confirmation of such cases.[13–16] At an interval of 3, 6, and 9 months, FISH should be performed preferably in bone marrow aspirate sample till a point where complete cytogenetic response (CCyR) is achieved. These should be followed by annual FISH testing in accordance with the current international guidelines for disease monitoring.[12,16,17] As per the European LeukemiaNet (ELN) 2020 recommendation, cytogenetic testing (including FISH) is useful for disease monitoring in CML patients harboring rare or atypical *BCR::ABL1* transcripts and atypical translocations that cannot be measured by quantitative polymerase chain reaction (PCR) techniques.[18]

Molecular Genetic Disease Monitoring

The detection and quantification of the chimeric *BCR::ABL1* fusion gene has been the most widely adopted approach in molecular genetics for CML patients. Multiple established and emerging molecular diagnostic platforms are at hand of clinicians, each having their own advantages, disadvantages, and technical nuances. We will endeavor to elaborate upon these molecular methods further.

Conventional Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction

The real-time quantitative reverse transcriptase PCR (RQ-PCR) is the most widely used method for CML monitoring currently, owing to its widespread availability and established standardization. This approach starts with extraction of RNA from peripheral blood sample or bone marrow aspirate, which is followed by cDNA conversion by using random hexamers and reverse transcriptase enzyme.[19] Both Moloney murine leukemia virus and SuperScript are suitable for reverse transcription.[20] The amplification of *BCR::ABL1* along with internal housekeeping gene (*ABL1* or *GUSB*) is performed on the cDNA. After this step, the quantification is done using the standards of known concentration.

The PCR components consist of majorly input template, fluorescent probes, and thermal cycler. At least 1 µg of RNA input is necessary for proper amplification of the transcript. Any deviation can lead to inaccuracies in the quantification.[20,21] Hydrolysis or hybridization probe is recommended for this assay, with TaqMan probes being the most popular. The choice of using a particular real-time thermal cycler depends on the throughput, sensitivity, and cost.

The quality parameters are of utmost importance in real-time PCR. For each PCR reaction, plasmid standard curves have to be generated that should cover the dynamics of test with at least four standard points.[15] In real-time quantification for *BCR::ABL1*, the recommendation is to run *BCR::ABL1* in triplicates and *ABL1* in duplicates. The recommended slopes for standard curves should lie between −3.20 and −3.60 (ideally close to −3.32) and R^2 (coefficient of correlation) should be >0.9815.[20,21] During the analysis of the *BCR::ABL1* copies, a constant threshold is to be strictly maintained (recommended range is between 0.05 and 0.1 depending on the PCR platforms used).[20] The Y intercept is also an important quality parameter and should ideally be 39.8 ± 1 for both *BCR::ABL1* and *ABL1*, respectively. Any major difference in Y intercept values between different runs and/or between *BCR::ABL1* and *ABL1* copies will lead to inaccurate quantification.[7,21]

The copy numbers are counted by mean value of the replicates. The Cq values of less than 0.5 between the highest and lowest replicates is an absolute requirement till 35 intercept value. The copy numbers detected outside 0.5 Cq to be excluded from quantification and mean of the remaining replicates can be used.[20,22] Any deviations from this mentioned quality parameters in real-time PCR should be rectified for correct quantification.

The treatment response of CML patients to TKIs should be assessed as the ratio of *BCR::ABL1* transcripts to *ABL1* transcripts or to other internationally accepted control transcripts (e.g., β glucuronidase, *GUSB*) using the international scale (IS). To bring uniformity among the laboratories for measuring *BCR::ABL1* copies, the IS was developed. A standard base line for this scale was calculated from the patients of the IRIS trial. The minimum number of reference gene for MRD monitoring used for *BCR::ABL1* reaction should be as per the ELN 2020 recommendation (►**Table 1**). According to ELN 2020 recommendations, the response evaluation to treatment in CML patients is tabulated here (►**Table 2**)[18,23,24] Due to its ready availability, high throughput, and robust standardization, RQ-PCR has been the most popular method of disease monitoring in CML till date.

Digital PCR-Based MRD Monitoring

The digital PCR (dPCR) is one of the latest generation PCR technologies that is based on the principle of partitioning the PCR reaction and provide an absolute quantity of copies in numerical digits. It is becoming a popular and widely accepted method for the MRD molecular monitoring in hemato-oncology,[25–28] which can be attributed to its increased accuracy and sensitivity. In comparison to RQ-PCR, the dPCR has the added advantage of providing absolute quantification without the requirement of reference standards.[29,30] The dPCR can more efficiently monitor CML patients in TFR trial.[31–35]

The droplet digital PCR (ddPCR) is a type of dPCR based on the separation of a standard PCR reaction into many thousand nanoliter single droplets (specifically 20,000 droplets). Most of the droplet contain either one (or more) or no target

Table 1 ELN recommended reference gene copy numbers for CML MRD monitoring

TKI treatment response	BCR::ABL1 ratio, % (IS scale)	Lowest required housekeeping gene copy numbers
Major molecular response	≤0.1%	10,000 *ABL1* or 24000 *GUSB*
Molecular response-4	≤0.01%	
Molecular response-4.5	≤0.0032%	32,000 *ABL1* or 77,000 *GUSB*
Molecular response-5	≤0.001%	1,00,000 *ABL1 or* 2,40,000 *GUSB*

Abbreviations: CML, chronic myeloid leukemia; ELN, European LeukemiaNet; IS, international scale; MRD, measurable residual disease; TKI, tyrosine kinase inhibitor.

Table 2 ELN 2020 recommendations for treatment response evaluation in CML patients by monitoring *BCR::ABL1%* on the international scale[18,23,24]

Time points	Optimal	Warning	Failure
Baseline	NA	High-risk ACA, high-risk ELTS score	NA
3 mo	≤10%	>10%	>10% if confirmed within 1–3 mo
6 mo	≤1%	>1–10%	>10%
12 mo	≤0.1%	>0.1–1%	>1%
Any time	≤0.1%	>0.1% loss of ≤ 0.1% (MMR)[a]	>1%, resistance mutations high-risk ACA

Abbreviations: ACAs, additional chromosomal aberrations; ELN, European LeukemiaNet; ELTS, EUTOS score for long-term survival; MMR, major molecular remission; NA, not applicable.
[a]Loss of MMR (BCR-ABL1 > 0.1%) indicates failure after TFR.

copy. In each single droplet, the PCR amplification is performed and the original target copy number is calculated from the proportion of positive and negative droplets generated using the Poisson distribution statistics. Moreover, ddPCR is more resistant to nonspecific amplifications.[5,36] The greater sensitivity of ddPCR has been established by a reasonable number of studies that have shown prompt detection of loss of deep molecular remission utilizing this technique in comparison to real-time PCR.[37–40] The ddPCR platform has shown great promise in MRD monitoring, especially for lesser common BCR::ABL transcripts (e.g., e1a2, e19a2).

Microfluidic-Based MRD Monitoring

In recent times, various other monitoring techniques based on RNA have become available. One of the most used among these techniques is by Cepheid Xpert *BCR::ABL1* ultra. It is a quantitative tests for *BCR::ABL1* p210 transcripts. This is a sensitive, fully automated cartridge-based technique, which is based on the GeneXpert technology.[41] The technology uses the principle of microfluidics in a cartridge and performs RNA extraction, reverse transcription, and *BCR::ABL1* quantification in a single reaction.[41,42] To achieve uniformity of result, this kit also provides a conversion factor. Various previous studies have reported inability of this system to detect the BCR::ABL1 transcript level below 0.01%. However, recent advancement in kits has resolved such issue.[43]

MRD Monitoring in CML with Nonclassical *BCR::ABL1* Transcripts

Rarely, in 1 to 2% of CML cases, the nonclassical transcript (e19a2, e13a3/e14a3, and e1a2) are encountered. Conventional karyotyping, FISH, and reverse transcription PCR can be useful for such rare transcript detection, as documented in literateure.[44,45] The probability of achieving CCyR and major molecular remission in such cases is less; in addition, lower rates of event-free survival and progression-free survival have also been reported.[45] The quantification of such transcripts poses a unique problem due to the lack of standardization among commercially available kits for MRD monitoring. Conventional cytogenetics, quantitative RQ-PCR, and ddPCR using patient-specific primers can provide an effective solution but it can be expensive.[46]

Flowcytometric-Based MRD Monitoring

The flowcytometric immunobead assay utilization for *BCR::ABL1* fusion proteins detection has been used by few centers.[47] A concordance between reverse transcription PCR of fusion gene transcripts by approach of utilizing the anti-*BCR* catching antibody adhered to immunobead and fluorescently tagged anti-*ABL1*antibody is published in the literature. The limit of detection (LOD) of 1% was derived using the sequentially diluting three different *BCR::ABL1*-harboring cell lines. The sensitivity is better than karyotyping (~5%), equals to FISH (~1–2%), and lower than the PCR-based platforms.[47–49] The proximity ligation assay is a flowcytometric-based approach that can quantify white blood cells having the *BCR::ABL1* fusion at the proteomic level. The other approach is by the enzyme dipeptidyl-peptidase-IV (DPPIV/CD26), which are being detected in a specific type of CD34+/CD38− leukemic cells along with CD26 positivity, which is not seen in normal hematopoietic stem cells (HSCs) and only present in CML leukemic stem cells (LSCs). This attribute is used in flowcytometry to capture CML-positive stem cells for MRD monitoring.[50–53]

Novel Techniques for MRD Monitoring

Single-cell sequencing (SCS) technologies using the single-cell gene amplification for identifying the heterogeneity among the LSCs is a novel approach for MRD monitoring.[54] This technique uses a multiomics work approach. The transcriptome of CML LSCs, which are resistant to TKI, is biologically different from the normal HSCs, as demonstrated in Smart-seq2 study using SCS.[55]

The study of microRNA (miRNA) in leukemogenesis is another area of interest in the present times. The translation of miRNAs from research to diagnostic setup in leukemogenesis is picking up the pace. The genomic profile of miRNAs responsible for oncogenesis ranges from expression analysis to mutation, deletion, and epigenetic changes.[56] The HSC differentiation and deregulated expression of several miRNAs such as miR-486–5p play a vital role in hematological malignancies, and these also get overexpressed in CML CD34+ progenitor cells.[57–59]

The monitoring of CML using DNA as an input template is sparsely utilized, and very few studies are published on this approach. Among the published literature, there are discordances documented among the detection of positive *BCR::ABL1* copies by RNA and DNA. The use of RNA as an input material is widely accepted method; however, DNA-based methods provide more sensitive and specific results.[60] The LOD for DNA based method (10^{-6}) is superior than RNA-based techniques. The disadvantage of DNA-based approach is requirement of pretesting characterization of breakpoints, which is a very laborious and time-consuming work. The newer techniques to overcome this problem have been under trial such as long-range PCR, multiplex PCR, Sanger sequencing, and next-generation sequencing (NGS).[61–65] Due to its greater sensitivity compared with RNA-based technique, it can be adopted for TFR trial in future.[66]

BCR::ABL1 TKI Resistance in CML

The first-generation TKI imatinib is offered upfront to majority of the newly diagnosed CML cases due to its widespread availability, efficacy, compliance, and cost. There is a significant number of CML cases that develop resistance to imatinib (10–15% of cases) and are bound to shift to higher generations of TKI. It becomes essential to identify imatinib resistance at the earliest, to benefit these patients with dose escalation, higher generation TKI, or in, certain cases, HSC transplant.[18,67]

Types of TKI Resistance

TKI resistance mechanism can be primarily segregated into two groups: innate (primary) resistance and acquired

(secondary) resistance. Innate resistance may be suspected in the following scenario during CML treatment:

- Absence of complete hematological response or FISH Ph positivity of more than 95% at 3 months.
- *BCR::ABL1* copies greater than 10% or FISH Ph positivity of more than 35% at 6 months.
- *BCR::ABL1* copies greater than 1%, FISH Ph positivity of more than or equal to 1%, or CCyR at 18 months.

Acquired resistance can be suspected whenever there is a forfeiture of a previously achieved hematological, cytogenetic, or molecular response during the course of TKI treatment.[68]

TKI Resistance due to *BCR::ABL1* Tyrosine Kinase Domain Mutation

Among many myriad mechanisms, tyrosine kinase domain (TKD) mutations accounts for the majority among the causes of TKI resistance. These may be detected in up to approximately 60% of patients with suboptimal TKI response. During the disease progression (accelerated/blast phase), these mutations are documented with higher frequencies. Recognition of these TKD mutation is very critical during the therapeutic phase of CML, as the change of TKI is predominantly dependent on the type of mutations.[69]

The *ABL1* TKD has components such as P-loop, catalytic domain, and A-loop.[70] The usual binding of the TKI takes place between the mentioned TKDs. The TKD mutations may lead to ineffective binding of the drug moiety and result in TKI resistance in majority. In the literature, there are hundreds of variants documented with varying response to TKI therapy based on the location of mutation. P-loop mutations are the most common, accounting for nearly 50% of the TKD mutations, and confer a poorer prognosis.[71] Compound TKD mutations are also documented, which are defined by the occurrence of more than one variant on the same DNA strand, and these are often associated with particularly high resistance to multiple generations of TKIs.[72,73] The most frequent TKD mutations along with their location and resistance profile have been depicted in ►**Table 3**.[74,75]

The timing of *BCR::ABL1* TKD mutations testing is critical, as early detection can be decisive. Various recommendations exist from ELN, European Society for Medical Oncology (ESMO), and National Comprehensive Cancer Network (NCCN) regarding the appropriate time point to perform the TKD mutation analysis and are summarized in ►**Table 4**.[76,77]

Table 3 Frequency of *BCR::ABL1* TKD mutations and their response profile to the approved inhibitors[74,75]

TKD variant	Site	Imatinib	Dasatinib	Nilotinib	Bosutinib	Ponatinib	Asciminib
Wild-type							
M244	P-loop						
L248							
G250							
Q252							
Y253							
E255							
V299	C-helix						
T315	Drug contact site						
F317							
A337	Catalytic-loop						
M351							
M355							
F359							
H396	Activation-loop						
W464	Myristate pocket						
P465							
V468							
I502							
	Sensitive						
	Intermediate sensitivity						
	Resistant						

Abbreviation: TKD, tyrosine kinase domain.

Table 4 Recommended time points for TKD mutation analysis[76,77]

Guidelines	Diagnostic time point	During first-line therapy with imatinib	During second-line therapy with dasatinib or nilotinib
European LeukemiaNet (ELN) and European Society for Medical Oncology (ESMO)	Patients with accelerated phase/blast phase CML	• Treatment failure • Suboptimal therapeutic response • Loss of MMR due to increment in BCR::ABL1 transcript levels • Prior to shifting to other TKIs/alternate therapies	In event of hematologic or cytogenetic failure, including: • No cytogenetic response at 3 mo • Minimal cytogenetic response at 6 mo • Not achieving partial cytogenetic response at 12 mo • Prior to shifting to other TKIs/alternate therapies
National Comprehensive Cancer Network (NCCN)	➢ Disease progression to accelerated phase/blast phase	• CML chronic phase with inadequate initial response (failure to achieve partial cytogenetic response or *BCR::ABL1/ABL1* (IS) ratio 10% or less at 3 mo or complete cytogenetic response at 12 mo and 18 mo • CML chronic phase with indication of loss of response (hematologic or cytogenetic relapse or greater than 1-log increase in BCR::ABL1 transcript levels and loss of MMR	

Abbreviations: CML, chronic myeloid leukemia; IS, international scale; MMR, major molecular remission; TKD, tyrosine kinase domain; TKI, tyrosine kinase inhibitor.

Acquired TKI Resistance Mechanisms

TKI resistances not associated with TKD mutations can also have significant contribution toward suboptimal therapeutic response and are grouped as secondary factors. Many such secondary factors can range from (but is not limited to) variables such as treatment compliance, drug bioavailability, altered pharmacodynamics and pharmacokinetics, genomic instability, and *BCR::ABL1* gene amplification/overexpression. Very rarely, causes such as alternate mechanism of signaling and mutations beyond the kinase domain of *ABL1* gene can also lead to acquired resistance.[78]

Methodologies to Assess TKI Resistance

Currently, many molecular platforms with a variety of assays to detect TKD mutational profile having a significant clinical impact are readily available. These assays have their own merits and drawbacks as summarized in ►**Table 5**. The most commonly used techniques for *BCR::ABL1* TKD mutation assays are discussed in brief in the following.

General Guidelines for *BCR:ABL1* TKD Mutation Analysis

The sample processing and RNA extraction should be done per the recommended standard protocols of laboratory. There should be a written policy to avoid cross contamination, especially for the nested PCR-based methods. Appropriate negative controls and NTC (no template controls) must be employed during each run. Generally, most TKD mutation detection strategies use methods that selectively amplify the *ABL1* component of the *BCR::ABL1* fusion product and should not amplify the nonmutated (wild-type *ABL1* gene). Multiple transcripts of *BCR::ABL1* fusion have been documented; therefore, it is of utmost importance to know the transcript of the patient before proceeding with these assays. The quality of the RNA should pass the recommended quality parameters. One of the important quality control parameters is that the sample should have *BCR::ABL1* and *ABL1* copy numbers >50 and >5,000, respectively; any suboptimal copies should not be tested and re-extraction is advised.[80]

Sanger Sequencing–Based TKD Mutation Analysis

Sanger sequencing is considered the gold standard assay for the detection and screening for TKD mutation screening. Due to recommendation by international guidelines and consensus panels, it is being employed in majority of laboratories. However, it has a drawback of relatively poor sensitivity (10–20%) and can lead to missing out some mutation (false negative). There are also limitations of Sanger sequencing since it cannot detect all existing mutations, such as compound, polyclonal mutations, and mutations present below the detection limit of the assay (variant allele frequency $\leq$ 20%). A common strategy employed is to selectively amplify the TK domain of *ABL1* (exons 4–10) and use bidirectional Sanger sequencing with overlapping primers. This strategy is more effective, as every base gets sequenced at least two times.[81]

PCR (dPCR)-Based TKD Mutation Analysis

dPCR can also be potentially applied in TKD mutation analysis. The dPCR assay detects targeted TKD mutations by using specific primers and probes. It is more useful when limited mutation analysis is desired; it is technically less demanding and has a shorter turnaround time. A single-tube dPCR assay for the detection and quantification of common TKD mutations has been recently developed.[82] The "drop-phase" dPCR is one of the modified versions of dPCR, which utilizes droplet-based dPCR to identify compound mutations. This platform uses mutation-specific dual-color probes using which compound mutations can be detected as an increase in double-positive droplets.[83]

NGS-Based TKD Mutation Analysis

NGS is a high throughput molecular diagnostic modality that is gaining wide-reaching popularity in the detection of TKD

Table 5 Advantages and drawbacks of various TKI mutation detection platforms[77,79,80]

Testing platform	Sensitivity	Advantages	Drawbacks
Sanger sequencing	15–20%	Widely available Economical Bidirectional confirmation possible Semiquantitative Short turnaround time	Relatively less sensitive Suboptimal RNA quality and quantity may affect accuracy Compound and polyclonal mutations cannot be detected Technically tedious
Digital PCR	0.01–0.02%	Highly sensitive Economical Rapid results	Only limited number of mutations can be investigated Lacks standardization Compound mutations may be detected only if the mutation partners are already known
NGS (ultra-deep sequencing)	0.1–1.0%	Entire TKD is analyzed Can detect and discriminate between complex mutations (polyclonal vs compound) Can monitor mutation dynamics Quantitative Better sensitivity and specificity	Not widely available Labor-intensive and needs expertise Not yet standardized Requires good sample volume to be economically feasible (batch assay) Clinical relevance of low-level TKD mutations not well established
Denaturing high-performance liquid chromatography	0.5–15%	High output Economical Good screening test	Limited availability Cannot characterize mutation Can generate nonspecific peaks False-negative results (in cases with high mutation burden)
Allele-specific oligonucleotide quantitative reverse transcription PCR	0.001–0.1%	Good sensitivity Quantitative analysis possible Wide availability Simple workflow	Limited to only few targetable mutations Compound variants not detected Low throughput High chances of false positives and false negatives Low output

Abbreviations: PCR, polymerase chain reaction; TKD, tyrosine kinase domain; TKI, tyrosine kinase inhibitor.

mutations in CML patients and is particularly advantageous in detecting TKD variants at very low allelic frequencies.[84]

NGS of amplicons encompassing the TKD is capable of detecting single nucleotide variant, insertion and deletion variants in the *BCR::ABL1* transcript. This approach can achieve sensitivities up to 1% or even deeper. "Deep" or "ultra-deep sequencing" (UDS-NGS) is an application of NGS that is optimized for TKD mutation analysis, where a genetic region of interest is sequenced many times (hundreds to thousands), thus enabling it to achieve very high sensitivities.[85] Multiple studies have documented UDS-NGS can detect TKD mutations (including T315I variant) earlier as compared with Sanger sequencing and other highly sensitive assays. UDS-NGS can identify all TKD mutations including novel variants and can detect patients who harbored more than one resistance mutation.[86] CML patients harboring compound variants can be distinguished from those with polyclonal variants by the variation in read distribution using assays with longer amplicons design. To rule out false-positive results due to sequencing artifacts and chimeric reads, modified sequencing strategies (error-corrected sequencing, single molecule consensus sequencing) may be employed.[87,88] In the year 2020, ELN advocated the use of NGS for those CML cases that did not adequately respond to standard TKIs.[18] More robust outcome-based evidence would further strengthen the importance of TKD mutation analysis by NGS and help in its wider utilization in the clinical setting.

D-HPLC-Based TKD Mutation Analysis

Denaturing high-performance liquid chromatography (D-HPLC) is another screening technique with a high-output capacity. This employs a heteroduplex formation by PCR products amplified from wild-type and mutant alleles. Subsequently, these heteroduplexes are then used to distinguish from homoduplexes under optimal denaturation conditions. D-HPLC is more sensitive compared with direct sequencing; however, it is not as widely available and prone to false-negative (homozygous) results at higher mutant cDNA concentrations. Positive results by the technique are required to be confirmed by sequencing; therefore, the main utility of D-HPLC seems to be as a screening method.[89]

TKD Mutation Analysis by Allele-Specific Oligonucleotide Reverse Transcription Quantitative PCR (ASO RQ-PCR)

ASO RQ-PCR is based on the principle of AS-PCR with subsequent quantification of the product in real time. This

can be used only for detection of single mutation, and it reasonably failed to quantify compound mutations. The ASO RQ-PCR is reported to have high specificity and sensitivity, but the drawback of low throughput and tedious work process makes it less preferable for use in routine diagnostics.[90]

Conclusion and Future Insights

CML is one of the most studied and well-characterized hematological neoplasm. Different generations of TKI have made it possible to achieve near-normal life expectancy among patients. Despite such therapeutic advancements, there are challenges posed by residual disease and TKI resistance. Hence, a constant evolution is happening among various testing modalities from past, to the present and into the future, which always aimed to mitigate these problems. Novel techniques are endeavoring to reach even better accuracy and sensitivities, thus allowing these patients to achieve TFR.

Studies with promising future perspective about the disease monitoring in CML patients have documented the utility of whole-exome/genome sequencing, copy-number detection, SCS, and/or RNA sequencing in detecting novel gene variants, gene rearrangements, isoforms, and transcriptome in newly diagnosed CML patients. Integrating genomic and transcriptomic analysis in future will help further refine patient-specific risk-adapted therapeutic approaches.

Conflict of Interest
None declared.

References

1 Bhutani M, Vora A, Kumar L, Kochupillai V. Lympho-hemopoietic malignancies in India. Med Oncol 2002;19(03):141–150
2 Bansal S, Prabhash K, Parikh P. Chronic myeloid leukemia data from India. Indian J Med Paediatr Oncol 2013;34(03):154–158
3 Shah NP. Advanced CML: therapeutic options for patients in accelerated and blast phases. J Natl Compr Canc Netw 2008;6 (Suppl 2):S31–S36
4 Chereda B, Melo JV. Natural course and biology of CML. Ann Hematol 2015;94(Suppl 2):S107–S121
5 Soverini S, Mancini M, Bavaro L, Cavo M, Martinelli G. Chronic myeloid leukemia: the paradigm of targeting oncogenic tyrosine kinase signaling and counteracting resistance for successful cancer therapy. Mol Cancer 2018;17(01):49
6 Kadam PR, Nanjangud GJ, Advani SH, et al. Chromosomal characteristics of chronic and blastic phase of chronic myeloid leukemia. A study of 100 patients in India. Cancer Genet Cytogenet 1991;51 (02):167–181
7 Nanjangud G, Kadam PR, Saikia T, et al. Karyotypic findings as an independent prognostic marker in chronic myeloid leukaemia blast crisis. Leuk Res 1994;18(05):385–392
8 Krishna Chandran R, Geetha N, Sakthivel KM, Suresh Kumar R, Jagathnath Krishna KMN, Sreedharan H. Impact of Additional Chromosomal Aberrations on the Disease Progression of Chronic Myelogenous Leukemia. Front Oncol 2019;9:88
9 Amare PS, Baisane C, Saikia T, Nair R, Gawade H, Advani S. Fluorescence in situ hybridization, highly efficient technique of molecular diagnosis, also a sensitive tool for prediction of future course of disease in patients with myeloid leukemias. Cancer Genet Cytogenet 2001;131:125–134
10 Luatti S, Castagnetti F, Marzocchi G, et al. Additional chromosomal abnormalities in Philadelphia-positive clone: adverse prognostic influence on frontline imatinib therapy: a GIMEMA Working Party on CML analysis [published correction appears in Blood. 2013 Jun 27;121(26):5259. Cambrin, Rege [corrected to Rege-Cambrin, Giovanna]]. Blood 2012;120(04):761–767
11 Perrotti D, Jamieson C, Goldman J, Skorski T. Chronic myeloid leukemia: mechanisms of blastic transformation. J Clin Invest 2010;120(07):2254–2264
12 Hughes TP, Hochhaus A, Branford S, et al; IRIS investigators. Long-term prognostic significance of early molecular response to imatinib in newly diagnosed chronic myeloid leukemia: an analysis from the International Randomized Study of Interferon and STI571 (IRIS). Blood 2010;116(19):3758–3765
13 Landstrom AP, Ketterling RP, Knudson RA, Tefferi A. Utility of peripheral blood dual color, double fusion fluorescent in situ hybridization for BCR/ABL fusion to assess cytogenetic remission status in chronic myeloid leukemia. Leuk Lymphoma 2006;47 (10):2055–2061
14 Kantarjian H, Schiffer C, Jones D, Cortes J. Monitoring the response and course of chronic myeloid leukemia in the modern era of BCR-ABL tyrosine kinase inhibitors: practical advice on the use and interpretation of monitoring methods. Blood 2008;111(04): 1774–1780
15 Jabbour E, Kantarjian H. Chronic myeloid leukemia: 2014 update on diagnosis, monitoring, and management. Am J Hematol 2014; 89(05):547–556
16 Hughes TP, Branford S. Monitoring disease response to tyrosine kinase inhibitor therapy in CML. Hematology (Am Soc Hematol Educ Program) 2009:477–487
17 Assouline S, Lipton JH. Monitoring response and resistance to treatment in chronic myeloid leukemia. Curr Oncol 2011;18(02): e71–e83
18 Hochhaus A, Baccarani M, Silver RT, et al. European LeukemiaNet 2020 recommendations for treating chronic myeloid leukemia. Leukemia 2020;34(04):966–984
19 Gabert J, Beillard E, van der Velden VH, et al. Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia - a Europe Against Cancer program. Leukemia 2003;17(12):2318–2357
20 Foroni L, Wilson G, Gerrard G, et al. Guidelines for the measurement of BCR-ABL1 transcripts in chronic myeloid leukaemia. Br J Haematol 2011;153(02):179–190
21 Costa JM, Ernault P, Olivi M, Gaillon T, Arar K. Chimeric LNA/DNA probes as a detection system for real-time PCR. Clin Biochem 2004;37(10):930–932
22 Baccarani M, Deininger MW, Rosti G, et al. European LeukemiaNet recommendations for the management of chronic myeloid leukemia: 2013. Blood 2013;122(06):872–884
23 Link-Lenczowska D, Pallisgaard N, Cordua S, et al. A comparison of qPCR and ddPCR used for quantification of the JAK2. V617F allele burden in Ph negative MPNs. Ann Hematol 2018;97(12):2299–2308
24 Hehlmann R. The new ELN recommendations for treating CML. J Clin Med 2020;9(11):3671
25 Bochicchio MT, Petiti J, Berchialla P, et al. Droplet digital PCR for *BCR-ABL1* monitoring in diagnostic routine: ready to start? Cancers (Basel) 2021;13(21):5470
26 Del Giudice I, Raponi S, Della Starza I, et al. Minimal residual disease in chronic lymphocytic leukemia: a new goal? Front Oncol 2019;9:689
27 Coccaro N, Anelli L, Zagaria A, et al. Droplet digital PCR is a robust tool for monitoring minimal residual disease in adult Philadelphia-positive acute lymphoblastic leukemia. J Mol Diagn 2018;20 (04):474–482

28 Hindson CM, Chevillet JR, Briggs HA, et al. Absolute quantification by droplet digital PCR versus analog real-time PCR. Nat Methods 2013;10(10):1003–1005
29 Baker M. Digital PCR hits its stride. Nat Methods 2012;9(06): 541–544
30 Jennings LJ, George D, Czech J, Yu M, Joseph L. Detection and quantification of BCR-ABL1 fusion transcripts by droplet digital PCR. J Mol Diagn 2014;16(02):174–179
31 Goh HG, Lin M, Fukushima T, et al. Sensitive quantitation of minimal residual disease in chronic myeloid leukemia using nanofluidic digital polymerase chain reaction assay. Leuk Lymphoma 2011;52(05):896–904
32 Franke GN, Maier J, Wildenberger K, et al. Comparison of real-time quantitative PCR and digital droplet PCR for BCR-ABL1 monitoring in patients with chronic myeloid leukemia. J Mol Diagn 2020;22 (01):81–89
33 Franke GN, Maier J, Wildenberger K, et al. Comparison of Real-Time Quantitative PCR and Digital Droplet PCR for BCR-ABL1 Monitoring in Patients with Chronic Myeloid Leukemia. J Mol Diagn 2020;22(01):81–89
34 Bernardi S, Malagola M, Zanaglio C, et al. Digital PCR improves the quantitation of DMR and the selection of CML candidates to TKIs discontinuation. Cancer Med 2019;8(05):2041–2055
35 Vogelstein B, Kinzler KW. Digital PCR. Proc Natl Acad Sci U S A 1999;96(16):9236–9241
36 Foskett P, Gerrard G, Foroni L. Real-time quantification assay to monitor BCR-ABL1 transcripts in chronic myeloid leukemia. Methods Mol Biol 2014;1160:115–124
37 Wang WJ, Zheng CF, Liu Z, et al. Droplet digital PCR for BCR/ABL (P210) detection of chronic myeloid leukemia: a high sensitive method of the minimal residual disease and disease progression. Eur J Haematol 2018;101(03):291–296
38 Mori S, Vagge E, le Coutre P, et al. Age and dPCR can predict relapse in CML patients who discontinued imatinib: the ISAV study. Am J Hematol 2015;90(10):910–914
39 Atallah E, Schiffer CA, Weinfurt KP, et al. Design and rationale for the life after stopping tyrosine kinase inhibitors (LAST) study, a prospective, single-group longitudinal study in patients with chronic myeloid leukemia. BMC Cancer 2018;18(01):359
40 Bernardi S, Foroni C, Zanaglio C, et al. Feasibility of tumor-derived exosome enrichment in the onco-hematology leukemic model of chronic myeloid leukemia. Int J Mol Med 2019;44(06): 2133–2144
41 Enjeti A, Granter N, Ashraf A, et al. A longitudinal evaluation of performance of automated BCR-ABL1 quantitation using cartridge-based detection system. Pathology 2015;47(06):570–574
42 Gerrard G, Foong HE, Mudge K, Alikian M, Apperley JF, Foroni L. Cepheid xpert monitor platform for the confirmation of BCR-ABL1 IS conversion factors for the molecular monitoring of chronic myeloid leukaemia. Leuk Res 2016;49:47–50
43 O'Dwyer ME, Swords R, Nagler A, et al. Nilotinib 300. mg BID as frontline treatment of CML: prospective analysis of the Xpert BCR-ABL monitor system and significance of 3-month molecular response. Leuk Res 2014;38(03):310–315
44 Qin YZ, Jiang Q, Jiang H, et al. Prevalence and outcomes of uncommon BCR-ABL1 fusion transcripts in patients with chronic myeloid leukaemia: data from a single centre. Br J Haematol 2018; 182(05):693–700
45 Duan MH, Li H, Cai H. A rare e13a3 (b2a3) BCR-ABL1 fusion transcript with normal karyotype in chronic myeloid leukemia: the challenges in diagnosis and monitoring minimal residual disease (MRD). Leuk Res 2017;59:8–11
46 Tong YQ, Zhao ZJ, Liu B, et al. New rapid method to detect BCR-ABL fusion genes with multiplex RT-qPCR in one-tube at a time. Leuk Res 2018;69:47–53
47 Raponi S, De Propris MS, Wai H, et al. An accurate and rapid flow cytometric diagnosis of BCR-ABL positive acute lymphoblastic leukemia. Haematologica 2009;94(12):1767–1770
48 Yujie W, Yu Z, Sixuan Q, et al. Detection of BCR-ABL fusion proteins in patients with leukemia using a cytometric bead array. Leuk Lymphoma 2012;53(03):451–455
49 Löf L, Arngården L, Olsson-Strömberg U, et al. Flow cytometric measurement of blood cells with BCR-ABL1 fusion protein in chronic myeloid leukemia. Sci Rep 2017;7(01):623
50 Herrmann H, Sadovnik I, Cerny-Reiterer S, et al. Dipeptidylpeptidase IV (CD26) defines leukemic stem cells (LSC) in chronic myeloid leukemia. Blood 2014;123(25):3951–3962
51 Valent P, Sadovnik I, Ráčil Z, et al. DPPIV (CD26) as a novel stem cell marker in Ph+ chronic myeloid leukaemia. Eur J Clin Invest 2014; 44(12):1239–1245
52 Bocchia M, Sicuranza A, Abruzzese E, et al. Residual peripheral blood $CD26^{+}$ leukemic stem cells in chronic myeloid leukemia patients during TKI therapy and during treatment-free remission. Front Oncol 2018;8:194
53 Herrmann H, Cerny-Reiterer S, Gleixner KV, et al. CD34(+)/CD38(-) stem cells in chronic myeloid leukemia express Siglec-3 (CD33) and are responsive to the CD33-targeting drug gemtuzumab/ozogamicin. Haematologica 2012;97(02):219–226
54 Giustacchini A, Thongjuea S, Barkas N, et al. Single-cell transcriptomics uncovers distinct molecular signatures of stem cells in chronic myeloid leukemia. Nat Med 2017;23(06):692–702
55 Warfvinge R, Geironson L, Sommarin MNE, et al. Single-cell molecular analysis defines therapy response and immunophenotype of stem cell subpopulations in CML. Blood 2017;129(17): 2384–2394
56 Huang XP, Hou J, Shen XY, et al. MicroRNA-486-5p, which is downregulated in hepatocellular carcinoma, suppresses tumor growth by targeting PIK3R1. FEBS J 2015;282(03):579–594
57 Gordon JE, Wong JJ, Rasko JE. MicroRNAs in myeloid malignancies. Br J Haematol 2013;162(02):162–176
58 Jiang M, Li X, Quan X, et al. MiR-486 as an effective biomarker in cancer diagnosis and prognosis: a systematic review and meta-analysis. Oncotarget 2018;9(17):13948–13958
59 Ninawe A, Guru SA, Yadav P, et al. miR-486-5p: a prognostic biomarker for chronic myeloid leukemia. ACS Omega 2021;6(11): 7711–7718
60 Waller CF, Dennebaum G, Feldmann C, Lange W. Long-template DNA polymerase chain reaction for the detection of the bcr/abl translocation in patients with chronic myelogenous leukemia. Clin Cancer Res 1999;5(12):4146–4151
61 Siebert PD, Chenchik A, Kellogg DE, Lukyanov KA, Lukyanov SA. An improved PCR method for walking in uncloned genomic DNA. Nucleic Acids Res 1995;23(06):1087–1088
62 Mattarucchi E, Spinelli O, Rambaldi A, et al. Molecular monitoring of residual disease in chronic myeloid leukemia by genomic DNA compared with conventional mRNA analysis. J Mol Diagn 2009;11 (05):482–487
63 Pagani IS, Spinelli O, Mattarucchi E, et al. Genomic quantitative real-time PCR proves residual disease positivity in more than 30% samples with negative mRNA-based qRT-PCR in chronic myeloid leukemia. Oncoscience 2014;1(07):510–521
64 Bartley PA, Martin-Harris MH, Budgen BJ, Ross DM, Morley AA. Rapid isolation of translocation breakpoints in chronic myeloid and acute promyelocytic leukaemia. Br J Haematol 2010;149(02): 231–236
65 Bartley PA, Latham S, Budgen B, et al. A DNA real-time quantitative PCR method suitable for routine monitoring of low levels of minimal residual disease in chronic myeloid leukemia. J Mol Diagn 2015;17(02):185–192
66 Bartley PA, Ross DM, Latham S, et al. Sensitive detection and quantification of minimal residual disease in chronic myeloid leukaemia using nested quantitative PCR for BCR-ABL DNA. Int J Lab Hematol 2010;32(6 Pt 1):e222–e228
67 Cortes J, Lang F. Third-line therapy for chronic myeloid leukemia: current status and future directions. J Hematol Oncol 2021;14 (01):44

68 Kaleem B, Shahab S, Ahmed N, Shamsi TS. Chronic myeloid leukemia–prognostic value of mutations. Asian Pac J Cancer Prev 2015;16(17):7415–7423

69 Baccarani M, Soverini S, De Benedittis C. Molecular monitoring and mutations in chronic myeloid leukemia: how to get the most out of your tyrosine kinase inhibitor. Am Soc Clin Oncol Educ Book 2014:167–175

70 Nardi V, Azam M, Daley GQ. Mechanisms and implications of imatinib resistance mutations in BCR-ABL. Curr Opin Hematol 2004;11(01):35–43

71 Bommannan KB, Naseem S, Binota J, Varma N, Malhotra P, Varma S. Tyrosine kinase domain mutations in chronic myelogenous leukemia patients: a single center experience. J Postgrad Med 2022;68(02):93–97

72 Khorashad JS, Kelley TW, Szankasi P, et al. BCR-ABL1 compound mutations in tyrosine kinase inhibitor-resistant CML: frequency and clonal relationships. Blood 2013;121(03):489–498

73 Zabriskie MS, Eide CA, Tantravahi SK, et al. BCR-ABL1 compound mutations combining key kinase domain positions confer clinical resistance to ponatinib in Ph chromosome-positive leukemia. Cancer Cell 2014;26(03):428–442

74 Alves R, Gonçalves AC, Rutella S, et al. Resistance to tyrosine kinase inhibitors in chronic myeloid leukemia-from molecular mechanisms to clinical relevance. Cancers (Basel) 2021;13(19): 4820

75 O'Hare T, Shakespeare WC, Zhu X, et al. AP24534, a pan-BCR-ABL inhibitor for chronic myeloid leukemia, potently inhibits the T315I mutant and overcomes mutation-based resistance. Cancer Cell 2009;16(05):401–412

76 NCCN. NCCN Clinical Practice Guidelines in Oncology. NCCN Chronic Myelogenous Leukemia Guidelines Version 4. NCCN; 2013

77 Soverini S, Branford S, Nicolini FE, et al. Implications of BCR-ABL1 kinase domain-mediated resistance in chronic myeloid leukemia. Leuk Res 2014;38(01):10–20

78 Patkar N, Ghodke K, Joshi S, et al. Characteristics of BCR-ABL kinase domain mutations in chronic myeloid leukemia from India: not just missense mutations but insertions and deletions are also associated with TKI resistance. Leuk Lymphoma 2016;57(11): 2653–2660

79 Lavallade H, Kizilors A. The importance of mutational analysis in chronic myeloid leukaemia for treatment choice. EMJ Oncol 2016; 4(01):86–95

80 Alikian M, Gerrard G, Subramanian PG, et al. BCR-ABL1 kinase domain mutations: methodology and clinical evaluation. Am J Hematol 2012;87(03):298–304

81 Sorel N, Mayeur-Rousse C, Deverrière S, et al. Comprehensive characterization of a novel intronic pseudo-exon inserted within an e14/a2 BCR-ABL rearrangement in a patient with chronic myeloid leukemia. J Mol Diagn 2010;12(04):520–524

82 Soverini S, Bernardi S, Galimberti S. Molecular testing in CML between old and new methods: are we at a turning point? J Clin Med 2020;9(12):3865

83 Vannuffel P, Bavaro L, Nollet F, et al. Droplet Digital PCR Phasing (DROP-PHASE): A Novel Method for Straightforward Detection of BCR-ABL1 Compound Mutations in Tyrosine Kinase Inhibitors Resistant Chronic Myeloid Leukemia (CML) and Acute Lymphoblastic Leukemia (ALL). Blood 2019;134 (Supplement_1): 4660

84 Soverini S, Abruzzese E, Bocchia M, et al. Next-generation sequencing for BCR-ABL1 kinase domain mutation testing in patients with chronic myeloid leukemia: a position paper. J Hematol Oncol 2019;12(01):131

85 Yohe S, Thyagarajan B. Review of clinical next-generation sequencing. Arch Pathol Lab Med 2017;141(11):1544–1557

86 Machova Polakova K, Kulvait V, Benesova A, et al. Next-generation deep sequencing improves detection of BCR-ABL1 kinase domain mutations emerging under tyrosine kinase inhibitor treatment of chronic myeloid leukemia patients in chronic phase. J Cancer Res Clin Oncol 2015;141(05):887–899

87 Parker WT, Phillis SR, Yeung DT, Hughes TP, Scott HS, Branford S. Many BCR-ABL1 compound mutations reported in chronic myeloid leukemia patients may actually be artifacts due to PCR-mediated recombination. Blood 2014;124(01):153–155

88 Schmitt MW, Pritchard JR, Leighow SM, et al. Single-Molecule Sequencing Reveals Patterns of Preexisting Drug Resistance That Suggest Treatment Strategies in Philadelphia-Positive Leukemias. Clin Cancer Res 2018;24(21):5321–5334

89 Deininger MW, McGreevey L, Willis S, Bainbridge TM, Druker BJ, Heinrich MC. Detection of ABL kinase domain mutations with denaturing high-performance liquid chromatography. Leukemia 2004;18(04):864–871

90 Wongboonma W, Thongnoppakhun W, Auewarakul CU. BCR-ABL kinase domain mutations in tyrosine kinase inhibitors-naïve and -exposed Southeast Asian chronic myeloid leukemia patients. Exp Mol Pathol 2012;92(02):259–265