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

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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

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 (ABL) 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.\textsuperscript{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.\textsuperscript{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 R² (coefficient of correlation) should be >0.9815,\textsuperscript{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).\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 (\textsuperscript{\textbullet}Table 1). According to ELN 2020 recommendations, the response evaluation to treatment in CML patients is tabulated here (\textsuperscript{\textbullet}Table 2)\textsuperscript{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 hematology,\textsuperscript{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.\textsuperscript{29,30} The dPCR can more efficiently monitor CML patients in TFR trial.\textsuperscript{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

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

<table>
<thead>
<tr>
<th>TKI treatment response</th>
<th>BCR::ABL1 ratio, % (IS scale)</th>
<th>Lowest required housekeeping gene copy numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major molecular response</td>
<td>≤0.1%</td>
<td>10,000 BCR1 or 24,000 GUSB</td>
</tr>
<tr>
<td>Molecular response-4</td>
<td>≤0.01%</td>
<td></td>
</tr>
<tr>
<td>Molecular response-4.5</td>
<td>≤0.0032%</td>
<td>32,000 BCR1 or 77,000 GUSB</td>
</tr>
<tr>
<td>Molecular response-5</td>
<td>≤0.001%</td>
<td>1,000,000 BCR1 or 2,400,000 GUSB</td>
</tr>
</tbody>
</table>

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\textsuperscript{18,23,24}

<table>
<thead>
<tr>
<th>Time points</th>
<th>Optimal</th>
<th>Warning</th>
<th>Failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>NA</td>
<td>High-risk ACAA, high-risk ELTS score</td>
<td>NA</td>
</tr>
<tr>
<td>3 mo</td>
<td>≤10%</td>
<td>&gt;10%</td>
<td>&gt;10% if confirmed within 1–3 mo</td>
</tr>
<tr>
<td>6 mo</td>
<td>≤1%</td>
<td>&gt;1–10%</td>
<td>&gt;10%</td>
</tr>
<tr>
<td>12 mo</td>
<td>≤0.1%</td>
<td>&gt;0.1–1%</td>
<td>&gt;1%</td>
</tr>
<tr>
<td>Any time</td>
<td>≤0.1%</td>
<td>&gt;0.1–1%</td>
<td>&gt;1%, resistance mutations high-risk ACAA</td>
</tr>
</tbody>
</table>

Abbreviations: ACAs, additional chromosomal aberrations; ELN, European LeukemiaNet; ELTS, EUTOS score for long-term survival; MMR, major molecular remission; NA, not applicable.

\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{43}

**MRD Monitoring in CML with Nonclassical BCR::ABL 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 literature.\textsuperscript{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.\textsuperscript{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.\textsuperscript{46}

**Flowcytometric-Based MRD Monitoring**

The flowcytometric immunobead assay utilization for BCR::ABL1 fusion proteins detection has been used by few centers.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{54} This technique uses a multomics 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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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).\textsuperscript{61–63} Due to its greater sensitivity compared with RNA-based technique, it can be adopted for TFR trial in future.\textsuperscript{64}

**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.\textsuperscript{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.58

**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 inhibitors74,75

<table>
<thead>
<tr>
<th>TKD variant</th>
<th>Site</th>
<th>Imatinib</th>
<th>Dasatinib</th>
<th>Nilotinib</th>
<th>Bosutinib</th>
<th>Ponatinib</th>
<th>Asciminib</th>
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<td>Wild-type</td>
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Abbreviation: TKD, tyrosine kinase domain.
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 ≤ 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 4 Recommended time points for TKD mutation analysis76,77

<table>
<thead>
<tr>
<th>Guidelines</th>
<th>Diagnostic time point</th>
<th>During first-line therapy with imatinib</th>
<th>During second-line therapy with dasatinib or nilotinib</th>
</tr>
</thead>
<tbody>
<tr>
<td>European LeukemiaNet (ELN) and European Society for Medical Oncology (ESMO)</td>
<td>Patients with accelerated phase/blast phase CML</td>
<td>• Treatment failure &lt;br&gt; • Suboptimal therapeutic response &lt;br&gt; • Loss of MMR due to increment in BCR::ABL1 transcript levels &lt;br&gt; • Prior to shifting to other TKIs/alternate therapies</td>
<td>In event of hematologic or cytogenetic failure, including: &lt;br&gt; • No cytogenetic response at 3 mo &lt;br&gt; • Minimal cytogenetic response at 6 mo &lt;br&gt; • Not achieving partial cytogenetic response at 12 mo &lt;br&gt; • Prior to shifting to other TKIs/alternate therapies</td>
</tr>
<tr>
<td>National Comprehensive Cancer Network (NCCN)</td>
<td>≥ Disease progression to accelerated phase/blast phase</td>
<td>• 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) &lt;br&gt; • 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)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CML, chronic myeloid leukemia; IS, international scale; MMR, major molecular remission; 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. 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. 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. 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. In the year 2020, ELN advocated the use of NGS for those CML cases that did not adequately respond to standard TKIs. 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.

### 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

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**Table 5** Advantages and drawbacks of various TKI mutation detection platforms

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

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

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