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REVIEW ARTICLE

# Molecular diagnosis and monitoring of chronic myelogenous leukemia: BCR-Abl and more

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## Summary

*The current treatment of chronic myelogenous leukemia (CML) is one of the most successful examples of molecularly targeted therapy in cancer. The identification of the fusion oncogene BCR-ABL allowed the discovery of small molecule inhibitors of its tyrosine kinase activity which, in turn, have literally revolutionized the treatment of this disease.*

*However, large part of a successful clinical management of CML relies on appropriate diagnosis, molecular monitoring and identification of mutations potentially leading to drug resistance. These issues are discussed here together with an overview on how patients treated with tyrosine kinase inhibitors should be monitored.*

**Key words:** chronic myelogenous leukemia, cytogenetic analysis, imatinib, molecular monitoring, mutational analysis, resistance

## Introduction

CML is a hematopoietic stem cell (HSC) disorder accounting for about 15-20% of all leukemias of the adult [1,2]. The main hematological features are represented by an increase in the number of circulating mature granulocytes and their precursors and, subsequently, by a secondary evolution in acute leukemia. Additionally, in over 95% of CML cases, it is possible to recognize a karyotype abnormality resulting from a reciprocal translocation involving chromosome 9 and chromosome 22, t(9;22), termed Philadelphia chromosome (cr. Ph) [3,4]. This balanced translocation leads to a fusion gene, the product of which is the constitutively active protein-tyrosine kinase, BCR-ABL. The biochemical signal transduction pathways stimulated by BCR-ABL kinase activity are responsible for $Ph^+$ CML oncogenesis [5-13].

Several BCR-ABL variants have been reported. In general, while in all chimeric proteins the breakpoint within ABL gene is consistently located upstream of exon 2 (a2), the breakpoint in the BCR gene varies in its localization [14]. Accordingly, different BCR-ABL isoforms with different molecular weights result [15]. A major breakpoint cluster region (M-bcr) and a minor breakpoint cluster region (m-bcr) have been defined [16]. The M-bcr maps to a 5.8 Kilobase (Kb) area spanning exons 12 through 16. The resulting fusion transcripts with ABL generate a 210-kDa protein named p210 which is the most common BCR-ABL form, being observed in 99% of the CML patients and in one-third of Ph-positive B-cell acute lymphoblastic leukemia ($Ph^+$ B-ALL) [17]. m-bcr localizes to a 54.4-kb area sited downstream of exon 1. It gives rise to a fusion transcript with ABL named p190. p190 is rarely observed in CML, but is the most frequent BCR-ABL isoform in $Ph^+$ B-ALL. Finally, 3' breakpoints downstream of BCR exon 19 have also been described and they give rise to a 230-kDa fusion protein (p230 BCR-ABL), which is typically found in chronic neutrophilic leukemia (CNL) [18].

The oncogenic potential of BCR-ABL derives from its capacity to activate intracellular signalling

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cascades that lead to uncontrolled cell proliferation, altered cell adhesion, and apoptosis inhibition [19,20]. Increased susceptibility to proliferate derives from BCR-ABL's capacity to activate mitogen activated protein kinase (MAPK) and JAK/STAT signalling; the interaction with SRC is responsible for increased cell motility; resistance to apoptosis is thought to result from BCR-ABL-mediated activation of phosphatidylinositol-3-phosphate kinase (PI3K) and thereby of Akt (Figure 1). Consistent with these molecular sequelae, BCR-ABL was shown to transform hematopoietic progenitor cells in *in vitro* and *in vivo* studies [21-23]. Recent reports identified a role for other signalling cascades in CML biology, including Hedgehog, Wnt and Ikaros, suggesting that pharmacological inhibitors of these pathways may find application in the treatment of CML [24-27]. Finally, also micro RNA (miRNA) regulation appears to apply to CML biology since miR-203, which would normally suppress BCR-ABL expression, is either mutated or epigenetically silenced in CML. In the latter type of condition, demethylating drugs such as 5-azacytidine and 4-phenylbutyrate were shown to restore miR-203 and to thereby decrease BCR-ABL expression and proliferation rate of $Ph^+$ human CML cell lines [28,29]. The definition of the molecular structure of BCR-ABL tyrosine kinase domain has led to development of potent and specific tyrosine kinase inhibitor (TKIs) [30,31]. TKIs such as imatinib mesylate (Gleevec™, Novartis) induce apoptosis in CML but not in healthy tissues, which is thought to result from addiction of CML cells to BCR-ABL signalling. Importantly, although TKIs do induce disease remissions in most CML patients, they are not curative because of their incapacity to eradicate CML stem cells. In this respect, the only curative approach for CML remains allogeneic bone marrow/peripheral blood stem cell transplantation [32,33]. Moreover, acquired resistance to imatinib is commonly observed and requires the prompt introduction of other TKIs that retain activity against BCR-ABL [34,35]. Therefore, a timely and accurate follow-up is crucial for the management of CML and for effective therapeutic decisions [36-44].

This review discusses the role of disease monitoring in the management of CML patients, the methods used, and the mechanisms of resistance to TKIs. The current recommendations on how patients treated with imatinib should be monitored are also summarized.

## CML monitoring

Routine CML diagnostics largely relies nowadays on traditional blood cell count, cytogenetic analysis (standard karyotype with or without fluorescence *in situ* hybridization-FISH), and real time quantitative polymerase chain reaction (RT-Q-PCR) for BCR-ABL messenger RNA (mRNA). These tests allow defining the hematological, cytogenetic, and molecular response to treatment, respectively [45,46].

The hematological response to treatment is assessed by peripheral blood cell counts and by spleen size, and is classified as:

1. *Complete hematological response (CHR)*: normalization of peripheral blood counts with no immature blood cells and with disappearance of any sign of disease
2. *Partial hematological response (PHR)*: presence of immature blood cells and/or persistent splenomegaly

The next level of response is the cytogenetic one (CyR), defined as a decrease in the number of $Ph^+$

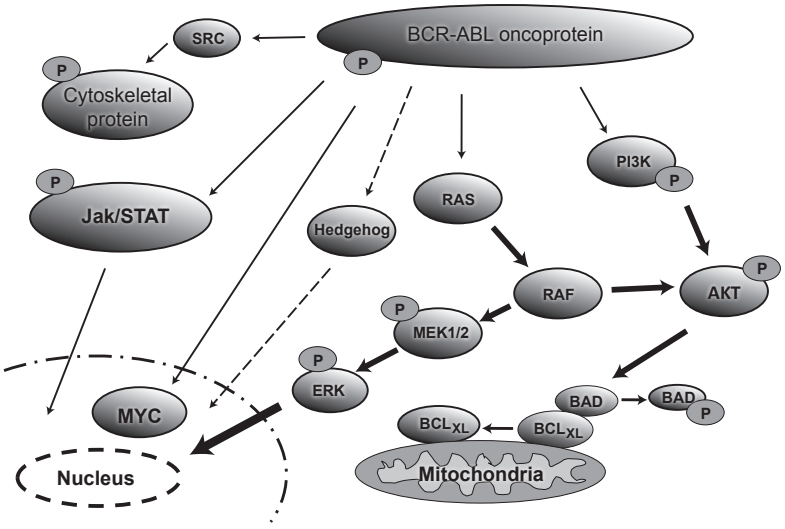


**Figure 1.** Molecular pathways involved in oncogenic BCR-ABL signaling. Schematic view of the signal transduction pathways in cells transformed by BCR-ABL. Multiple pathways are activated, including the RAS-mitogen activated protein (MAP) kinase signaling cascade, Phosphatidyl inositol 3' kinase (PI3'K), Jak/Stat, and Myc. The net effects of these molecular alterations include inhibition of apoptosis, increased cell proliferation, aberrant interaction with the bone marrow stroma, and genetic instability. Many of the molecules involved in BCR-ABL-mediated cell transformation are potential drug targets.

metaphases in a bone marrow aspirate (using ≥ 20 metaphases). This is categorized as :

1. *Complete cytogenetic response (CCyR):* 0% $Ph^+$ metaphases
2. *Partial cytogenetic response (PCyR):* 1-35% $Ph^+$ metaphases
3. *Minor cytogenetic response*: 36-65% $Ph^+$ metaphases
4. *Minimal cytogenetic response*: 66-95% $Ph^+$ metaphases

CCyR or PCyR configure a *major cytogenetic response* (MCyR).

Finally, residual leukemia cells (minimal residual disease, MRD) can be detected using RT-Q-PCR. Particularly, the molecular response is defined as a decrease of the BCR-ABL to control gene transcript ratio according to the International Scale *(IS)* (see below):

1. *Complete molecular response (CMR):* undetectable level of chimeric transcript
2. *Major molecular response (MMR):* reduction in transcript levels of at least 3-log from standard baseline level (which represent 100% on the International Scale) or ≤1%.

## Cytogenetics and FISH

The Ph chromosome can be detected by standard cytogenetic techniques in the vast majority of patients [47]. In patients who are cytogenetically Ph chromosome negative ($Ph^-$), molecular techniques such as FISH and RT-Q-PCR may be useful in detecting BCR-ABL. Cytogenetic analysis is typically performed by chromosome banding of at least 20 bone marrow cells in metaphase allowing to identify the t(9:22) translocation [48]. In addition, cytogenetics also allows to define any additional chromosomal abnormality (i.e. additional Ph chromosome, isochromosome 17q, trisomy 8, or trisomy 19), thereby providing additional prognostic information. Baccarani et al. recommend that, at diagnosis, two cytogenetic analyses are performed in order to increase the sensitivity of the method. Furthermore, if less than 20 metaphases are visualized, the cytogenetic analysis should be validated by FISH or by RT-Q-PCR (see below) [49]. Importantly, in 5% of CML cases no cytogenetically-detectable Ph chromosome can be demonstrated, since in these cases (about 2-3% of all CMLs) the BCR-ABL fusion oncogene derives from a submicroscopic genetic fusion. In these cases, FISH or RT-Q-PCR will demonstrate the presence of the specific genetic abnormality.

Traditional FISH uses 5' BCR and 3' ABL fluorescent probes of different colors while more recent FISH reagents use 3-4 probes (D-FISH). Such probes can detect the variant translocations leading to Ph chromosome formation and are also associated with low false positive rates [50-55]. Interphase or hypermetaphase FISH can be performed on peripheral blood specimen or bone marrow aspirates, respectively. Interphase FISH is applicable to a larger population of cells since does not require cycling cells. On the other hand, this technique is associated with a background signal greater than 1-5% (depending on the specific probe used in the assay) [56-59]. Hypermetaphase FISH is applicable only to dividing bone marrow cells [60]. This approach is more sensitive and can analyze up to 500 metaphases at a time.

Usually, FISH results correlate with traditional cytogenetic analysis and with RT-Q-PCR results, thus remaining a convenient and sensitive diagnostic tool (see below).

## PCR-based approaches to CML monitoring

Nested reverse transcriptase PCR can detect one CML cell in a background of ≥ 100.000 normal cells [61]. However, it remains a purely qualitative assay which is only capable of demonstrating the presence or absence of CML cells. Nested-PCR is normally only used to confirm the achievement of CMR. RT-Q-PCR methods are less sensitive than qualitative PCR (by 0.5-1 order of magnitude) but they have the advantage of determining the actual percentage of BCR-ABL transcripts and can therefore be used to track changes in the number of leukemic cells over time [62-66]. Currently, RT-Q-PCR for BCR-ABL is the recommended approach for routine follow-up of CML patients and is considered the gold standard test for routine therapeutics decision.

The BCR-ABL transcript levels are expressed as a percentage ratio of BCR-ABL compared to ABL transcripts. ABL acts as control gene to compensate for variations in the quality of the RNA and for differences in the efficiency of the reverse transcription reaction. The last years have seen numerous efforts to standardize the molecular approaches to CML monitoring as well as their interpretation criteria. In order to harmonize the results across laboratories worldwide, a standard pre-treatment baseline value for each laboratory was established. Thus, a molecular response is defined by reductions from an absolute baseline (common to all) rather than a relative baseline (individualized). This ensures that patients with the same level of response have the same degree of residual disease. Additionally, under- or over-estimation of the extent of response due to individual variations is avoided by using a common standard baseline. According to the international reporting scale (IS) the absolute

BCR-ABL value to define major molecular response is standardized at 0.1% (or 3 log) reduction from the laboratory-specific pretreatment standard baseline [67-69]. A value of 1.0% is approximately equivalent to the achievement of a CCyR and a CMR is achieved when transcripts are undetectable [70-72].

Because of its high sensitivity, CML monitoring by RT-Q-PCR enables to define an early loss of response once CCyR has been achieved [73,74]. Additionally, early molecular monitoring after initiation of treatment helps to identify patients at higher risk of relapse after pharmacological treatment onset as well as after allogeneic bone marrow transplantation [75-77].

Finally, another advantage of CML monitoring by RT-Q-PCR is the feasibility of this method on peripheral blood samples. In a large cohort of patients monitored to BCR-ABL mRNA levels after allogeneic bone marrow transplantation, we found that peripheral blood and bone marrow samples perform equally well in terms of sensitivity in relapse detection and show a very good correlation of results. Thus, molecular monitoring of CML with RT-Q-PCR can be performed using peripheral blood samples instead of bone marrow [78]. The drawbacks of this method include a substantial incidence of false negative tests, which on the other hand, is strongly reduced when serial evaluations are performed.

Nowadays, RT-Q-PCR monitoring is included as integral part of the management of CML patient treated with TKIs and must be performed every 3 months even in patients in MMR. An increase in BCR-ABL levels of 2 to 5 fold is an early sign of relapse, and suggests the need to switch to another type of treatment as soon as possible.

## Mechanisms of resistance

A growing problem in the treatment of CML is resistance to treatment since most patients in chronic phase initially respond to TKIs but subsequently relapse and/or progress to accelerated phase or blast crisis [34,35]. Primary resistance or, perhaps more appropriately, primary refractoriness (typically BCR-ABL independent), is defined as the failure to achieve initial response to therapy and is only seen in approximately 5% of newly diagnosed patients in chronic phase of CML [79]. Acquired resistance is more common (10-15% of patients) and it is defined as the loss of previous response. Resistance to TKIs may be primary or secondary and is usually classified in BCR-ABL-dependent or -independent. The BCR-ABL-dependent mechanisms include reactivation of BCR-ABL signaling through mutations in the ABL kinase domain (KD), and increased production of BCR-ABL at the genomic (gene amplification) or transcript (overexpression) levels [80-82]. Conversely, BCR-ABL-independent resistance mechanisms involve i) a drop in the intracellular drug concentration through expression of drug efflux (such as multidrug-resistant P-glycoprotein MDR-1) [83,84] or drug influx (such as hOCT1 that affects intracellular drug availability) [85] genes; ii) activation of Src family of kinases (SFKs); and iii) acquisition of additional chromosomal abnormalities in addition to the Ph-chromosome [86-88]. Although gene amplification occurs more frequently than point mutations ($10^{-4}$ per cell division vs. $10^{-9}$), clinical resistance is much more likely to be due to a point mutation in the BCR-ABL TK domain than to BCR-ABL amplification [89]. To date more than 50 mutations have been identified, each of which arises at variable frequencies and with different consequences [90-103]. Mutations may occur in various ATP-binding sites, such as the phosphate-binding loop (P-loop), activation site, catalytic site, or other areas in the BCR-ABL structure. Depending on the mutation site, resistance to imatinib will either be absolute or relative, or it will be clinically irrelevant.

Earlier studies have associated P-loop mutations and the T315I mutation with the worst outcomes [104]. Mutations within the P-loop site are found in 30-40% of the resistant cases and reduce susceptibility to imatinib by 70 to 100 folds. The T315I mutation in BCR-ABL occurs in 0.16-0.32% of newly diagnosed patients in chronic phase, leading to substitution of threonine 315 with isoleucine. This "gatekeeper" mutation also affects the response to the currently existing second-generation TKIs. Therefore, upon its identification, patients should be considered for alternative pharmacological treatments or for allogeneic bone marrow transplantation.

## Mutational analysis

A careful mutational screening allows the timely identification of potential mutant clones and suggests the most suitable second-line treatment based on the *in vitro* sensitivity of the specific mutation. The technologies used to identify and quantify the ABL KD mutations include: direct sequencing [42], subcloning and sequencing, denaturing-high performance liquid chromatography analysis (DHPLC) [97], pyrosequencing and allele specific oligonucleotide PCR. Direct sequencing represents the most widespread method used for routine monitoring. Its main drawback is the low sensitivity (20%) which is responsible for false negative results. Fluorescent-based allele-specific oligonucleotide PCR (ASO-PCR) assays have higher sensitivity (0.1%), although their main drawback is that the search for specific mutations does not include screening of the

entire KD region of the BCR-ABL gene. Nowadays, numerous groups perform DHPLC to monitor CML patients, followed by a sequence analysis to confirm the data. DHPLC has a sensitivity of 1-5% [105]. Mutation studies might be performed on peripheral blood or bone marrow although a direct comparison of these two types of samples has not been done yet.

The search for BCR-ABL mutations should be performed, according to NCCN CML guidelines [106], in the following conditions:

1. Progression to accelerated or blast phase
2. Treatment failure
3. Suboptimal therapeutic responses
4. Increasing BCR-ABL levels (5 to 10 fold in mRNA)

## Scheduling CML diagnostics and monitoring (Figure 2)

An effective CML monitoring entails an appropriate follow up-schedule [107]. Evidence obtained in clinical trials has prompted experts to formulate consensus recommendations to assess the response to treatment in patients with $Ph^+$ CML [108].

In the diagnostic setting, bone marrow cytogenetics is recommended before initiation of treatment. Additionally, a nested PCR confirms the diagnosis of CML and establishes the type of BCR-ABL fusion transcript present. Bone marrow cytogenetics is able to detect chromosomal abnormalities that FISH is not able to detect. However, if bone marrow collection is not feasible, FISH on peripheral blood specimen with dual probe (BCR and ABL genes) is a suitable tool to confirm the diagnosis. Subsequently, the cytogenetic evaluation is recommended at 6 and 12 months from the beginning of treatment. If a CCyR is achieved at 6 months, it is not necessary to repeat the cytogenetic evaluation at 12 months. If the patients is not in a CCyR at 12 months, a cytogenetic evaluation should be repeated at 18 months. Once cytogenetic remission is achieved, residual disease should be monitored using BCR-ABL transcript levels by RT-Q-PCR, which is the most sensitive technique to monitor BCR-ABL. The hybrid transcript levels should be measured every 3 months at the beginning of treatment and then every 3-6 months since a CCyR is achieved.

A steady decline in BCR-ABL transcripts indicates an ideal response to therapy. A rising level of BCR-ABL transcript (1 log increase) following the achievement of a MMR mandates to repeat the molecular analysis after 1 month [107]. If the result is confirmed, bone marrow cytogenetics should be performed, BCR-ABL quantifications by RT-Q-PCR should be scheduled every month, and a kinase domain mutational analysis should also be done [43].

The evaluation of the hematologic response foresees that, starting from treatment onset, blood cell counts are performed every 2 weeks until a stable CHR is achiev-

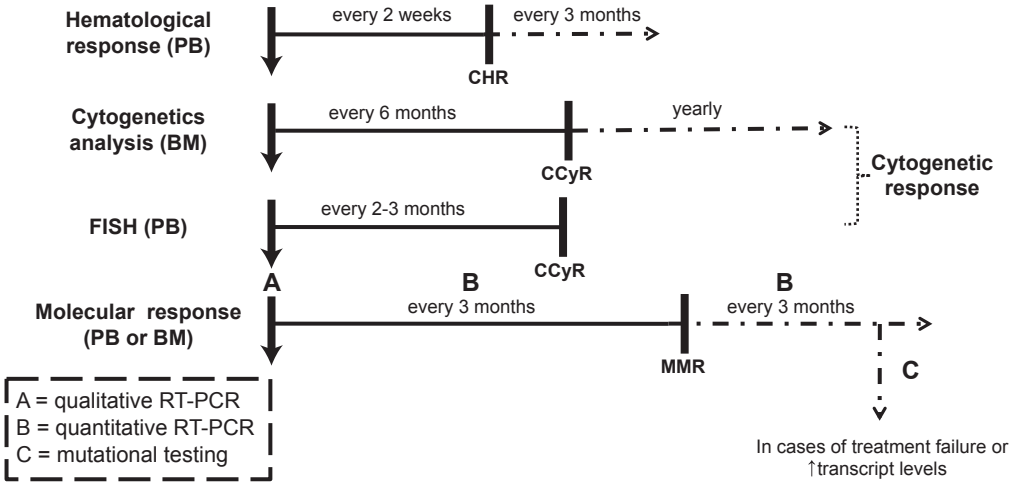


**Figure 2.** Proposed algorithm for CML monitoring according to the National Comprehensive Cancer Network guidelines. The arrow pointing down indicates testing at the time of diagnosis, followed by interval testing as indicated until the response is documented (CHR: complete hematologic response; CCyR: complete cytogenetic response; MMR: major molecular response; BM: bone marrow; PB: peripheral blood). After a response is achieved, the dotted arrow indicates recommended intervals for continued testing. The use of PB FISH for initial monitoring is optional, but does not appear to be superior to RT-Q-PCR. Once CCyR has been attained, there is no indication to perform FISH (on either PB or BM). These recommendations are adapted from the 2010 National Comprehensive Cancer Network panel recommendations.

ed, then every 3 months [109]. If the patient fails to achieve CHR by 3 months, the treatment is generally regarded as a failure, indicating the need to consider alternative therapeutic strategies.

In summary, the international guidelines recommend that:

1. Hematologic responses should be assessed every 2 weeks until a CHR is achieved, then every 3 months.
2. Cytogenetic responses should be assessed every 6 months until a CCyR is achieved, then every 12 months.
3. Molecular responses should be assessed every 3 months, or monthly if an increasing BCR-ABL transcript level is detected.

## Conclusions

Molecular tools have become fundamental not only in the diagnostic evaluation but even in the management of CML patients. While traditional cytogenetics with or without FISH and qualitative nested-PCR are essential for the diagnosis of CML, serial RT-Q-PCRs are the mainstay of therapeutic monitoring and MDR assessment [45]. In cases of treatment failure highlighted by increasing BCR-ABL levels and/or by loss of hematologic and cytogenetic responses, mutational analysis to identify KD mutations should be considered in order to meet the better treatment decisions (i.e. use alternative TKIs or stem cell transplantation) [46]. Additionally, an early identification of treatment failure increases the chance that alternative treatments will be effective [110].

All these said, despite the numerous advantages of modern technologies, it is important to continue interpreting laboratory data within the clinical context of the patient in order to effectively and inexpensively utilize current and nascent laboratory tools.

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