Molecular Monitoring and Treatment of Chronic Myeloid Leukemia (CML)

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Abstract

The advances in the treatment strategies in CML epitomize the concept of personalized medicine. These rapid developments towards cure of CML are acutely dependent on our ability to monitor therapeutic response to the drugs and fine-tune the therapy to the challenges posed by drug resistance from development of target site mutations. The fundamental requirement for success of targeted therapy in CML is standardization of methods for molecular monitoring such that there is a uniform understanding of response therapies and results of clinical trials. This review discusses the aspects of molecular monitoring of CML in the context of recent advances in its treatment strategies. It provides guidelines to both clinicians and laboratory physicians in choosing the appropriate methodology for laboratory monitoring and in interpretation of the results for effective therapeutic decision-making.

Keywords: CML; Targeted therapy; Tyrosine kinase inhibitors; Drug resistance; Molecular monitoring

Introduction

In 1960, Peter Nowell and David Hungerford made the seminal observation of an abnormally small chromosome in patients with Chronic Myeloid Leukemia (CML), which they named the Philadelphia or Ph1 chromosome [1] for the city where the discovery was made. In the early 1970’s, Janet Rowley identified the underlying non-random balanced chromosomal translocation between chromosomes 9q34 and 22q11 [2]. Molecularly, this translocation fuses the 5’ end of the BCR gene on chromosome 22 with 3’ end of the ABL1 gene on chromosome 9 and gives rise to the chimeric BCR-ABL gene [3]. Ph1 was the first disease-specific chromosomal abnormality described in cancer and is the disease defining abnormality for Chronic Myeloid Leukemia (CML). The BCR-ABL1 chimeric gene on derivative chromosome 22 has since been established to drive the oncogenesis.

In the fifty years since the first identification of the Ph1 chromosome, the sequence of discoveries and inventions made in understanding the biology, detection strategies, and treatment of CML exemplify the concept of molecular medicine. The increase in understanding of the disease has resulted in a paradigm shift in treatment from the traditional combination of Hydroxyurea, interferon-alpha, and Ara-C to a targeted approach by imatinib mesylate, (Glivec/Gleevec; formerly STI571; Novartis Pharmaceuticals, East Hanover, NJ), a tyrosine kinase inhibitor (TKI) that targets the breakpoint cluster region-v-abl, the Abelson murine leukemia viral oncogene homolog 1, also known as ABL1, and by competitive inhibition, disrupts the downstream oncogenic signals. In the IRIS (International Randomized Study of Interferon and STI571) trial, imatinib proved superior to a combination of interferon-alpha and cytarabine in having statistically significant higher rates of hematologic and complete cytogenetic responses, lower risk of progression to advanced disease states, and increased progression free survival [4]. Imatinib has thus revolutionized the treatment and outcome of CML, making it the paradigm of molecularly targeted cancer therapies. The success of imatinib in the IRIS trial necessitated the development of sensitive laboratory methods for molecular monitoring of the disease. This review summarizes the recent advances in the treatment of CML and the current appropriate methods for establishing diagnosis and molecular monitoring of the disease.

Natural History of the Disease

CML is characterized by uncontrolled proliferation of primarily the granulocytic and megakaryocytic lineages resulting in accumulation of immature granulocytic precursors in the one marrow, blood and other organs. If left untreated, the natural history of CML follows a course progressing through a chronic phase to an accelerated phase (in most cases) and terminating in blast crisis over 3-5 years. Disease progression is heralded by increasing genomic instability and acquisition of additional chromosomal abnormalities, the most common of which are +8, +19, del(9)(q34), isochromosome 17, and additional Ph1 chromosome. Some recent studies have shown emergence of the RUNX1 mutations during progression of CP-CML to blast crisis [5]. The blast transformation is myeloblastic in two-thirds, and lymphoblastic (precursor B-) in one-third of the patients. Blasts with mixed phenotypes representing different lineages are not uncommon, consistent with the stem cell origin of the disease [6-8].

Molecular Pathogenesis

Studies in mice have shown that translocation between BCR and v-ABL is sufficient to drive oncogenesis [9]. The chimeric BCR-ABL gene encodes for an abnormal, constitutively active tyrosine kinase, which phosphorylates and activates downstream signaling pathways including P13K, AKT, SRC-kinase, and STAT that in turn modulate transcription factors related to cell proliferation, survival, protection from apoptosis, and differentiation. In addition, the BCR-ABL protein causes oxidative damage to the DNA by induction of reactive oxygen species and also interferes with the DNA-repair pathway [10], thus making the cells genetically unstable and prone to accumulate deleterious mutations. Nearly 80% of patients with CML have additional chromosomal abnormalities, many of which are associated with disease progression.

Laboratory Diagnosis of CML

The WHO classification has laid down definite criteria for the...
chronic phase, accelerated phase, and blast crisis of CML. Whereas demonstration of the defining BCR-ABL translocation is a must for establishing the diagnosis of CML, the diagnosis can easily be made from the peripheral smear in most cases. Leukocytosis with circulating granulocytic precursors, absolute basophilia, and thrombocytosis form the backbone of morphological diagnosis of chronic phase CML (CP-CML). In CP-CML, the peripheral blood and bone marrow have less than 10% blasts. Accelerated disease is associated with increasing leukocyte count, increase in circulating or bone marrow blasts, basophilia of >20% in the peripheral blood, thrombocytosis or thrombocytopenia, and bone marrow fibrosis with proliferation and clustering of abnormal megakaryocytes. The last mentioned morphological feature, while not a definitive criterion, is often observed with the other defined WHO criteria for accelerated disease. For details on the clinical features and diagnostic criteria of the different stages of disease evolution, the reader is referred to the WHO Blue book on the Classification of tumors of the hematopoietic and lymphoid system [6].

Demonstration of the translocation t(9;22)(q34;q22) or the BCR-ABL1 chimeric gene product is quintessential to establish the diagnosis. The Ph1 chromosome can be demonstrated by G-banded metaphase cytogenetics or fluorescent in-situ hybridization (FISH), and the abnormal BCR-ABL transcript by RT-PCR (reverse transcriptase-PCR). Each of these methods has its pros and cons as a diagnostic tool or for subsequent monitoring of the disease.

Conventional metaphase cytogenetics and fluorescent in-situ hybridization

In 95% of patients, the Ph1 chromosome can be detected by metaphase cytogenetics or G-banding [11]. Analysis of 20-25 metaphases for presence of Ph1 chromosome is standard practice. A bone marrow sample is required for obtaining metaphases from dividing cells, entailing the risk associated with an invasive procedure. In about 5% cases the result may be negative due to the overgrowth of normal bone marrow cells or inadequate metaphases for analysis. The specific translocation may be masked in the presence of a complex translocation involving three or more chromosomes, or when there is no alteration in the size of chromosome 22 due to cryptic interstitial insertions of the ABL1 within the BCR gene [12]. The main advantage of G-banding is that no a priori information about the chromosomal abnormalities is required and it can detect abnormalities that may be present in addition to the Ph1 chromosome. The commonest secondary abnormalities in CML are an additional Ph1 chromosome, +8, and isochromosome 17q. Several previous studies had reported rapid disease progression and worse outcome in the 10-15% of patients who develop deletions of the derivative chromosome 9 [13-15]; in recent studies however, authors show that the adverse effect of this chromosomal abnormality on the EFS in chronic phase patients can be mitigated by second generation TK inhibitors [4-16]. Fluorescent in-situ hybridization (FISH) has a higher sensitivity for detection of Ph1 than metaphase cytogenetics. The dual-color, dual-fusion FISH probe set is very sensitive and has high specificity of 1.0 to 0.1%. The major advantage of FISH is that it can be performed on interphase, non-dividing cells from peripheral blood, precluding requirement for the invasive, costly, and labor intensive bone marrow procedure. The results obtained from the peripheral blood sample are comparable in sensitivity to those obtained from the bone marrow cells [17]. FISH should however not be considered an alternative for metaphase cytogenetics. Current guidelines recommend that cytogenetic analysis be performed at baseline, and then at 3 to 6 month intervals after initiation of TK inhibitor therapy [18,19]. Attaining the defined levels of cytogenetic responses at specific time points correlates with response to therapy and likelihood of subsequent relapse or disease progression. The definitions for hematological, cytogenetic and molecular remission are depicted in 9 (Table 1). (Table 2) shows the NCCN and ELN recommendations for time points for metaphase cytogenetics, FISH, and quantitative PCR. Patients with a major cytogenetic response (MCyR) including those with a partial response (1-34% Ph1 positive metaphases), or a complete cytogenetic response (CCyR: no Ph1 positive metaphases) are less likely to progress to advanced phase CML. Achieving CCyR at 12 months is a major landmark in therapeutic response to imatinib [20-21].

Qualitative and quantitative PCR

Qualitative testing by PCR analysis of the BCR-ABL transcript is required to establish diagnosis only in the rare situations when interphase FISH is inconclusive due to failure of probe hybridization or sample inadequacy. At the molecular level, the translocation t(9;22)(q34;q22) consistently involves exon 2 of the ABL gene, but occurs in different exons of the BCR gene. The fusion involving the major breakpoint region (MBR) between exons 12 and 13 (or e1a2; formerly called b2a2) or 13 and 14 (or e1a2; formerly called b3a2) leads to expression of a transcript that codes for a 210kD fusion protein (p210BCR-ABL). The breakpoint in the minor breakpoint region (MB) between alternate exon 1 and exon 2 (e1a2) results in a smaller transcript coding for a 190 kD protein (p190BCR-ABL) [22,23]. Most adult CML have the b2a2 or b3a2 transcript (p210BCR-ABL), whereas the e1a2 (p190BCR-ABL) is usually present in acute lymphoblastic leukemia and the lymphoid blast crisis of CML. Chronic phase CML with p190 BCR-ABL1 have a myelomonocytic morphology. Less commonly, the break point involves the μ-BCR (micro-BCR) or exon 19(e19a2) resulting in a p230BCR-ABL 1kD product [23]. Co-expression of the p210BCR-ABL and p190BCR-ABL encoding transcripts can occur as a result of alternative splicing in the M-BR of BCR [24-27]. Quantitative monitoring is the basis of molecular monitoring of CML and is discussed in a later section.

Treatment Strategies

Prior to the advent current therapeutic modalities, the treatment for CML was restricted to lowering the high cell counts by chemotherapy, or managing the symptoms arising from an enlarging spleen with local radiotherapy [28]. Interferon-alpha (IFNa) based regimens were the

<table>
<thead>
<tr>
<th>Hematological</th>
<th>Complete (CHR)</th>
<th>WBC&lt; 1x10^9/L; basophils &lt;5%; no circulating granulocyte precursors; platelet count &lt;450x10^11/L; no splenomegaly</th>
</tr>
</thead>
<tbody>
<tr>
<td>Partial (PHR)</td>
<td>Levels less than the above</td>
<td></td>
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</table>

| Cytogenetic | Partial (PCyR) | 1%-35% Ph1 positive metaphases |
| Major (MCyR) | Combination of PCyR and CCyR (≤35% Ph1 positive metaphases) |
| Complete (CCyR) | No Ph1 positive metaphases detected in 2 successive bone marrow samples after counting 30 metaphases |

| Molecular | Major (MMR) | 3-log reduction in transcript level based on 2-consecutive molecular analyses |
| Complete (CMR) | 2 consecutive samples with no detectable transcripts provided that control gene copy numbers are adequate |

Table 1: Definitions for hematological, Cytogenetic and Molecular remission.
first to offer the possibility of eliminating the malignant clone, with 20-30% of patients achieving CCyR [29] and were distinctly superior to the primarily leukostatic hydroxyurea [30,31]. However, the benefit with IFNa was mostly in patients with a low Sokal risk score, and that too at the expense of profound toxicity.

Allogeneic hematopoietic stem cell transplant is curative, especially when performed early during the disease course and was very popular in the 1990s. The advantage from HSCT was however restricted to the younger patients with a suitable donor. The outcome in HSCT depends on several factors including the age of the patient, the stage of the disease, time since diagnosis, type of donor, source of stem cells, post-transplant management of infections and graft versus host disease [32]. Younger patients in chronic phase disease with matched related donors have the best outcome [33,34]. Better supportive care has largely mitigated the negative impact of age in the matched related transplant group, but not for those with matched unrelated transplants [35]. With advances in HLA typing and GVHD management, the results for fully matched unrelated transplants are now nearly similar to that for matched related donor transplants [36]. Currently, the survival following HSCT is nearly 80% in chronic phase patients, about 40%-50% in accelerated phase, and 20% in patients who receive it after blast crisis sets in [37]. The therapeutic landscape for CML has undergone a paradigm change with the advent of TK inhibitors. HSCT is no longer the choice for first-line treatment even in younger patients. At this time, HSCT is recommended for patients who develop resistant mutations that fail to respond to second line TK inhibitors as well, or for patients in the advanced stage of disease [37,38].

**Imatinib as first-Line therapy**

Imatinib was initially approved over a decade ago for treatment of CML patients who had failed prior therapy with IFNa. The results of this Phase I study demonstrated a remarkable response with 53/54 patients achieving complete hematological remission (CHR), and 31% achieving MCyR (major molecular remission) (13%) with CCyR [39]. In the subsequent Phase II study, the efficacy of imatinib was established in patients in all stages of the disease. The response in CP-CML was more impressive (CHR 95%, MCyR 60%, CCyR 41%) than in AP-CML and BP-CML in whom CHR was obtained in 34% and 8% patients respectively [40]. Shortly thereafter, the International Randomized Study of Interferon and STI571 (IRIS) study (Phase III, randomized, double blind crossover study) demonstrated the superiority of imatinib over the IFNα/Ara-C combination in newly diagnosed chronic phase patients [20]. Results of the IRIS study showed 87% MCyR rate in patients on imatinib versus 35% on IFNα-Ara-C (P < 0.001), and a CCyR of 76% in the imatinib group compared with 15% in the IFNα/Ara-C group (P < 0.001). The progression free survival (PFS) at 18 months was significantly higher in the imatinib group versus IFNα/Ara-C (97% vs. 91.5%; P < 0.0001) [20]. Based on the impressive results of the IRIS trial, imatinib received a fast approval by FDA for use as first line therapy of CML patients in May 2001. FDA also approved imatinib for treatment of AP and BP-CML, relapsed CML following HSCT, and Ph1 positive acute lymphoblastic leukemia. Long term follow up of the IRIS trial confirmed that the responses obtained with imatinib were durable over time and clearly improved the overall survival (OS) and PFS in patients with CML [41].

**Management of imatinib resistance**

Whereas the majority of patients achieve sustained remission with imatinib, an 8-year follow up study showed that it had to be discontinued in 30%-40% of patients because of intolerance or refractoriness to the drug [42]. Resistance to imatinib is more commonly acquired and mostly due to additional genetic abnormalities, or resistant mutations in the ABL kinase domain. Primary resistance is less common and may be due to polymorphisms in drug metabolizing enzymes [43]. Secondary resistance or refractoriness to imatinib is most often due to ABL kinase mutations, from alterations in the intracellular drug availability due to overexpression of the multidrug resistance (MDR1) gene product P-glycoprotein, or decreased expression of the octamer binding protein (OCT1) gene [44].

Increasing the dose of imatinib is a rational first step in countering resistance. The effect of many of the earlier mentioned factors for resistance, including some of the ABL kinase mutations (e.g., M351T, V299L, L384M, G398R, M244V, M244I, Q252H, E355G, L387M), can be overcome by escalation of the dose from the standard 400 mg daily to 600 mg or even 800 mg per day. However, in these dose-escalation trials only a small number of patients achieved CCyR, which is predictive of a sustained favorable outcome [45-47]. The T315I is a highly resistant mutation that fails to respond to imatinib dose escalation as well as to second generation TKIs, as discussed further.

**Second-generation TK inhibitors**

Second line TK inhibitors- dasatinib and nilotinib are effective in patients who fail to respond to dose escalation or develop adverse effects to it. Dasatinib and nilotinib are structurally different from imatinib, hence are able to overcome the resistance induced as a result of conformational change in the molecule by ABL1 kinase mutations. In addition, dasatinib and nilotinib do not require the OCT1 drug transport protein and are not affected by a decrease in its level [48,49]. Dasatinib is several hundred times more potent than imatinib and is also effective against the Src Family Kinases. Nilotinib is 30 times more effective than imatinib against BCR-ABL1 kinase. Both dasatinib and nilotinib can counter the negative effect of many of the ABL kinase mutations including several that do not respond to escalation of imatinib dose (e.g., G250E, E255K, E255V, F486Y, V253H, V253F, F317L, F317L) [50]. Clinical trials using dasatinib and nilotinib have demonstrated durable overall response rates, improved PFS rates, and tolerability of these second line TK inhibitors in patients with imatinib resistant or intolerant CML in CP, AP, and BP-CML resulting in FDA-approval for use of dasatinib and nilotinib in treatment of

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**Table 2: NCCN and European LeukemiaNet criteria for remission, suboptimal response and failure for previously untreated chronic-phase CML patients on standard dose of imatinib.**

<table>
<thead>
<tr>
<th>Response</th>
<th>3 months</th>
<th>6 months</th>
<th>12 months</th>
<th>18 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CHR</td>
<td>PCyR</td>
<td>CCyR</td>
<td>MMR</td>
</tr>
<tr>
<td>Suboptimal response</td>
<td>&lt;CHR</td>
<td>&lt;PCyR</td>
<td>&lt;CCyR</td>
<td>&lt;MMR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(≤35% Ph1 positive metaphases)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Failure</td>
<td>No hematological response, stable disease, or disease progression</td>
<td>&lt;CHR or no CyR (≤35% Ph1 positive metaphases)</td>
<td>&lt;PCyR (≤35% Ph1 positive metaphases)</td>
<td>&lt;CCyR</td>
</tr>
<tr>
<td>Failure</td>
<td>At any time: loss of CHR, or loss of CCyR, or Abl kinase Mutation</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

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patients resistant or intolerant CML in all stages of the disease [51-54]. The choice between dasatinib and nilotinib is generally based on the adverse effect profile of these drugs in the context of a specific patient, and the nature of the specific mutations [55]. Secondary resistance from additional mutations may develop under drug pressure from second line TK inhibitors [56,57]. Mutations such as Q252H, E255K/V, V299L, F317L, and T315A are more likely to develop resistance to dasatinib, whereas others including Y253H, F359C/V, and E255K/V to nilotinib. The little overlap between mutations insensitive to both drugs allows, replacing one by the other to restore effectiveness against the mutation. The highly resistant T315I mutation however fails to respond to both dasatinib and nilotinib, requiring recourse to allogenic BMT or other alternative therapies for subsequent management [58].

Third-generation TKI

A future option may be a third-generation TK inhibitor ponatinib. Ponatinib is an oral pan-BCR-ABL inhibitor developed to specifically counter the steric hindrance induced by the T315I mutation. Ponatinib overlaps the gatekeeper region of the ATP-binding site, and has an extensive network of high-potency hydrogen and triple carbon bonds that form multiple high-affinity contact points preventing destabilization of the ABL-kinase domain by the mutation [59]. It is effective against several common BCR-ABL1 mutations as well. In a cell-based mutagenesis screen, Ponatinib in therapeutic doses was able to inhibit growth of resistant BCR-ABL1 mutations, offering the possibility that it may prevent emergence of resistant mutations [60]. Initial results of a 3-month evaluation in the pivotal phase II trial of ponatinib (AP24534) in patients with refractory CML show MCyR in 57% of the 23 patients with the T315I mutation, of whom 11 achieved CCyR [59].

Second-generation TK Inhibitors as frontline therapy for CML

Results of recent clinical trials comparing the efficacy of dasatinib and nilotinib with standard dose imatinib in the first-line setting in CP-CML clearly demonstrate significantly higher rates and depth for achieving CCyR and MMR with both nilotinib and dasatinib compared with standard dose imatinib, with lesser rates of progression to accelerated phase or blast crisis, and with similar safety profiles [61,62]. Compared with imatinib, the time to attain CCyR and MMR was significantly shorter with dasatinib (p < 0.0001) [62]. FDA has approved both dasatinib and nilotinib for front line treatment of CP-CML.

Overall, for most patients, the treatment options for CML are rendering it more of a chronic disease with the median survival estimated at greater than 20 years [63]. With improved overall survival for most patients, quality of life issues require more attention. In addition to common class-related adverse effects, each TK kinase inhibitor is characterized by a unique side effect profile [64]. The importance of being cognizant of these adverse effects and taking them along with the patient-specific comorbidities into account while tailoring the TK inhibitor therapy cannot be overemphasized. Early management of adverse effects is important in preventing non-compliance and to ensure maintenance of remission status in patients [65].

Molecular monitoring of CML

With the current molecularly targeted therapy for CML the majority of patients in chronic phase achieve CCyR at the end of one year [20,66]. At the time of CCyR the tumor burden in the bone marrow may be as high as 10^6 leukemic cells. In the IRIS study, quantitative real time PCR testing for the BCR-ABL transcript was performed in all patients who achieved CCyR [66]. Results of the study showed that the likelihood of progression free state at 24 months was 100% in patients who additionally achieved a 3-log reduction in the BCR-ABL transcript at 12 months, compared with 95% in those who did not achieve 3-log reduction (P < 0.001) [66]. These results made it incumbent on laboratories to develop more sensitive, reproducible, and standardized methods for subsequent monitoring of the disease. Quantitative reverse transcriptase PCR (RQ-PCR) performed on real time platforms can detect presence of abnormal transcripts with a sensitivity of 1 in 10^3 or 1 in 10^4 cells, and is currently the most sensitive laboratory technique for detection of the BCR-ABL transcript [67-70]. RQ-PCR is particularly useful for subsequent monitoring of patients who have achieved CCyR. The assay is equally sensitive on peripheral blood and bone marrow samples and either specimen can be used for the assay, as long as the follow up is done using the same sample type for comparable results [71]. The levels of BCR-ABL transcript in the peripheral blood by RQ-PCR show excellent congruity with those of metaphase cytogenetics [71]. Quantitative reverse-transcriptase PCR (RQ-PCR) involves extraction of total RNA from the peripheral blood or bone marrow specimen, reverse-transcription of the mRNA so obtained into cDNA, and quantitative (real time) co-amplification of the target BCR-ABL cDNA and cDNA of an internal control gene (to control for RNA integrity, sample preparation, extraction and loading). Standard curves are constructed by serial dilutions of known amount of cloned plasmid containing the fusion DNA, or from serial dilutions of K562 cells in normal DNA. The value of BCR-ABL transcript extrapolated from the standard curve is expressed as a normalized ratio of the BCR-ABL transcript to the control gene transcript [72,73]. Despite the fact that RQ-PCR is currently performed by most laboratories nation-wide, there is a lack of uniformity in the way the results are obtained and the data expressed. This inherent variability is due to several factors that affect the performance of RQ-PCR including methods of specimen transport, storage, RNA extraction procedures, reverse transcription and PCR efficiency, and the type of real-time platform used. The use of different control genes by the laboratories can also significantly alter the BCR-ABL/control gene ratios. To develop international treatment guidelines it is essential that the molecular end-points for therapy protocols be comparable. For harmonizing the molecular monitoring of CML by RQ-PCR, recommendations were made at the CML meeting at the National Institutes of Health in Bethesda in 2005 to establish guidelines for specimen collection and transport, appropriate RNA quality, methodology for reverse transcription, PCR amplification efficiency, use of suitable control genes, test sensitivity for reporting negative results, quality assurance of the assay, generation of international reference, and a standardized method for expressing the result on an international scale [71]. To achieve this objective, a proposal was made to develop laboratory-specific conversion factors to convert the results obtained locally to a standardized international scale (expressed as % or IS units) [66]. The international scale was derived from the IRIS study in which the baseline median transcript level in 30 pre-treatment patient samples by RQ-PCR performed independently in the three principal laboratories was defined as 100% BCR-ABL level. Using this international scale, 1% BCR-ABL level correlated with the state of complete cytogenetic remission, and 1 log below this level (0.1% BCR-ABL) was defined as major molecular remission (MMR). In the IRIS study, no patient in MMR had positive Ph chromosome karyotyping [71]. According to the current guidelines laid by the NCCN and the European Leukemia Net [18,19], optimum response to imatinib therapy is indicated by attainment of MMR by 18 months of start of therapy. The NCCN and ELN criteria for remission, suboptimal
response and failure for previously untreated patients on standard dose of imatinib are shown in (Table 3). Data from several recent studies have shown that time to response is important and the probability of a favorable long-term outcome is best for patients who have the best responses at 3 months after the start of therapy [74-76]. Using receiver-operated characteristic curves, [76] authors have confirmed that the BCR-ABL transcript ratio of less than 9.84% at 3 months correlates with best outcome, and compared with current NCCN and ELP cytogenetic milestones (Table 2), molecular determination of transcript levels at 6 and 12 months levels was the only independent of overall survival (p < 0.001 for both time points). These data have not yet been translated into guidelines for testing. It is clear that the success of these predictive time points depends on standardization of methodology for determining the BCR-ABL transcript and subsequent reporting of the results. The first World Health Organization International Genetic Reference Panel for quantitation of BCR-ABL mRNA was approved by the Expert Committee on Biological Standardization of the World Health Organization in November 2009 and is now available commercially [77,78]. It consists of 4 dilutions of lyophilized preparations of K562 cells with assigned, fixed BCR-ABL/control gene values according to the IS. This development is a major milestone towards standardized molecular monitoring of therapeutic response to tyrosine kinase inhibitors in CML.

Mechanism of resistance to imatinib and molecular testing for ABL kinase inhibitors

The majority of CML patients in chronic phase achieve an optimum response with the standard recommended dose of imatinib. A minority (20%), patients develop primary resistance to imatinib, defined by failure to achieve the different levels of response i.e. hematological, cytogenetic, or molecular, at the expected time points [19]. The resistance is ‘acquired’ in the majority of patients, with loss of a previously obtained hematological or cytogenetic remission, or loss of MMR fifty- ninety percent of the acquired resistance in CML is due to BCR-ABL-kinase domain mutations. According to a recent publication from 2011, nearly 100 different ABL kinase mutations have been identified [79]. The mutations involve the catalytic domain, the highly conserved p-loop, or the activation loop, with two different mechanisms of causing resistance [58,80,81]: direct interference in binding of the drug (eg, T315I, F317L, and F369C/V), or by a causing a conformational change in the ABL kinase ATP-binding site that prevents access to the drug (eg, G250E, Q252H, Y253H, E255K/V) [80]. The mutations have different level of sensitivity or resistance to the different TK inhibitors based on the in vitro IC_{50} determinations. Mutations resistant to imatinib may be sensitive to second-generation TK inhibitors, underscoring the importance of mutation testing. The T315I mutation present in 4-15% of imatinib-resistant patients however, is resistant to imatinib as well as the second-generation TK inhibitors dasatinib and nilotinib [58]. Other mechanisms of acquired resistance include increased dosage of the BCR-ABL1 transcript by amplification or additional copies of the Ph' chromosome, less commonly by involvement of different pathways such as the SRK kinase pathway that are not inhibited by imatinib, or drug efflux mechanisms that decrease the effective concentration of the drug within the cells [82,83].

The Pertinent Questions Regarding Mutation Testing for BCR-ABL1 Are Discussed Below

When should the testing be done and who should be tested?

Treatment naive patients: There are a few reports of ABL1 kinase mutations present in newly diagnosed treatment naïve patients. Most of them are in patients who present in accelerated phase or blast crisis at diagnosis [84-87]; they are rare in chronic phase treatment naïve patients [88,89]. Given the rare occurrence, and lack of any clinical evidence of an adverse outcome from mutations in chronic phase patients, routine testing of all chronic-phase patients for mutations is not recommended at this time. The present recommendation to perform BCR-ABL (kinase domain) KD mutations in treatment naïve patients is restricted to those who present with advanced disease in accelerated phase or blast crisis [87].

In patients on imatinib first line therapy: Per the NCCN practice guidelines [18] and ELN [19], testing for BCR-ABL KD mutations in patients on imatinib as first line therapy is to be performed in the following circumstances: 1) Failure to respond or suboptimal response at the defined time points i.e. complete hematological remission at 3 months, minor cytogenetic remission at 6 months, and CCyR at 12 months of initiation of imatinib therapy [1,8,19]. Twenty-nine percent of chronic phase non-responders and 16% of patients with suboptimal response harbor BCR-ABL1 KD mutations [90-92]. 2) Confirmed loss of previously attained optimum response demonstrated by loss of MMR or disease progression [86]. Fluctuations in levels of BCR-ABL transcript may occur when the levels are very low and are not considered clinically significant. A trend of increasing BCR-ABL transcript level by analysis of sequential samples, or a two-fold (or one log) increase in BCR-ABL transcript level is more consistent with definitive loss of MMR and predictive of relapse [93].

Mutation analysis in patients on second-line TKIs: KD mutations arise under selective drug pressure. Thus, it is not unexpected to see

<table>
<thead>
<tr>
<th>Test</th>
<th>Time Point</th>
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<tbody>
<tr>
<td>A. Metaphase cytogenetics</td>
<td>1. At diagnosis and 6, 12, and 18 months until CCyR achieved, if CCyR achieved at an earlier time point, then there is no need to perform metaphase cytogenetics in a stable patients.</td>
</tr>
<tr>
<td>B. FISH (peripheral blood sample is acceptable if bone marrow specimen not available) using dual color, dual fusion probes and standard technique</td>
<td>2. Rising level of BCR-ABL1 transcript (1 log increase) without achieving MMR</td>
</tr>
<tr>
<td>QRT-PCR for BCR-ABL transcript</td>
<td>1. At diagnosis to establish baseline transcript level.</td>
</tr>
<tr>
<td></td>
<td>2. Every 3 months thereafter until patient is responding to TK inhibitors, and every 3-6 months after achieving CCyR</td>
</tr>
<tr>
<td></td>
<td>3. If level of BCR-ABL1 transcript is rising (1 log increase) after achieving MMR, then QRT-PCR should be performed every 6 months</td>
</tr>
<tr>
<td>Kinase domain mutation testing</td>
<td>1. At time of suboptimal response or failure</td>
</tr>
<tr>
<td></td>
<td>2. Before switching to another TK inhibitor</td>
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</table>

Table 3: Recommendations of NCCN and European LeukemiaNet for monitoring of CML patients on Tyrosine kinase inhibitors.
emergence of additional resistant mutations in patients who are put on second-generation TK inhibitors following resistance to imatinib [94]. Mutations resistant to dasatinib (V299L, T315A, F317L/V/I/C) or to nilotinib (E255K/V, Y253H, F359V/C/I) have been described in patients developing secondary response failure with second-generation TK inhibitors [95,96]. The data on resistant mutations to the second generation TKIs is limited; currently KD mutation testing is done in event of failure to respond, or suboptimal response, similar to that for imatinib. Preliminary data show a better projected one-year survival in patients receiving dasatinib as second or subsequent-line therapy if they attain MCyR at 12 months, and suggest alternative therapies if there is no cytogenetic response in 3 to 6 months [97,98].

Methods for Mutation Analysis

Many different approaches can be applied for mutation detection with sensitivities varying from 20% (one mutant allele in a background of 5 normal alleles) by Sanger sequencing to 0.01%-0.1% by allele-specific PCR (AS-PCR) [71-79,99-101]. Despite the low sensitivity of Sanger sequencing, it is the most commonly used method because of its availability in most molecular laboratories, and its ability to detect a large number of mutations in the activation-loop, the P-loop, as well as the catalytic loop [71-79,99,100]. Bidirectional sequencing is recommended to avoid false positive calls [102]. A two-step process of initial screening by DHPLC (Denaturing High Performance Liquid Chromatography) increases the sensitivity of subsequent sequencing [99-101]. The threshold of detection at 20% is acceptable since the clinical significance of detecting very low-level mutations is unclear [101]. According to a recent study, sensitive detection methods such as mass-spectrometry can predict a sub group of poor-responders to dasatinib/nilotinib at the time of switchover, by detecting multiple low-level mutations that are not detected by routine direct sequencing method [103]. The clinical validity of these results is yet to be seen in large multicenter studies. The sensitivity of detection will be greatly increased when the high-throughput deep sequencing technology is established in the clinical laboratory.

Reporting of Results and Interpretation

The purpose of mutation detection is to guide further management using one of the second or third-generation TK inhibitors. The report should indicate the specific mutation detected, the location in the specific region of the BCR-ABL kinase gene, the nucleotide change, and the resultant amino acid change. In addition, the report should include the specific BCR-ABL isoform-p190, p210 or rarely p230, and the RQ-PCR results leading to the testing. Several laboratories have developed large databases that include a list of mutations reported previously and the sensitivity to the different TK inhibitors (IC₅₀). Soverini et al. [96] provide a meta-analysis of published IC₅₀ values of imatinib, dasatinib, and nilotinib for the most commonly occurring mutations as compared with the IC₅₀ for the wild type BCR-ABL. A reference for these databases is useful for making clinical decisions for second-line TKIs, and is a useful addition to the report.

Current Consideration

The durable remissions now possible with TK inhibitors enable us to ask the pertinent question whether therapy can be stopped in patients in stable remission. This question has been addressed by at least two studies with similar conclusions that at least 40% of patients who have been in complete molecular remission (CMR: level of BCR-ABL1 transcript 1-2 logs below the detection limit of the current sensitive qRT-PCR method of detection) for at least 2 years, will remain in CMR for 2 years after discontinuing imatinib [103,104]. Longer-term data are not yet available. Early molecular relapse in 60% of these patients with stable CMR, relapses occurring after nearly a decade in patients who underwent allogenic stem cell transplant [105] clearly indicate persistence of leukemic cells at a very low level. Indeed, the unique genomic BCR-ABL1 fusion of the original leukemic clone could be detected by patient-specific, highly sensitive, DNA quantitative PCR in patients who were transfected negative and in ‘functional remission’ [106,107]. The factors determining sustained remission, or relapse in a patient after stopping TK inhibitor therapy are presently unclear. It is believed that low level persistence of leukemic stem cells that are independent of BCR-ABL1 for their survival, and hence immune to the TK inhibitors, are responsible for subsequent relapses [108], implying that ‘cure’ in CML is dependent on eradication of this resistant leukemic stem cell clone. Three hypothetical models of operational cure have been proposed that include depletion of the stem cell clone by prolonged imatinib therapy in combination with therapies that target pathways other than those mediated by BCR-ABL1, exhaustion of the stem cell from asymmetric division by depleting the mutable mature leukemic clone that replenishes the self renewing cells, and third by boosting the host T- cell dependent cancer surveillance by immunomodulation to maintain the initially established remission [103].

In summary, the current management of CML patients is able to provide sustained CCyR and MMR in a significant number of patients. The goals are to maintain the remission status in these patients, recognize causes of relapse in the remaining subset of patients, and eventually gear towards a ‘cure’ for this disease. An approach to achieve PCyR or reducing the BCR-ABL1 transcript to 10% within 3 months by newer second-generation TK inhibitors, assiduous monitoring for early detection of resistance by standardized sensitive methodology, judicious use of alternate TK inhibitors and HSCT to counter resistant mutations, and ensuring compliance by management of adverse effects is required to meet these goals.

References


59. Initial findings from the PACE trial: A pivotal phase 2 study of ponatinib in patients with CML and Ph+ALL resistant or intolerant to dasatinib or nilotinib, or with the T315I mutation.


