Invited review

Implications of BCR-ABL1 kinase domain-mediated resistance in chronic myeloid leukemia

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A B S T R A C T

Patients with chronic myeloid leukemia develop resistance to both first-generation and second-generation tyrosine kinase inhibitors (TKIs) as a result of mutations in the kinase domain (KD) of BCR-ABL1. A wide range of BCR-ABL1 KD mutations that confer resistance to TKIs have been identified, and the T315I mutant has proven particularly difficult to target. This review summarizes the prevalence, impact, and prognostic implications of BCR-ABL1 KD mutations in patients with chronic myeloid leukemia who are treated with current TKIs and provides an overview of recent treatment guidelines and future trends for the detection of mutations.

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1. Introduction

Chronic myeloid leukemia (CML) is characterized by the presence of the Philadelphia chromosome (Ph+), which contains the oncogenic BCR-ABL1 fusion gene resulting from a translocation between chromosomes 9 and 22 [1,2]. This disease accounts for 15% of adult leukemias, with an estimated 5920 new cases and 610 deaths due to this disease in the United States (US) in 2013 [3].

Five BCR-ABL1 tyrosine kinase inhibitors (TKIs)—imatinib [4], dasatinib [5], nilotinib [6], bosutinib [7], and ponatinib [8]—have been approved in the US for the treatment of CML patients. Imatinib, dasatinib, and nilotinib are indicated for both newly diagnosed patients and patients with relapsed or refractory disease, whereas bosutinib and ponatinib are indicated for patients with resistance or intolerance to prior TKIs. Recently, omacetaxine, a protein synthesis inhibitor, has been approved in the US for CML patients resistant to and/or intolerant of two or more BCR-ABL1 TKIs [9]. Although BCR-ABL1 TKIs have not been formally compared with omacetaxine, TKIs are generally the preferred option [3]. Interferon-α alone is no longer considered as initial therapy for newly diagnosed CML patients [3,10], although it can be used during pregnancy [10–12], and clinical trials evaluating its use in combination with TKIs are ongoing [13]. It can also be considered as an option for patients who are unable to tolerate TKI therapy [3]. Until recently, imatinib was the only first-line option; therefore, the majority of newly diagnosed CML patients living today have been treated with imatinib. Now that three TKIs (imatinib, dasatinib, and nilotinib) are approved first-line options, the choice of initial agent depends on many different factors [3,10]. Second-generation TKIs are more potent and are associated with lower rates of blast transformation on study treatment [14,15], along with a lower level of mutation vulnerability than imatinib [16]. On the other hand, there is longer experience with imatinib than with second-generation TKIs with respect to side effects and safety [14,15,17].

Despite high response rates to BCR-ABL1 TKIs, primary resistance and secondary resistance have been observed: primary (intrinsic) resistance is defined as lack of initial response, and secondary (acquired) resistance (relapse) is defined as loss of an established response during TKI treatment [18]. Resistance has been defined using the European LeukemiaNet (ELN) criteria for failure of first-line TKI therapy: less than a complete hematologic response (CHR) and/or no cytogenetic response (CyR; defined as Ph+ bone marrow metaphases >95%) at 3 months, BCR-ABL1 transcript levels above 10% and/or less than a partial CyR (PCyR; defined as ≤35% Ph+ metaphases) at 6 months, BCR-ABL1 transcript levels above 1% and/or less than a complete CyR (CCyR; defined as no Ph+ metaphases) at 12 months, or loss of a CHR or CCyR or confirmed loss of MMR, mutations, or clonal chromosome abnormalities in Ph− cells at any subsequent time during therapy [10,19]. According to the National Comprehensive Cancer Network (NCCN) guidelines, resistance is defined as the detection of BCR-ABL1/ABL1 transcript levels above 10% on the International Scale or failure to achieve PCyR at 3 months, or failure to achieve CCyR at 12 months or 18 months [3]. Several mechanisms have been associated with resistance, including BCR-ABL1-dependent and BCR-ABL1-independent mechanisms [20]. BCR-ABL1 kinase domain (KD) mutations and BCR-ABL1 genomic amplification are the best-characterized mechanisms conferring resistance to TKI therapy [21]. Numerous mutations have been identified (Fig. 1) [22]. Mutation analysis is usually performed after patients experience TKI treatment resistance; the results of mutation analysis may guide the selection of subsequent TKIs [3,10,19,22]. Currently, few mutations are known to confer clinical resistance to nilotinib (Y253H, E255K/V, and F359V/C/I) [23,24] or dasatinib (V299L, T315A, F317I/L/I/V/C) [24–27] or both (T315I). Patients already harboring mutations have higher likelihood of developing additional mutations [21,24], leading to the appearance of multiple mutations, which can be associated with poor prognosis [26,28,29]. Compound mutations are defined as two or more codon changes in the same BCR-ABL1 mRNA molecule, and thereby within a single clone; polyclonal mutations are defined as two or more codon changes across different BCR-ABL1 mRNA molecules, and therefore presumably belonging to different mutant clones [21]. As patients are sequentially treated with different TKIs, new mutations may emerge [24]. In a series of 17 imatinib-resistant CML patients treated sequentially with imatinib and dasatinib at UCLA, 30% had compound mutations (detected by subcloning and sequencing) at the time of relapse with dasatinib that were not detected prior to dasatinib treatment [26]. Based on an analysis conducted in 47 CML patients on TKI therapy, the majority (70%) of double mutations detected by direct sequencing represent compound mutations.

2. Emergence of BCR-ABL1 KD mutations with current TKIs

2.1. Resistance and mutations with first-line imatinib

While treatment with first-line imatinib is associated with high hematologic response rates, a proportion of patients may fail to experience cytogenetic responses (primary resistance) [18]. Across the historical trials of imatinib, approximately 65–90% of chronic-phase (CP) CML patients experience CCyR at by 12–24 months (Table 1) [14,16,30–38]. Resistance to imatinib treatment can also occur after patients have achieved an initial response (secondary resistance). Based on the 6-year update of the IRIS trial, the discontinuation rate was 34% and the estimated cumulative annual event rate, including loss of CHR, loss of McCyR, progression to accelerated phase (AP) or blast phase (BP), or death during treatment, was 18%, of which an estimated 7% account for patients who progressed to AP/BP [17].
Among the 456 patients who achieved CCyR, the estimated annual rates of treatment failure were 5% in the first year, 2% in the second year, 1% in the third year, and 0.3% in the fourth year after achieving that response. The corresponding annual rates of AP/BP progression were 2%, 0.7%, 0.3%, and 0%, respectively. The event-free survival (EFS) rate was substantially worse in patients with no CyR (59%) and minimal/minor CyR (58%), compared with those with P CyR (85%) and CCyR (91%).

Findings from several studies indicate that BCR-ABL1 mutations are detected with a frequency ranging from 12% to 63% in CML patients who experienced imatinib resistance (Table 2). The most common mutations with imatinib were T315I, G250E, M345V, M351T, and E255K/V (Table 2). In most studies, more patients with secondary resistance developed mutations during imatinib treatment than those with primary resistance (Table 2). Between 21% and 48% of cases of primary resistance and 10–68% of cases of secondary resistance were associated with mutations (Table 2) [39–49].

### 2.2. Resistance and mutations with second-line dasatinib or nilotinib

Thirteen percent of CP-CML patients treated with dasatinib 100 mg once daily, the approved dose, discontinued the CA180-034 trial as second-line treatment because of disease progression, at a minimum follow-up of 24 months [50]. In this trial, disease progression was defined as confirmed AP or BP, loss of a previous CHR or MCyR, ≥30% increase in Ph+ metaphases, increasing

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**Table 1**

CCyR rates from clinical trials of imatinib in the first-line setting.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Daily imatinib dose</th>
<th>N</th>
<th>CCyR rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRIS [30]</td>
<td>400 mg</td>
<td>553</td>
<td>74% by 18 months</td>
</tr>
<tr>
<td>MD Anderson Cancer Center [31]</td>
<td>800 mg</td>
<td>114</td>
<td>90% by 18 months</td>
</tr>
<tr>
<td>Hammersmith Hospital [32]</td>
<td>400 mg</td>
<td>224</td>
<td>65% by 15.5 months</td>
</tr>
<tr>
<td>TIDEL-I [33]</td>
<td>600 mg</td>
<td>103</td>
<td>88% by 12 months</td>
</tr>
<tr>
<td>RIGHT [34]</td>
<td>800 mg</td>
<td>115</td>
<td>90% by 24 months</td>
</tr>
<tr>
<td>TOPS [35]</td>
<td>400 mg</td>
<td>157</td>
<td>66% by 12 months</td>
</tr>
<tr>
<td>SPIRIT [36]</td>
<td>800 mg</td>
<td>319</td>
<td>70% by 12 months</td>
</tr>
<tr>
<td>GIMEMA CML WP trials (CML/021, CML/022, CML/023) [37]</td>
<td>400 mg</td>
<td>159</td>
<td>70% by 12 months</td>
</tr>
<tr>
<td>German CML study IV [38]</td>
<td>800 mg</td>
<td>160</td>
<td>65% by 12 months</td>
</tr>
<tr>
<td>ENEStnd [16]</td>
<td>400 mg</td>
<td>160</td>
<td>65% by 12 months</td>
</tr>
<tr>
<td>DASSIGN [14]</td>
<td>800 mg</td>
<td>311</td>
<td>75% by 18 months</td>
</tr>
<tr>
<td></td>
<td>400 mg</td>
<td>283</td>
<td>77% by 24 months</td>
</tr>
<tr>
<td></td>
<td>400 mg</td>
<td>260</td>
<td>82% by 24 months</td>
</tr>
</tbody>
</table>

CCyR, complete cytogenetic response; IRIS, International Randomized Study of Interferon and STI571; TIDEL-I, Therapeutic Intensification in De-novo Leukaemia; RIGHT, Rationale and Insight for Gleevec High-dose Therapy; TOPS, Tyrosine kinase Inhibitor Optimization and Selectivity; SPIRIT, STI571 Prospective Randomized Trial; GIMEMA CML WP, Italian Group for Hematological Malignancies of the Adult CML Working Party; ENEStnd, Evaluating Nilotinib Efficacy and Safety in clinical Trials—newly diagnosed patients; DASSIGN, DASatinib versus Imatinib Study In treatment-Naive CML patients.

* Estimated response rate.
* 83% of evaluated patients.
* Confirmed CCyR was 74%.
Table 2
Summary of mutation analysis conducted at imatinib failure in a series of studies of imatinib with or without previous interferon-α, in which at least 50 patients were analyzed.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Frequency of BCR-ABL1 mutations (n/N)</th>
<th>Frequency of mutations among patients with mutations</th>
<th>CML phase</th>
<th>Primary vs secondary resistance (n/N)</th>
<th>Method used</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIMEMA CML WP [39]</td>
<td>43% (127/293)</td>
<td>E255K/V (17%) M351T (11%) T315I (12%)</td>
<td>CP; LBC/Ph+ ALL; MBC; AP</td>
<td>30% (45/152) vs 57% (82/145)</td>
<td>D-HPLC and direct sequencing</td>
</tr>
<tr>
<td>MD Anderson Cancer Center [40]</td>
<td>54% (61/112)</td>
<td>G250E (20%) T315I (16%)</td>
<td>CP; AP; BP</td>
<td>NA</td>
<td>Direct sequencing</td>
</tr>
<tr>
<td>Argentinean study [41]</td>
<td>23% (36/154)</td>
<td>G250E (17%) M351T (14%) T315I (11%)</td>
<td>CP; AP; BP</td>
<td>21% (16/77) vs 34% (20/59)</td>
<td>Direct sequencing</td>
</tr>
<tr>
<td>Chinese study [42]</td>
<td>58% (74/127)</td>
<td>M244V (16%) Y253H (14%) G250E (11%)</td>
<td>CP; AP; BP</td>
<td>48% (31/64) vs 68% (43/63)</td>
<td>Direct sequencing</td>
</tr>
<tr>
<td>Korean study [43]</td>
<td>63% (70/111)</td>
<td>T315I (24%) E255K (11%) G250E (10%)</td>
<td>CP; AP; BP</td>
<td>NA</td>
<td>ASO-PCR and direct sequencing</td>
</tr>
<tr>
<td>Polish MAPTEST study [44]</td>
<td>12% (11/92)</td>
<td>T315I (27%) M351T (18%)</td>
<td>CP; AP; BP</td>
<td>40% (2/5) vs 10% (9/87)</td>
<td>Direct sequencing</td>
</tr>
<tr>
<td>Indian study [45]</td>
<td>33% (25/76)</td>
<td>T315I (16%) M244V (16%) Y253H (12%)</td>
<td>CP; AP; BP</td>
<td>NA</td>
<td>Direct sequencing</td>
</tr>
<tr>
<td>Hungarian study [46]</td>
<td>36% (27/74)</td>
<td>M244V (19%) T315I (15%)</td>
<td>CP; AP; BP; Ph+ ALL</td>
<td>NA</td>
<td>Direct sequencing</td>
</tr>
<tr>
<td>French study [47]</td>
<td>NA</td>
<td>T315I (19%) M244V (10%) M351T (10%)</td>
<td>CP; AP; BP</td>
<td>NA</td>
<td>Direct sequencing</td>
</tr>
<tr>
<td>Australian study [48]</td>
<td>19% (27/144)</td>
<td>M351T (30%) E255L (19%) Q252H (19%)</td>
<td>CP; AP</td>
<td>NA</td>
<td>Direct sequencing</td>
</tr>
<tr>
<td>Australian study [49]</td>
<td>NA</td>
<td>T315I (14%) M351T (12%) G250E (12%)</td>
<td>CP; AP; LBC/Ph+ ALL; MBC</td>
<td>NA</td>
<td>Direct sequencing</td>
</tr>
</tbody>
</table>

CML, chronic myeloid leukemia; GIMEMA CML WP, Italian Group for Hematological Malignancies of the Adult CML Working Party; CP, chronic phase; LBC/Ph+ ALL, lymphoid blast crisis/Philadelphia chromosome positive acute lymphoblastic leukemia; MBC, myeloid blast crisis; AP, accelerated phase; D-HPLC, denaturing high-performance liquid chromatography; NA, not applicable; ASO-PCR, allele-specific oligonucleotide-polymerase chain reaction.

\(^a\) The percentage indicates the number of patients with mutations divided by the total number of patients with mutations; the most frequent mutations are reported.
\(^b\) The percentage indicates the number of patients with mutations divided by the total number of patients who showed clinical resistance (definition of resistance was different across these studies).
\(^c\) A total of 54 patients had received interferon-α prior to imatinib.
\(^d\) 69 patients had received interferon-α prior to imatinib.
\(^e\) 76 patients had received interferon-α prior to imatinib.
\(^f\) A total of 25 patients had received interferon-α prior to imatinib.
\(^g\) A total of 18 patients had received hydroxyurea or interferon-α alone or in combination with arabinoside cytosine prior to imatinib.
\(^h\) A total of 34 patients had received interferon-α prior to imatinib.
\(^i\) The percentage of BCR-ABL1 mutations in CML patients only was 32% (22/69).
\(^j\) Patients had received 6 or more months of imatinib therapy or had developed resistance and ceased therapy before 6 months.
Table 3
Summary of mutation analysis conducted in a series of clinical trials of dasatinib/nilotinib in the second-line setting.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Frequency of BCR-ABL mutations (n/N)*</th>
<th>Frequency of mutations among patients with mutationsa</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dasatinib second-line</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>START-C, START-R, and CA180-034 [23]</td>
<td>27% (47/174)b</td>
<td>T315I (33%) F317I (21%) V299L (15%)</td>
</tr>
<tr>
<td><strong>Nilotinib second-line</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase II nilotinib registration trial [23]</td>
<td>24% (47/192)c</td>
<td>E255KV (28%) T315I (26%) F359C (15%) G250E (15%) Y253H (13%)</td>
</tr>
<tr>
<td><strong>Dasatinib/nilotinib second-line</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MD Anderson Cancer Center [40]</td>
<td>21% (23/110)d</td>
<td>Y253H (13%) F317I (13%) T315I (9%)</td>
</tr>
<tr>
<td>Italian study [24]</td>
<td>33% (31/95)e</td>
<td>T315I (36%) F317I (20%) E255KV (11%) V299L (9%) Y253H (9%)</td>
</tr>
</tbody>
</table>

* n is the number of patients with mutations and N is the number of patients in whom mutation analysis was performed.

† The percentage indicates the number of patients with a specific mutation divided by the total number of patients with mutations; the most frequent mutations are reported.

‡ Mutation analysis was performed at the time of progression or discontinuation.

§ Only patients resistant to imatinib have been included.

¶ Mutation analysis was performed during nilotinib therapy.

†† Frequency of new BCR-ABL1 mutations in patients who experienced progression: 25/64 (39%); most frequent mutations: E255KV (28%), T315I (28%), F359C/E450K (12%).

∥ Mutation analysis was performed after treatment with a second TKI.

‡‡ Mutation analysis was performed during treatment with a second TKI.

* Includes patients who relapsed on dasatinib or nilotinib as second or third TKI.

2.4. Resistance and mutations with ponatinib

Ponatinib has shown preclinical and clinical activity against a spectrum of mutants including T315I [56,57]. In a phase 1 study, among 43 heavily pretreated CP-CML patients, 3 (7%) discontinued due to disease progression; all 12 of the patients carrying the T315I mutation at baseline remained in the study [56]. Nine of the 12 patients (75%) who had the T315I mutation achieved CCyR. Ten of the 15 patients (67%) who had other mutations at baseline (M244V, G250E, F317I, M351T, F359C/I/V) achieved CCyR. Six of the 13 patients (46%) with no mutation at baseline had CCyR. Kaplan-Meier analysis estimated that 91% of the CP-CML patients with the T315I mutation who experienced MCyR would maintain response at 1 year. No data on emerging mutations are available to date.

2.5. Resistance and mutations with first-line dasatinib or nilotinib

Based on the DASISION, S0325 (US Intergroup study of dasatinib versus imatinib in the first-line setting), and ENESTnd trials, dasatinib and nilotinib first-line are associated with fewer failures than imatinib first-line [14–16,58]. In the 2-year follow-up of the DASISION trial, 9% (22/258) of CP-CML patients discontinued first-line dasatinib because of disease progression or treatment failure, compared with 11% (28/258) treated with imatinib [14]. In the S0325 study, 2% (2/123) of CP-CML patients discontinued first-line dasatinib because of disease progression or treatment failure, compared with 7% (8/123) treated with imatinib [58]. In the 2-year follow-up of the ENESTnd trial, 9% (26/282) and 3% (9/281) of CP-CML patients discontinued first-line nilotinib (300 mg and 400 mg bid arms, respectively) because of disease progression, treatment failure or suboptimal response, compared with 17% (48/283) treated with imatinib [16]. At the 3-year follow-up of the ENESTnd trial, 10% (28/282) and 5% (15/281) of CP-CML patients discontinued first-line nilotinib (300 mg and 400 mg bid arms, respectively) because of disease progression, treatment failure or suboptimal response, compared with 20% (57/283) treated with imatinib [15]. The definition of progression and the duration of follow-up prior to censoring were not uniform among these studies.

In the DASISION trial, mutation analysis was performed at the time of disease progression, treatment failure or end of treatment [59]. The frequency of emerging mutations among patients who discontinued treatment was similar between dasatinib-treated and imatinib-treated patients. At the 2-year follow-up, 26% (10/38) of patients treated with dasatinib who had an evaluable mutation analysis performed at discontinuation had a mutation compared with 21% (10/48) of patients treated with imatinib [14]. There were 6 cases of nonevaluable samples in the dasatinib group and one case in the imatinib group. Treatment failure was defined as disease progression, no hematologic response at 3 months, no CHR or no CyR at 6 months, no PCyR at 12 months, or no CCyR at 18 months [59]. Specifically, the 10 dasatinib-treated patients carrying mutations had the following mutations: T315I (n = 7), F317I (n = 2), and F317I/V299L (n = 1) [14]. In the US Intergroup S0325 study, mutation analysis was successfully conducted in 22 patients with evidence of resistant disease or with hematologic or cytogenetic relapse or progression; 1 of 9 (11%) dasatinib-treated patients and 2/13 (15%) of imatinib-treated patients had a mutation [58].

The registration trial of bosutinib was conducted in 288 imatinib-resistant or imatinib-intolerant CP-CML patients [54]. Among imatinib-resistant patients, 23% (45/200) discontinued due to disease progression or unsatisfactory response; among imatinib-intolerant patients, 7% (6/88) discontinued due to disease progression or unsatisfactory response. BCR-ABL1 mutation status at baseline was available for 115 patients, 65 (57%) of whom had at least one mutation. The most common mutations were M351T (n = 7), F359V (n = 7), F317L (n = 4), L248V (n = 4), G250E (n = 3), M244V (n = 3), and T315I (n = 3). Similar rates of CHR or MCyR were observed between patients with and without mutations. In a subanalysis of 118 patients who had received imatinib followed by dasatinib and/or nilotinib, emerging mutations were observed in nine patients (one had 2 emerging mutations): V299L (n = 4), L248V (n = 2), T315I (n = 2), F359C (n = 1), and G250E (n = 1) [55].

The white blood cell count, or death from any cause [51]. Thirty percent of CP-CML patients discontinued the nilotinib registration trial CAMN107A2101 as second-line treatment because of disease progression, although 70% of the patients discontinued for any reason, at a follow-up of 48 months [52]. In this trial, disease progression was defined as hematologic or cytogenetic relapse, or transformation to AP or BP [53].

Mutation analyses performed in several studies indicate that approximately 14–33% of CML patients treated with second-line dasatinib or nilotinib develop new BCR-ABL1 mutations (Table 3). The most common mutations with dasatinib were T315I, F317L, and V299L (Table 3). The most common mutations with nilotinib were E255KV, T315I, F359C/V, G250E, and Y253I (Table 3) [23–25,40].
In the ENESTnd trial, mutation analysis was performed in all patients at baseline. In patients with no baseline mutations, mutation analysis was performed when patients experienced either lack of response or loss of response (defined as failure to achieve MMR at 12 months; confirmed loss of MMR during the study; or rise in BCR-ABL1 transcript level by >5-fold from the lowest value achieved on study), and at the end of study. In patients with baseline mutations, mutation analysis was performed every 3 months [60,61]. The frequency of emerging mutations during treatment was lower in the nilotinib arm than in the imatinib arm of the ENESTnd trial. At the 3-year minimum follow-up, 5% (11/228) and 5% (11/215) of patients with at least one postbaseline mutation analysis treated with nilotinib (300 mg and 400 mg bid arms, respectively) had a mutation compared with 9% (21/237) treated with imatinib [60]. The 11 patients in the 300 mg bid nilotinib group carrying new mutations (3 patients had multiple mutations) had the following mutations: Y253H (n = 4), F359V (n = 4), T315I (n = 3), G250E (n = 1), E255K (n = 1), E459K (n = 1) [60]. The 11 patients in the 400 mg bid nilotinib group carrying new mutations (2 patients had multiple mutations) had the following mutations: Y253H (n = 4), E255K/V (n = 3/1), T315I (n = 2), F359V (n = 2), Q252H (n = 1).

3. Prognostic implications of BCR-ABL1 KD mutations

3.1. Imatinib

Emergence of new BCR-ABL1 KD mutations during imatinib treatment predicts loss of CCyR, shorter progression-free survival (PFS), shorter time to AP/BP progression, and shorter overall survival (OS) during first-line imatinib treatment [62–65].

In a series of 319 CP-CML patients treated with imatinib at the Hammersmith Hospital and with mutation analysis available, BCR-ABL1 KD mutations were identified in 37 patients (12%) [62]. Of these 319 patients, 171 patients received first-line imatinib therapy. Thirty of 214 patients (14%) who achieved CCyR lost their response during follow-up, and multivariate analysis revealed that emergence of BCR-ABL1 KD mutations was highly predictive of loss of CCyR (RR = 3.8; P = 0.005). Patients who had developed a mutation (n = 23) by 2 years of imatinib treatment had a significantly lower 5-year PFS compared with those who had not developed a mutation (n = 227; 65% vs 86%; P = 0.0001).

In a series of 150 CP-CML patients treated with imatinib at the Knight Cancer Institute (most of whom had received prior treatment with interferon-α), mutation screening was undertaken in 101 patients in whom BCR-ABL1 RNA had increased at least 3-fold during treatment [63]. Mutations were identified in 53 patients (52%). Median PFS was significantly shorter (P < 0.001) for patients with mutations (10.4 months) compared with those without mutations (32.4 months); with a hazard ratio of 3.2 (95% CI, 1.9–5.4).

A retrospective molecular analysis was conducted on 40 late CP-CML patients intolerant or resistant to interferon-α and with cytogenetic resistance to imatinib, who were enrolled in the CML002/STI571 GIMEMA trial conducted before the advent of dasatinib and nilotinib [65]. All 40 patients failed to obtain MCyR at 12 months, and mutations were found in 19 (48%). The presence of BCR-ABL1 KD mutations was associated with significantly shorter time to AP/BP progression (P = 0.0002) and shorter survival (P = 0.001).

In a series of 40 CML patients who were resistant to imatinib and intolerant or resistant to interferon-α, mutations were found in 18 patients (45%) [64]. The 2-year PFS was significantly inferior for patients with mutations compared with those without mutations (72% vs 95%, P = 0.0045).

3.2. Second-line dasatinib and nilotinib

Several studies found that CP-CML patients who harbored mutants less sensitive to dasatinib or nilotinib in vitro experienced lower CCyR rates and PFS rates than patients harboring mutants with high or unknown in vitro sensitivity to dasatinib or nilotinib [23,25,66].

Patients with CP-CML treated with second-line dasatinib, who participated in the trials START-C, START-R, and CA180-034, were analyzed at 2-year follow-up [25]. Among 121 patients who had a BCR-ABL1 mutant with an intermediate in vitro sensitivity to dasatinib (IC50 >3 nM), 32% had CCyR and PFS was 67%. Among patients who had a mutant with a high in vitro sensitivity to dasatinib (IC50 ≤3 nM, n = 176) or unknown IC50 (n = 84), 53% and 51% achieved CCyR, and the PFS rates were 75% and 80%, respectively.

Patients who participated in the phase 2 nilotinib second-line registration trial were analyzed at the 1-year follow-up [23]. Among 26 patients who had a BCR-ABL1 mutant with an intermediate in vitro sensitivity to nilotinib (IC50 >150 nM), none achieved CCyR and the PFS was 31%. Among patients who had a mutant with a high in vitro sensitivity to nilotinib (IC50 ≤150 nM, n = 45) or unknown IC50 (n = 29), 40% and 48% achieved CCyR, and the PFS was 64% and 59%, respectively.

In a study conducted at the MD Anderson Cancer Center in 86 CML patients with baseline mutations (30 with CP-CML, 41 with AP-CML, and 15 with BP-CML) treated with dasatinib or nilotinib after imatinib failure, response rates were lower in CP-CML patients carrying intermediate IC50 mutations (25% for both MCyR and CCyR), compared with those with low IC50 mutations (87% and 73%, respectively) [66]. The 2-year EFS rates were lower in CP-CML patients carrying intermediate or high IC50 mutations (22% and 0%, respectively), compared with those with low or not reported IC50 mutations (78% and 67%, respectively), as were the 2-year OS rates (70% and 75%, respectively, for intermediate or high IC50 mutations), versus 100% for patients with low or not reported IC50 mutations.

3.3. T315I mutations

Prior to the availability of ponatinib, several studies have shown that CML patients harboring the T315I mutation have a poor prognosis [47,49,67,68]. In a series of 386 imatinib-treated patients with mutations, T315I mutations were more frequently detected in patients who had progressed to BP [49]. A retrospective analysis from the French CML intergroup conducted in 89 imatinib-resistant CML patients showed that across all phases of CML, median OS was 12.6 months for the T315I mutation (n = 15), and not reached for other mutations (n = 47; P = 0.004045) [47]. Patients with CP-CML and with T315I (n = 8) mutations had worse OS and PFS than patients with other mutations (n = 38 and n = 37, respectively; P = 0.014 for each). A retrospective observational study in 176 CML patients carrying the T315I mutation who received first- or second-generation TKIs revealed that survival of patients harboring a T315I mutation is dependent on the disease phase at the time of mutation detection, with worse outcome for BP-CML patients [68]. Median OS rates from the time of T315I detection were 22.4, 28.4, and 4.0 months for CP-, AP-, and BP-CML, respectively. In a matched pairs analysis, 64 CP-CML patients resistant to imatinib and carrying the T315I mutation were compared with 53 CP-CML patients resistant to imatinib with no detectable T315I mutation [67]. This study confirmed that the T315I mutation has a negative impact on OS and failure-free survival (FFS). Median OS since imatinib resistance was 48 months in patients carrying the T315I mutation, while it was not reached in patients without the T315I mutation (P = 0.006). Median FFS since imatinib resistance was 35 months in patients carrying
the T315I mutation, while it was not reached in patients without the T315I mutation ($P=0.003$).

### 3.4. Low-level mutations

Low-level mutations are mutations that are below the detection limit of conventional direct sequencing and are detected only with more sensitive methods. The clinical significance of low-level mutations in CML patients has long been debated and remains, at present, unclear. Retrospective studies have suggested that mutations found in rare Ph+ cells may fail to expand and their detection does not consistently predict relapse [69,70]. One of these studies was conducted in patients with stable CCyR to imatinib by direct sequencing and denaturing high-performance liquid chromatography (D-HPLC) [69]; the other was conducted in imatinib-naïve patients by allele-specific oligonucleotide-polymerase chain reaction and direct sequencing [70].

In a mutation analysis conducted in 220 CML patients at imatinib failure, before starting nilotinib or dasatinib therapy, 281 mutations were detected in 131 patients by sensitive mass spectrometry [29]. Of these 281 mutations, 132 were not detected by sequencing. Mutants that were resistant to nilotinib and/or dasatinib were detected in 32% of the patients by mass spectrometry, compared with 23% by sequencing. Therefore, mass spectrometry allowed the detection of an additional 9% of patients who could benefit from selecting the most appropriate therapy after imatinib failure.

This study also showed that low-level nilotinib-/dasatinib-resistant mutations are associated with poor response and high risk of failure. Patients with low-level mutations resistant to nilotinib or dasatinib, detected upon imatinib failure, achieved low CCyR rates (16%) with nilotinib or dasatinib therapy, compared with patients with other mutations (41%) or patients with no mutations (49%). Patients with low-level resistant mutations had extremely poor failure-free survival at 18 months after therapy with nilotinib or dasatinib (0%), compared with 51% for patients with other low-level mutations and 45% for those with no mutations.

The apparently contrasting results regarding the correlation of low-level mutations with subsequent relapse in the studies presented may be due to the fact that different techniques were used and different patient populations were analyzed. It is possible that a lower detection limit (to be identified) exists, below which mutations may not necessarily expand for biological or clinical reasons. This hypothesis needs further studies.

### 3.5. Multiple mutations

Studies have shown that patients with multiple mutations have a poorer prognosis than those with no or one mutation [28,71]. In a study conducted at the MD Anderson Cancer Center, 7 of 207 imatinib-resistant CML patients (3%) treated with a second-generation TKI (nilotinib, dasatinib, bosutinib, or the investigational TKI bafetinib) had more than one mutation detected by direct sequencing [71]. Of 102 CP-CML patients, 4 (4%) had multiple mutations. This study showed that patients with more than one mutation have a significantly worse outcome than those with no or one mutation. The 4-year PFS rates were 56% (no mutation), 49% (1 mutation), and 0% (more than 1 mutation; $P=0.02$).

In a study of 220 patients treated with nilotinib or dasatinib after imatinib resistance, mutation analysis was conducted by sequencing and by mass spectrometry [28,29]. By sequencing, multiple mutations were detected in 31 patients (14%) after imatinib failure, with one case with mutations clinically resistant to both nilotinib and dasatinib; by mass spectrometry, 60 patients (27%) had multiple mutations, with 5 cases with mutations known to confer clinical resistance to both nilotinib and dasatinib [29]. In patients without mutations clinically resistant to nilotinib or dasatinib, multiple sensitive mutations detectable only by mass spectrometry and detected after failure of imatinib treatment were associated with lower CCyR rates achieved after second-line TKI therapy, compared with no mutation or one mutation (21% vs 50%, $P=0.003$) [28].

### 3.6. Dasatinib and nilotinib first-line

Based on the 2-year follow-up of the DASISION trial, the development of $\textit{BCR-ABL1}$ KD mutations may predict progression to advanced phase. Among the 10 dasatinib-treated patients who developed a mutation, transformation to AP/BP occurred in 4 patients while among the 10 imatinib-treated patients who developed a mutation, transformation to AP/BP occurred in 3 patients [14].

Based on the 3-year follow-up of the ENEStnd trial, fewer nilotinib-treated patients who developed mutations during treatment had treatment failure, suboptimal response, or lost response on treatment, than imatinib-treated patients who developed mutations [60]. Among the 11 patients in the 300 mg bid nilotinib group who developed a mutation, 4 patients experienced treatment failure, 6 patients had suboptimal response, and one patient progressed after discontinuation; among the 11 patients in the 400 mg bid nilotinib group who developed a mutation, 6 patients experienced treatment failure, 2 patients had suboptimal response, 2 patients had confirmed loss of MMR, and one patient had an unconfirmed loss of MMR but regained MMR later; among the 21 patients in the imatinib group who developed a mutation, 16 patients experienced treatment failure and 5 patients had suboptimal response. Three of 22 patients with mutations in the nilotinib arms had not discontinued treatment at the 3-year follow-up. As mentioned previously, the trigger for mutation analysis in clinical studies has not been uniform.

### 4. Guidelines

#### 4.1. When to perform mutation analysis based on ELN, ESMO, and NCCN recommendations

The ELN, European Society for Medical Oncology (ESMO), and NCCN provide recommendations about when $\textit{BCR-ABL1}$ KD mutation analysis should be performed in CML patients treated with TKIs; these are summarized in Table 4 [3,22,72].

According to the ELN and the ESMO recommendations, mutation analysis is recommended at diagnosis only in AP-/BP-CML patients, before starting first-line therapy [22,72]. However, in the absence of prospective studies based on mutation screening up front and the low rate of mutation detection, it is questionable whether this is sensible or cost-effective. The NCCN recommends mutation analysis in TKI-pretreated AP-/BP-CML patients [5].

The ELN and the ESMO recommendations differentiate between first-line and second-line treatment, while the NCCN guidelines do not differentiate across lines of treatment. According to the ELN and the ESMO recommendations, $\textit{BCR-ABL1}$ KD mutation analysis should be performed during second-line treatment in case of hematologic or cytogenetic failure, whereas based on the NCCN guidelines, mutation analysis should also be performed in case of loss of response, inadequate response, or increased $\textit{BCR-ABL1}$ transcript levels.

#### 4.2. Mutation status aids therapeutic decisions

The results of the mutation analysis should guide the choice of subsequent therapy, as per ELN and NCCN recommendations
ELN, European LeukemiaNet; ESMO, European Society for Medical Oncology; AP, accelerated phase; BP, blast phase; CML, chronic myeloid leukemia; MMR, major molecular response; CyR, cytogenetic response; PCyR, partial cytogenetic response; CP, chronic phase; NCCN, National Comprehensive Cancer Network; CyR, complete cytogenetic response.

(Table 5) [3,22], ELN recommendations based on mutation analysis were published in 2011, before the approval of bosutinib, ponatinib, and omacetaxine [22]. The 2013 ELN recommendations for the management of CML are specific only for the T315I mutation; the recommendations otherwise focus on the targeted desired response, regardless of which TKI inhibitor is used [10].

5. Future trends in detection of BCR-ABL1 mutations

5.1. Current techniques for mutation analysis

Direct sequencing is currently the most extensively used technique to detect BCR-ABL1 mutations in clinical practice, and is recommended by ELN and ESMO [22,72]. Direct sequencing allows detection of mutations present in ≥20% of Ph+ cells [22]. Denaturing high-performance liquid chromatography can be combined with direct sequencing; D-HPLC allows prescreening for sequence variations, reducing the number of samples to be sequenced [22] and improves the limit of detection to 1%, but alone does not allow characterization of the precise sequence [73]. Subcloning prior to sequencing is a well-established, relatively “old” and inexpensive approach capable of detecting compound mutations, but is very labor intensive and highly prone to contamination and therefore not suitable for routine diagnostics [73]. Sensitive detection of low-level mutations after imatinib failure may help to inform the selection of subsequent therapy, although this should be confirmed by independent studies.

5.2. Newer technologies with higher sensitivity

Newer technologies to detect mutations at a higher sensitivity are still experimental, not yet incorporated into clinical practice. Some of these new technologies include mass spectrometry, digital PCR, and NGS (or ultra-deep sequencing).

Mass spectrometry is a very sensitive technique, with a detection limit ranging from 0.05% to 0.5% depending on the mutation (0.2% on average) [28,29]. It has been used to detect 31 specified most frequent and clinically relevant mutations, including all of the nilotinib- and/or dasatinib-resistant mutations and the most common imatinib-resistant mutations (detected in ≥1% of patients with mutations) [28,29]. However, more mutations can be included in the multiplex screening.
The Fluidigm quantitative real-time digital PCR is a nanofluidic-based method characterized by partitioning the sample into thousands of independent reaction chambers, increasing the detection of rare mutations [74]. This technology can assess the kinetics of mutation development, allowing earlier detection of mutations and potentially allowing correlating kinetics of mutation development with outcomes. It has been used to identify and quantify the T315I mutation in CML patients. Samples from 28 patients taken both before and at the time of relapse were analyzed and the T315I mutation was detected before relapse in all 8 patients in whom it was detected at relapse.

The unique advantage of NGS over other methods is conjugating high sensitivity with the detection of any (known/unknown) mutation within the KD of BCR-ABL1. The Roche GS Junior system utilizes emulsion PCR to densely decorate beads with monoclonal DNA templates followed by pyrosequencing [75]. The Ion Torrent system utilizes semiconductor technology to directly detect hydrogen ions released during base incorporation allowing sequencing-by-synthesis [76]. The Illumina system utilizes a sequencing-by-synthesis approach with a reversible terminator-based method to detect single bases as they are incorporated into DNA strands [77].

A BCR-ABL1 KD mutation screening approach has recently been set established on the Roche GS Junior system and has proven reliability and reproducibility to detect mutations with an abundance as low as 1% [78]. In a retrospective analysis of patients who had failed multiple lines of TKI therapy, this technology showed that the BCR-ABL1 mutation status as detected by conventional methods may represent just the tip of the iceberg [78]. In 55% of the samples, mutations undetectable by direct sequencing were found by NGS. In addition, NGs revealed that samples harboring multiple mutations often represent a complex mosaic of clones with both compound and polyclonal mutations, indicating that these types of mutations are not mutually exclusive. However, the GS Junior chemistry can only allow reconstruction of clonal architecture only if multiple mutations map in a region <450 bp. The recently developed Pacific Biosciences system using Single Molecule Real-Time (SMRT®) circular consensus sequencing technology affords single-molecule, real-time DNA synthesis and provides longer reads. This eliminates potential amplification errors and allows the sequencing single strands of DNA including the entire KD of BCR-ABL1. SMRT® technology has been reported to have a detection sensitivity of less than 0.1% [79].

6. Conclusion

Based on the literature reviewed, a current unmet medical need in CML treatment is the detection of evolving multidrug-resistant compound mutations, which may present formidable challenges for targeted agents. Although early clinical data from randomized studies comparing nilotinib or dasatinib with imatinib in newly diagnosed CP-CML patients show superior activity for second-generation TKIs, the failure rate is not zero; whether the third-generation “pan-BCR-ABL1 inhibitor” ponatinib is capable of further minimizing selection of drug-resistant mutants and preventing the selection of compound mutations, and ultimately, disease progression, remains to be seen. However, as our ability to treat drug-resistant KD mutations improves, it will also become increasingly important to gain better understanding of resistance mechanisms in the absence of KD mutations.

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Conflicts of interest statement

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