Detection of Drug-Resistant Clones in Chronic Myelogenous Leukemia Patients during Dasatinib and Nilotinib Treatment

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BACKGROUND: Imatinib effectively inhibits the tyrosine kinase activity conferred by the BCR-ABL gene [fusion gene of BCR (breakpoint cluster region) and ABL1 (c-abl oncogene 1, receptor tyrosine kinase)] and thereby appreciably improves outcomes for chronic myelogenous leukemia (CML). A small percentage of patients relapse because of the proliferation of escape clones; such relapses can be treated with second-generation drugs. Early detection and monitoring of resistant clones may provide clinical benefit. We describe the development and testing of a new approach for quantitative monitoring of CML resistance.

METHODS: We designed mutation-specific assays that use hydrolysis probes and an array of allele-specific primers containing nucleotides mismatched at various positions. All assays were tested with plasmids containing corresponding mutant or wild-type sequences, allowing identification of optimal assays for specific and effective amplification of the target template. Clinical samples were then used to compare the results of selected assays with those of standard genotyping.

RESULTS: We used a modified amplification refractory mutational system approach and testing with plasmid constructs to design assays that allowed highly selective detection of resistance for all target mutations. By taking advantage of single-step performance and high PCR efficiency, we were able to quantitatively track the absolute amount of resistance conferred by a specific mutation over 4 orders of magnitude. Moreover, we designed an integrated test for dasatinib resistance that uses multiple primers simultaneously.

CONCLUSIONS: These single-step, closed-tube assays specifically target mutations associated with resistance to dasatinib or nilotinib. Compared with standard genotyping, such biased genotyping improves the detection of resistance or alternative features via quantitative analysis of the absolute amount of resistance.

Imatinib therapy has improved outcomes for patients with chronic myelogenous leukemia (CML) (1). A small percentage of patients, however, relapse after an initial response, mainly because of the selection of cells harboring kinase domain mutations (2). Consequently, research into treatments for relapsed patients has led to the development of novel drugs. Two of these drugs, dasatinib and nilotinib, are second-generation tyrosine kinase inhibitor drugs (TKIs) that have recently been registered for clinical use. Both drugs are apparently less prone to the development of resistance (3–5). In addition, the development of second-generation TKIs has been accompanied by the development of novel assays for detecting CML resistance (6–13). We describe a novel approach for the quantitative analysis of drug-resistant clones associated with treatment by second-generation TKIs.

Nine mutations associated with the substitution of 6 amino acid residues [M244V (ATG–GTG), V299L (GTG–CTG, GTG–TTG), F311I (TTC–ATC), F317L (TTC–TTG, TTC–TTA, TTC–CTC), E355G (ATG–GTG), and F359V (TTC–GTG)] are highly relevant with respect to resistance to second-generation TKIs (3–5, 14, 15); however, they were not part of our previous imatinib mutation panel (10) and therefore were also targeted in this study. RNA was extracted from peripheral blood and subsequently reverse-transcribed to cDNA as previously described (16). Standard quantitative analyses of BCR-ABL [fusion gene of BCR (breakpoint cluster region) and ABL1 (c-abl oncogene 1, receptor tyrosine kinase)] and the total number of ABL transcripts were performed as previously described (17). Genotyping was conducted by denaturing HPLC (D-HPLC) in combination with direct sequencing (D-HPLC/DS) of the entire BCR-ABL kinase domain (11).

We based the selectivity of our approach on the design of primers in which the sequence targeting the point mutation of interest was placed at the 3′ end. In our experience, however, manipulation of the primers has been necessary to minimize off-target amplification. For that reason, we deliberately substituted mis-

4 Nonstandard abbreviations: CML, chronic myelogenous leukemia; TKI, tyrosine kinase inhibitor drug; BCR-ABL, fusion gene of BCR and ABL1; D-HPLC, denaturing HPLC; D-HPLC/DS, D-HPLC combined with direct sequencing; ARMS, amplification refractory mutational system; Cq, quantification cycle.

5 Human genes: BCR, breakpoint cluster region; ABL1, c-abl oncogene 1, receptor tyrosine kinase; GUSB, glucuronidase, beta.
matching nucleotides according to the amplification refractory mutational system (ARMS) described by Newton et al. (18). We also optimized the impact of mismatches by carefully testing our assays for target selectivity and PCR efficiency. Specifically, we designed an array of 10 primers per mutation, each of which complemented the mutation of interest at the 3’ end; the primers differed, however, in overall length and with respect to the presence of a mismatching nucleotide. To evaluate off-target amplification, we used TOPO TA Cloning kits (Invitrogen) to prepare plasmid templates containing the relevant gene fragments. Plasmids were linearized and evaluated for DNA mass by spectrophotometry (NanoDrop; Thermo Scientific). Finally, mass values were converted to copy number values as follows: copy number = [mass \times (Avogadro’s number)]/[(mean molecular weight of a base) \times (template length)], where mass is expressed in grams, Avogadro’s number is 6.0221415 \times 10^{23}, and template length is the number of nucleotides in the gene fragment. We then amplified 10^4 copies each of a matching template and a mismatching plasmid template with assays containing our test primers. We evaluated the resulting amplification curves for quantification cycle (Cq) and then calculated a ΔCq value to characterize the ability of the assay to preferentially amplify the matching plasmid template without amplifying the mismatched plasmid template (Table 1). Each real-time PCR analysis was performed on the Stratagene Mx3000 real-time PCR platform (Stratagene/Agilent Technologies) in 25-μL assay volumes containing 12.5 μL MasterMix (Eurogentec), 2.5 μL of each primer (final concentration, 2 μmol/L), 2.5 μL probes (1 μmol/L), 2 μL template (corresponding to 8 ng RNA), and 3 μL H2O. Thermal cycling conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 30 s. Cq was calculated with a fixed fluorescence threshold.

We next used cDNA samples to evaluate the degree of clinical off-target amplification. We analyzed 20 negative samples (with respect to the presence of mutations) for each assay, and we deliberately defined a threshold of significance for mutational finding as 3 cycles less than the lowest observed Cq.

To quantify each mutation, we analyzed plasmid calibrators that we had serially diluted 10-fold and evaluated the slope and y intercept in a graph of Cq vs log copy number. We then converted positive Cq values to copy number values as follows: log copy number = [Cq − (y intercept)]/slope. Next, copy number values for mutant transcripts were related to the expression of a reference gene (total ABL copy numbers) in accordance with standard procedures established for monitoring of BCR-ABL transcript number (17). Our end point characterizes the resistance burden conferred by a specific mutation and might be compared directly with the leukemic burden assessed with the BCR-ABL ratio.

We were able to design assays for all target mutations that amplify almost exclusively transcripts harboring the mutations of interest (Table 1). The ΔCq values, a measure of the degree of selectivity, differed among the individual mutations. These differences reflected the varying annealing conditions, but Cq values

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**Table 1. Primer, ΔCq, and increase in target affinity for specific targets.**

<table>
<thead>
<tr>
<th>Target</th>
<th>Discriminative primer (5’→3’)a</th>
<th>Increase of target affinity by ARMS</th>
<th>Preclinical evaluation</th>
<th>y Intercept, Cq</th>
</tr>
</thead>
<tbody>
<tr>
<td>M244V (GTG)</td>
<td>GTAGTGCTGGCTGTTCAC</td>
<td>&gt;100</td>
<td>-3.41</td>
<td>95</td>
</tr>
<tr>
<td>V299L (CTG)</td>
<td>CAGACCCCAAGAGCTACAGb</td>
<td>&gt;100</td>
<td>-3.52</td>
<td>92</td>
</tr>
<tr>
<td>V299L (TTG)</td>
<td>AGACCCCAAGAGCTACAC</td>
<td>&gt;100</td>
<td>-3.49</td>
<td>94</td>
</tr>
<tr>
<td>F311I (ATC)</td>
<td>GAACGTAGATGATGATGATGATGAT</td>
<td>9.1</td>
<td>10–100</td>
<td>90</td>
</tr>
<tr>
<td>F317L (ATG)</td>
<td>AGAGGTCCCTGGAGTTATAC</td>
<td>14.5</td>
<td>&gt;100</td>
<td>3.31</td>
</tr>
<tr>
<td>F317L (ATG)</td>
<td>AGAGGTCCCTGGAGTTATAC</td>
<td>14.5</td>
<td>&gt;100</td>
<td>3.34</td>
</tr>
<tr>
<td>F317L (CTC)</td>
<td>GTTCCTGAGTCGTGAC</td>
<td>14.3</td>
<td>&gt;100</td>
<td>3.16</td>
</tr>
<tr>
<td>E355G (GTG)</td>
<td>TGTGATGAAGGTTTTTTCTTG</td>
<td>13.7</td>
<td>&gt;100</td>
<td>3.45</td>
</tr>
<tr>
<td>F359V (GTC)</td>
<td>AGACGGCTCTGTGAGA</td>
<td>8.9</td>
<td>10–100</td>
<td>3.45</td>
</tr>
</tbody>
</table>

a Mutation-specific primers were manipulated by including the mismatching nucleotides shown in boldface.

b Used in combination with forward primer (5’-AGGTCAGGAGAGGTGTG-3’) and probe (5’-FAM-TGATGCTCCTCTAGCTACAG-TAMRA-3’). FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

c Used in combination with forward primer (5’-TGCAGTCATGAAAGATCAAA-3’) and probe (5’-FAM-ACCCCTAAGCTATGCGAAGCTC-TAMRA-3’).

d Used in combination with forward primer (5’-GAACCTGATTCTGTGACTG-3’) and probe (5’-FAM-TACATGGCCTAGATCTCGTA-TAMRA-3’).
exceeded 9.9 cycles in most cases. Thus, the primers used in the study bind to template with the target mutation at least 1000 times more frequently than to off-target templates. Moreover, because our experience has shown that implementation of matching nucleotides is instrumental to the success of this approach, we also showed that mismatches increase the preferential amplification of the mutated allele by a mean factor of 100 ($\Delta C_q > 6.6$) compared with otherwise identical primers without any mismatches (increase of target affinity by ARMS, Table 1). Furthermore, by focusing on quantifying resistance, we tried to avoid affecting PCR efficiency for the sake of increased primer selectivity. The preferential use of “weak” mismatches in the study (A vs C or T vs G at a distance of 3 or 4 nucleotides from the 3′ end) did not compromise PCR efficiency, as is shown in the analysis of calibration curves of the 10-fold serially diluted plasmid solutions (slope and PCR efficiency values in Table 1). Consequently, the assays listed in Table 1 were highly qualified for detecting and quantitatively monitoring mutant clones vs a stable reference gene, such as total $ABL$ or $GUSB$ (glucuronidase, beta), in a single-step procedure. We have previously shown that similar assays can reach a detection level of 0.1% (10, 19). Plasmid-dilution experiments for our novel assays yielded similar levels of detection (data not shown).

For the purpose of clinical evaluation of our assay, we retrospectively analyzed cDNA samples derived from samples obtained from 23 CML patients at the time of clinical resistance to imatinib (baseline) and after 12 months of second-generation TKI treatment (follow-up). Patients were included in the clinical trials NCT00109707, NCT00384228, NCT00101660, NCT00101647, and NCT00101816 (http://clinicaltrials.gov), and the patients’ characteristics have previously been described (20). Samples were subjected to D-HPLC/DS and ARMS assays that targeted 17 mutations (see Table 1 in the Data Supplement that accompanies the online version of this Brief Communication at http://www.clinchem.org/content/vol56/issue3). We detected 45 mutations by D-HPLC/DS, 27 at baseline and 18 at follow-up. Of these 45 mutations, 36 (80%) were part of the ARMS panel, and all of them were confirmed by ARMS. An additional 23 mutations were positive by ARMS only (in boldface in Table 1 of the online Data Supplement). The median resistance burden measured by quantitative ARMS of D-HPLC/DS–negative and ARMS-positive mutations was markedly lower than that of D-HPLC/DS–positive and ARMS-positive mutations [median, 0.6 (range, 0.01–26.1) vs 25.5 (range, 1.25–243)], indicating that the observed increased rate of finding mutations with ARMS compared with D-HPLC/DS was due to the ability of ARMS to detect mutations at lower levels. In addition, the fact that the majority of D-HPLC/DS–negative and ARMS-positive mutations were detected by D-HPLC/DS at baseline (for divergent results at follow-up) or at follow-up (for divergent results at baseline) indicates the high specificity of the ARMS method.

To evaluate the quantitative changes induced by second-generation TKIs, we used our novel assays to assess the kinetics of mutated cell subsets (Fig. 1). We were able to detect measurable amounts of 17 mutations at both baseline and follow-up. We were also able to track mutations over a concentration range of almost 4 orders of magnitude, thereby permitting the monitoring of mutated clones from a low concentration to concentrations in which the majority of cells are $BCR-ABL$ positive. Alternatively, we were able to track mutated clones that dominate the sample at baseline to levels at follow-up that represent only a minor fraction.
of the population. In 3 samples, mutated BCR-ABL clones persisted throughout the treatment period.

Mutations associated with resistance to dasatinib are located in a gene cluster sufficiently small to be within the range of a single PCR reaction. We therefore set out to evaluate whether multiple PCR assays designed to detect dasatinib resistance could be used together in a single well. For this purpose, we mixed 6 mutation-specific primers for the detection of V299L, T315I, and F317L, together with 1 forward primer and 1 probe flanking the gatekeeper cluster. This combination permitted the amplification of a template spanning 212 bp. We subjected 23 clinical follow-up samples to a multiplexed amplification procedure that was the same as the standard protocol described above except for a final volume of 50 μL to accommodate the simultaneous use of 7 primers. Notably, all mutations covered by the assay and detected by D-HPLC/DS were confirmed with the integrated ARMS assay (see Table 1 in the online Data Supplement), demonstrating that biased mutation-specific PCR assays optimized according to the ARMS principle can even be used in a multiplex setting.

Our single-step real-time assays for monitoring of CML resistance are based on a novel use of the ARMS principle (19). Our approach clearly differs from the previously described standard genotyping approaches for CML with respect to the following unique features: (a) The assay obtains its high selectivity for the mutated allele without repetitive preamplification steps, thereby allowing true single-step, closed-tube performance; (b) the assays are performed on a widely available platform used for standard CML monitoring, thereby allowing screens for key resistance mutations to be performed in real time or according to the time frame of BCR-ABL transcript quantification; and (c) although selectivity is obtained by manipulating primer design, PCR efficiency is largely unaffected, meaning that results can be related directly to other highly effective PCR assays, as, for example, in the analysis of reference genes. Thus, this feature enables a quantitative analysis of the absolute amount of resistance burden conferred by a specific cell subset, rather than an analysis of the resistant subpopulation in relation to the total tumor burden. The latter approach represents the current prevailing method of quantification (9, 12, 13). Absolute measures, however, have advantages because leukemic burden is a fluctuating quantity. For example, a 10% resistance value in a sample with a 3-log reduction in leukemic burden represents a situation very different from that of a 10% resistance in a sample drawn at diagnosis or relapse, which consists of a very high proportion of BCR-ABL–positive cells.

The major disadvantage of our approach is that we find only the mutations we look for (i.e., the method cannot detect novel mutations). After several years of intensive translational research, however, we have acquired intimate knowledge regarding which mutations induce resistance to the available drugs, meaning that the panel of resistance mutations is not likely to increase. Therefore, it appears feasible to reduce future genotyping efforts to a restricted number of candidate mutations that confer resistance to a specific drug (e.g., so that a panel of assays can be created specifically for monitoring nilotinib or dasatinib resistance). Moreover, results of a previous study with 200 blinded samples showed that ARMS results are closely comparable with those of another technique (ligation PCR) (20). ARMS increased the number of detected mutations and indicated a high prevalence of low-level mutations. It is important to mention, however, that the clinical significance of low-level mutations is still unknown and needs further investigation. Furthermore, the detection of mutations is still experimental and is not recommended for routine use.