The role of serial *BCR-ABL* transcript monitoring in predicting the emergence of *BCR-ABL* kinase mutations in imatinib-treated patients with chronic myeloid leukemia

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BCR-ABL kinase mutations may confer resistance to imatinib in patients with chronic myeloid leukemia (CML), and may predict a poor outcome. We investigated whether rises in *BCR-ABL* transcript levels predicted mutation development in 82 CML patients receiving imatinib. Eleven mutations were detected in 10 patients. A single 2-fold or greater rise in *BCR-ABL* transcript did not predict mutations. However, a mutation was detectable in five of six cases with progressively rising levels of transcripts. In contrast, consecutive rises were not seen in any of 33 stable responders. Rising *BCR-ABL* transcript levels can identify patients who develop *BCR-ABL* mutations. A serial rise is more reliable than a single rise.

Key words: leukemia, CML, *BCR-ABL, BCR-ABL* kinase mutations, imatinib mesylate.

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Imatinib is now widely used for the treatment of chronic myeloid leukemia (CML). However, as clinical experience grows, it is becoming clear that some patients may not respond, and additional patients may lose an initial cytogenetic or hematologic response. Several mechanisms of resistance to imatinib have now been described. The most important in clinical samples appears to be the development of mutations in the kinase domain of *BCR-ABL.* Patients with mutations in the adenosine triphosphate (ATP) binding loop (P-loop) have a significantly worse survival than those with mutations outside the P-loop.[1,2] These data suggest that knowing the *BCR-ABL* kinase mutation status may be clinically useful, since it may prompt a change in therapy. However, routine screening of patients' samples for *BCR-ABL* kinase mutations is laborious and expensive, and it would be helpful to have simpler techniques for screening patients at high risk of emerging mutations. We used LightCycler technology for serial quantitative real time polymerase chain reaction (QRT-PCR) assessment of BCR-ABL transcript levels, and direct sequencing of the *BCR-ABL* kinase domain to detect mutations.

Design and Methods

All 82 patients commencing imatinib at our center between October 2000 and May 2004 were included in this study. The median age of these patients was 55 years. There were 58 patients in first chronic phase, 18 in accelerated phase, and six in blast crisis. Accelerated phase and blast crisis were defined by conventional hematologic and morphological criteria.[3] Twenty-three of the patients received imatinib as their first definitive CML therapy, whereas 59 were prescribed imatinib after other CML therapy had failed (including transplantation in six cases. All patients received imatinib at a stable dose of 400mg (chronic phase) or 600 mg (accelerated phase and blast crisis) daily. Cytogenetic responses were assessed by standard marrow metaphase cytogenetics on at least 20 metaphases. In occasional cases previously in complete cytogenetic remission (CCR), interphase fluorescent *in situ* hybridization was used to confirm ongoing CCR, using standard techniques. Cytogenetic responses were defined conventionally as: complete (absence of Philadelphia chromosome positive (Ph+) cells); partial (1-34% Ph⁺ cells) or nonresponse (persistence of at least 35% Ph+ cells). Disease progression was defined as present if a patient in chronic phase progressed to accelerated phase or blast crisis, or if a patient in accelerated phase developed a blast crisis.

Peripheral blood white cells were collected at the commencement of imatinib therapy, and at 3-monthly intervals there after. RNA extraction, cDNA synthesis and QRT-PCR for measuring *BCR-ABL* transcripts were performed as previously described.[4] For sequencing, nested PCR was used to amplify the complete *BCR-ABL* kinase domain. The first round PCR primers amplified only intact *BCR-ABL,* and were located at *BCR* gene exon 13 (B2A 5'TTCAGAAGC-TTCTCCCTGACAT3') and *ABL* gene exon 10 (A10R1 5'TGAGGCATCTCAGGCACG-TC 3'). The amplicon size for b3a2 *BCR-ABL* transcripts is 1,754 base pairs. In the first round, 1 μL of cDNA was used with 35 cycles each consisting of denaturation at

Table 1. Details of the *BCR-ABL* kinase domain mutations. Two mutations were detected in patient #8.

Case number	Mutation	Codon	Location	Start of treatment	Response to imatinib	Current status
1	TAC→CAC	Y253H	P-loop	CP	CCR, relapse	Died in blast crisis
2	TTC→ATC	F359I	Active site	CP	PR, relapse	Molecular remission post allo-BMT
3	TTG→ATG	L387M	A-loop	AP	CCR, relapse	Died during allo-BMT
4	CTG→ATG	L384M	A-loop	AP	CCR, relapse	Died of blast crisis
5	GAC→GGC	D276G	C-helix	CP	CCR, relapse	Stopped imatinib; currently well in chronic phase
6	CTG→TTG	T315I	Active site	BC	NR, blast crisis	Died
7	TTC→GTC	F359V	Active site	AP	NR, blast crisis	Died
8	GAG→GTG	E255V	P-loop	AP	NR, accelerating	Died in blast crisis
8	GTC→CCT	A397P	A-loop			
9	CTG→TTG	T315I	Active site	BC	CCR, relapse	Died
10	GGG→GAG	G250E	P-loop	BC	NR, blast crisis	Died

CCR: complete cytogenetic responder; PR: partial responder; NR: cytogenetic non-responder; relapse: loss of an initial cytogenetic response. BC: blast crisis; CP: chronic phase; AP: accelerated phase; BMT: bone marrow transplantation.

95°C for 30 seconds, annealing at 62°C for 30 seconds, and extension at 72°C for 2 minutes. In the second round, 1 µL of a 1 in 100 dilution of first round PCR product was used with primers spanning the *ABL* kinase domain. The PCR conditions were 32 cycles of denaturation at 95°C for 30 seconds, annealing at 62°C for 30 seconds, and extension at 72°C for 90 seconds. The second round PCR primers were located at *ABL* gene exon 4 (A4F 5'CCAAAGCGCAACAAGCCCAC3') and *ABL* gene exon 10 (A10R2 5'ACAGCCCCACGGACGC-CTTG 3') to cover the whole *BCR-ABL* kinase domain. The resultant PCR product was run on 1% agarose gel and the expected 905 bp product was purified by using a GenEluteTM Agarose Spin Column (Sigma, Poole, Dorset, UK). This fragment was directly sequenced using A4F and A10R2 (Lark Technologies Inc. Essex, UK). Only sequence results confirmed by both primers were accepted. Mutation analysis was performed on the latest samples from all 82 patients. If a mutation was identified, serial previous samples from that patient were analyzed for that mutation. In addition, samples that showed, in QRT-PCR, a 2-fold or greater increase of *BCR-ABL* transcript compared to the previous sample were also analyzed for *BCR-ABL* kinase mutations, irrespectively of whether a mutation was detected in the latest available sample. In total, 114 individual samples were analyzed.

Results and Discussion

All 82 patients achieved a complete hematologic response (normal blood count and disappearance of splenomegaly) within 3 months of commencing imatinib. By 6 months of treatment, 38 had achieved CCR, 5 a partial response, and 39 were cytogenetic non-responders. At the latest follow-up, 33 of the 38 patients who had achieved a CCR remain stable and five have undergone disease progression. The response was lost in one of the five partial responders. None of the cytogenetic non-responders at 6 months has subsequently had a cytogenetic response, and 21 have undergone disease progression. Overall, 19 patients have died (18 due

to disease progression), and three have undergone allogeneic transplantation. The median follow-up for the remainder was 15 (range 6-41) months. Overall, 11 mutations were detected in ten patients (Table 1). One of these 11 mutations, L384M, has not been previously described. Ten (37%) of 27 patients with disease progression after an initial response had mutations. Mutations were detected in all six cases that lost an earlier CCR (5 patients) or partial response (1 patient). Mutations were detected in four of 39 cytogenetic non-responders, including two in blast crisis and two in accelerated phase. The mutation rate in cytogenetic non-responders whose disease progressed after an initial hematologic response was 19% (4/21). No mutations were detected in any of 33 patients who remained in CCR at their latest study point. Mutations were detected in all six patients who achieved a major cytogenetic response (5 CCR; 1 partial response) that was subsequently lost. Details are given in Figure 1. Samples were available at 3-monthly or more frequent intervals in five of these six cases, and their mutations were first detected between 7 and 18 months from the start of imatinib therapy. All six cases underwent disease progression (3 to accelerated phase and 3 to blast crisis). In five of these six, the mutations were first detectable 2-22 months before disease progression (Figure 1). The sensitivity of LightCycler and nested PCR was assessed by serial log dilutions of K562 cells by the *BCR-ABL* negative human cell line CV727. The limit of LightCycler PCR detection was 1 K562 cell in 10^6 CV727 cells, and for nested PCR was 1 K562 cell in 10^7 CV727 (*data not shown*). The sensitivity of mutation analysis was evaluated by diluting mutated *BCR-ABL* cDNA transcripts with unmutated transcripts, at ratios of 50% mutant: 50% unmutated, 25%:75%, 20%:80%, 15%:85%, 10%:90%, 5%:95% and 1%:99%. Mutant transcripts were reliably detected at a dilution of 10%:90%, but not invariably at 5%:95% or 1%:99%. Our assay can therefore reliably detect mutant transcripts when they constitute 5-10% of the total *BCR-ABL* transcripts. The reproducibility of the LightCycler PCR assay was investigated on samples containing high, intermediate and low levels of *BCR-ABL* tran-
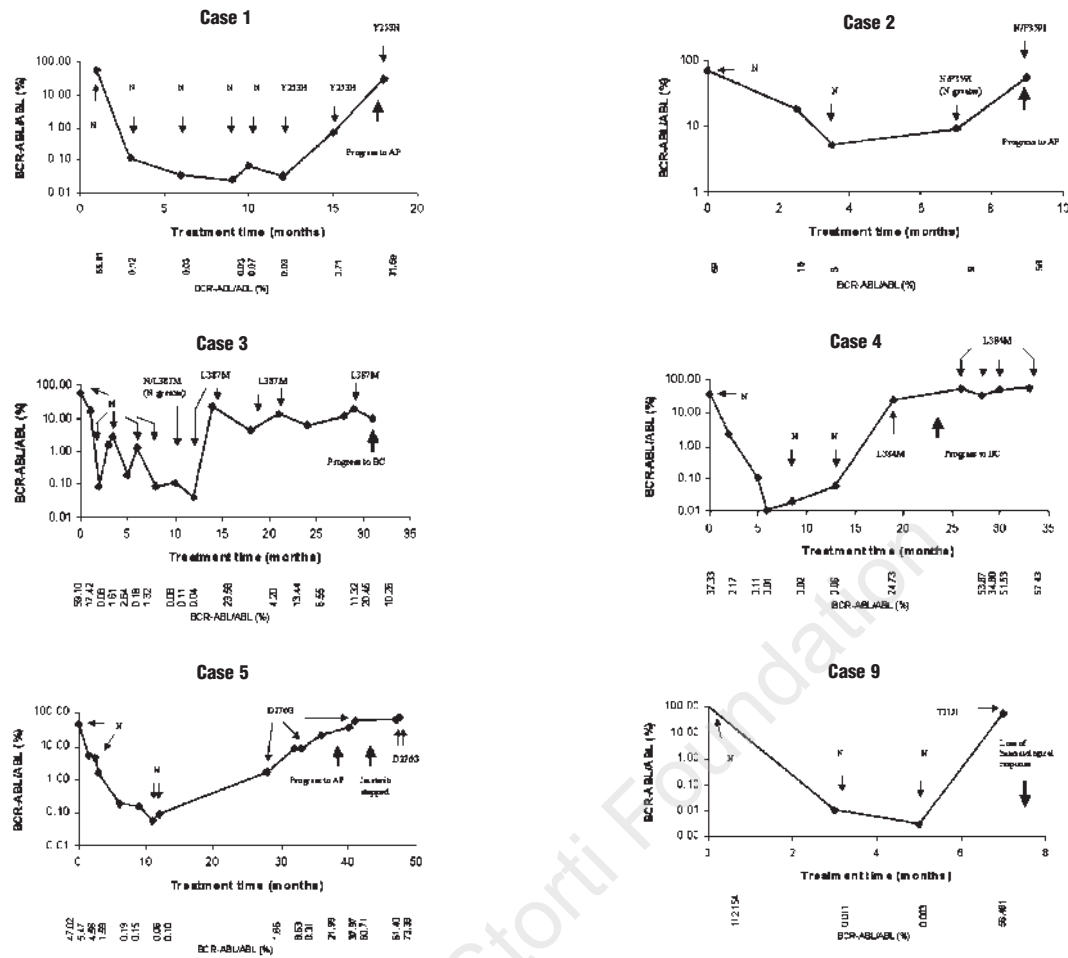
Figure 1. Pattern of *BCR-ABL* transcripts in the six patients with mutations, who lost an earlier cytogenetic response. The x-axis denotes duration of imatinib therapy. All patients received imatinib throughout, except patient n. 5 as annotated (*see text*). Arrows indicate the points at which mutation analysis was carried out; N: wild type sequence only.
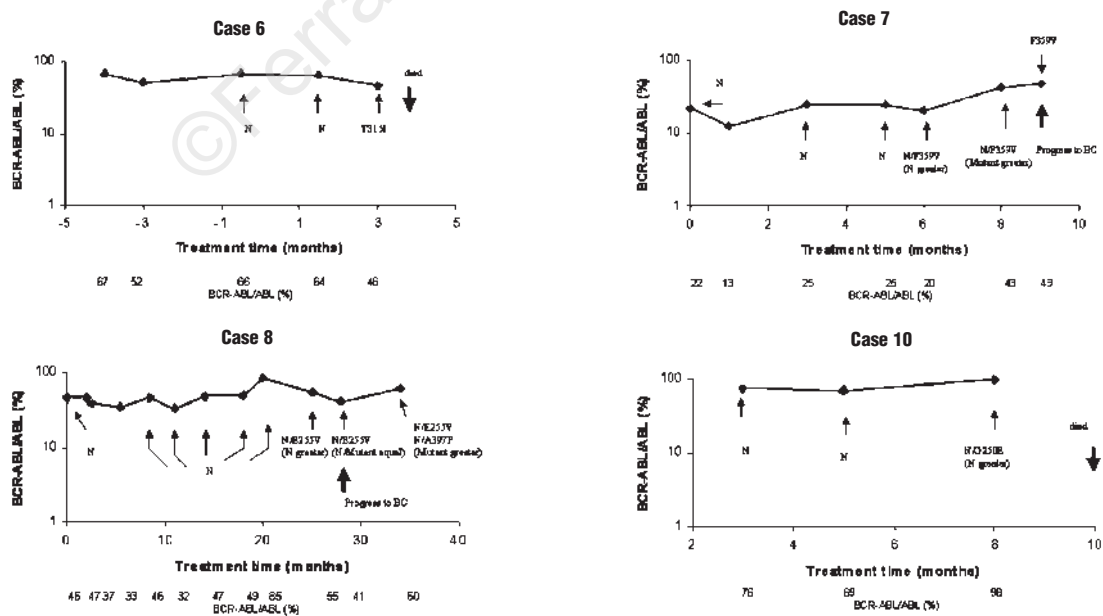

Figure 2. Pattern of *BCR-ABL* transcripts in the four patients who did not achieve a cytogenetic response to imatinib. Annotations are as for Figure 1.

scripts. Ten replicates were carried out for each. The coefficient of variation (CV) for the sample containing a higt level of BCR-ABL sample was 8% (mean BCR-ABL ± 2 SD = 66.71 to 90.36%). For the intermediate level the CV was 13% (mean±2 SD=2.68 to 4.64%), and for the low level, the CV was 17% (mean±2 SD=0.012 to 0.025%). The CV is therefore greatest at low *BCR-ABL* transcript levels. At this level, a CV of 17 % means that 95% of the replicate observations will lie within 2 SD of the mean, i.e. between 66% and 134% of the mean. This is approximately a 2-fold variation. Thus, an apparent 2-fold (or greater) rise in *BCR-ABL* transcripts has a less than 5% probability of being due to the variability of the assay. We therefore further explored the relevance of a two-fold or greater rise in transcript levels for predicting *BCR-ABL* kinase mutations.

All samples in which *BCR-ABL* transcripts had risen by at least 2-fold (compared with a sample 1-6 months earlier) were studied for *BCR-ABL* kinase mutations. Thirteen of 33 patients who remained in CCR throughout had a single transcript rise of at least 2-fold. No mutations were detected in any of these 13 CCR samples, nor in the most recent sample available for each case. Similarly, no mutations were detected among any of ten cytogenetic non-responding patients who showed a single rise of at least 2-fold. Conversely, only two of ten assessable cases in which a mutation was ultimately detected had their mutation detectable for the first time at the time of a 2-fold rise. A single 2-fold or greater transcript rise therefore appears a poor predictor of mutation evolution. In contrast, a *BCR-ABL* kinase mutation was detectable in five of six major cytogenetic responders who showed a *consecutive* transcript rise of at least 2-fold on at least two serial occasions. This contrasts with the absence of consecutive rises in any of 33 patients who remained in stable CCR at their latest follow up. However, the data in Figure 1 show that the mutations were detectable earlier than the transcript rise in three of the five assessable patients. A serial rise of greater than 2-fold over at least two consecutive assessments may therefore be a better predictor of mutation detection than a single rise. However, a mutation is typically present several months before the upward trend in *BCR-ABL* levels. Here, we report that routine QRT-PCR monitoring can be used to identify patients with a high probability of developing *BCR-ABL* kinase mutations. We have included all our imatinib-treated patients over a 42-month period. This absence of selection may explain our apparently lower incidence of mutations. We also demonstrated the reproducibility of our LightCycler assay, by showing acceptable CV values at various levels of BCR-ABL expression. Branford and colleagues reported that 61% of patients with a single rise of at least 2-fold in the BCR-ABL mRNA level had detectable mutations. This contrasted with only one mutation among 158 cases with stable or decreasing levels of *BCR-ABL* transcripts. A single 2-fold change in *BCR-ABL* expression was reliably detected at the level of a 3-log reduction in transcript from baseline, but not at levels below this.[5] We confirm two findings

of Branford and colleagues.[5] Firstly, we report ten further examples of previously reported mutations, and one mutation (L384M) that has not previously been reported in clinical samples. BaF3 cells transfected with *BCR-ABL* containing L384M had enhanced growth in the presence of 5 µM of imatinib.[6] Secondly, mutations were mostly seen in patients with secondary acquired rather than primary intrinsic imatinib resistance. Patients with mutations had inferior progression-free and overall survival compared with unmutated cases (*data not shown*).

Although we also confirmed that rising levels of *BCR-ABL* transcripts are predictive of a mutation, our data differ from those of Branford and colleagues[5] in that we found that a single 2-fold or greater rise was a poor predictor of mutation detection. However, among 39 patients in CCR and five patients in partial response, five of the six patients with serial rises in transcripts had a mutation, compared with no mutations in any of the 33 patients with a CCR and four with a partial response without serial rises. Next, we examined the temporal relationship between the initial detection of a mutation, the serial rise in transcript levels, and the time of overt disease progression. Although a rise in transcript levels preceded disease progression, a *BCR-ABL* mutation had typically been present for several months before *BCR-ABL* transcripts began to rise. Waiting for serial rises in transcripts may, therefore, delay the detection of emerging mutations.

Several techniques may have greater sensitivity than direct sequencing for detecting specific *BCR-ABL* kinase mutations,[7,8,9] but these are too laborious for routine screening, and the exact mutations and their locations may still need confirmation by direct sequencing. We, therefore, opted here for direct sequencing, since although this is less sensitive, it can detect single or multiple mutations, and is readily applicable for general testing. We acknowledge that it may miss infrequent clones, and also small clones of potential clinical significance. Direct sequencing may, therefore, delay the detection of mutations in comparison with more sensitive techniques.

In conclusion, we confirm that serial monitoring of *BCR-ABL* transcripts may be useful for predicting the development of *BCR-ABL* kinase mutations in imatinib-treated patients with CML. A serial rise in transcript levels may be a more reliable predictor than a single rise. However, the levels of transcript rise relatively late in the emergence of a mutant clone, and reliance on rising transcript levels may delay mutation detection in comparison with direct PCR product sequencing or more sensitive molecular screening techniques for specific mutations. It is important to test whether serial monitoring of transcript levels may lead to mutation detection early enough for treatment modification to be effective. Large longitudinal studies, pegged to standardized treatment schedules, are required to optimize the early detection of clinically relevant BCR-ABL kinase domain mutations in imatinib-treated patients with CML.

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