

## Clinical Significance of ABL Kinase Domain Mutation in Chronic Myeloid Leukemia under Imatinib Therapy

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**Abstract:** Background: Despite durable responses to imatinib in chronic myeloid leukaemia (CML), mutations in Bcr-Abl kinase domain (KD) are known to induce imatinib resistance and cause poor clinical outcome. Methods: We characterized Bcr-Abl KD mutations in 20 Egyptian CML patients with imatinib resistance (n = 12) or responsive (n = 8) using allele specific oligonucleotide polymerase chain reaction (PCR) and direct sequencing. The frequency of mutations in patients with increasing *BCR-ABL* transcript levels, and those with stable or decreasing levels was determined using allele specific oligonucleotide - polymerase chain reaction (ASO-PCR). Results: Six out of the twelve patients with primary or secondary resistance had detectable mutations, whereas none of the eight responders who achieved suboptimal, complete or major response had any detectable mutation. The presence of a mutation was significantly associated with a greater likelihood of subsequent progression to accelerated phase / blast crisis and shorter survival. Patients harboring P-loop mutations showed poor overall survival compared with patients harboring non-P-loop mutations (93% vs 67%  $P=0.001$ ). Conclusion: These data suggest that a rise in *BCR-ABL* transcript levels of > 2-fold can be used as a primary indicator to test patients for *BCR-ABL* kinase domain mutations and that ASO-PCR is a valuable tool allowing a timely detection of mutations. Moreover, early detection of *BCR-ABL* mutations may play a role in identifying patients who are likely to become resistant to imatinib therapy, for which alternative therapeutic options should be considered.

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### 1- Introduction

Chronic myeloid leukemia (CML) is a hematopoietic malignancy associated with the translocation t(9;22)(q34;q11), resulting in the Philadelphia chromosome (Ph) and the presence of the constitutively activated tyrosine kinase BCR-ABL.[1]

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 [2].

Five BCR-ABL1 tyrosine kinase inhibitors (TKIs)—imatinib [3], dasatinib [4], nilotinib [5], bosutinib [6], and ponatinib [7]—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 [8]. Although BCR-ABL1 TKIs have not been formally compared with omacetaxine, TKIs are generally the preferred option [9].

Despite the efficacy of imatinib in patients with Ph-positive (Ph1) CML in chronic phase (CP), approximately 15% of patients display resistance to imatinib [10,11]. 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 [12].

Several mechanisms have been associated with resistance, including BCR-ABL1-dependent and BCR-ABL1-independent mechanisms [13]. BCR-ABL1 kinase domain (KD) mutations and BCR-ABL1 genomic amplification are the best-characterized mechanisms conferring resistance to TKI therapy [14]. Mutation analysis is usually performed after patients experience TKI treatment resistance; the results of mutation analysis may guide the selection of subsequent TKIs [2, 15-17].

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. The most common mutations with imatinib were T315I, G250E, M244V, M351T, and E255K/V. In most studies, more patients with secondary resistance developed mutations during imatinib treatment [9].

## 2- Patients and methods

### Study group

Twenty newly diagnosed adult patients with Philadelphia-positive (Ph<sup>+</sup>) chronic phase - chronic myeloid leukemia (CP-CML); 12 males and 8 females were enrolled in this study and treated at Tanta university hospital from January 2011 to september 2012. Diagnosis of all patients was based on standard clinical criteria including peripheral blood and bone marrow smears examination, karyotyping to detect the presence of Philadelphia chromosome and polymerase chain reaction to detect the presence of *BCR-ABL* fusion gene. Inclusion criteria were (1) morphologic and cytogenetic evidence of Ph<sup>+</sup>- CML in early chronic phase (defined as < 12 months from diagnosis), chronic phase was defined as less than 10% blasts in peripheral blood or bone marrow, less than 20% blasts plus promyelocytes in the peripheral blood or bone marrow, less than 20% basophils, and no extramedullary involvement; (2) age 18 years or older; (3) normal renal, hepatic and cardiac function. Women of childbearing age were required to have a negative pregnancy test before starting imatinib, and all patients at risk were required to use contraception during therapy. Exclusion criteria included previous treatment for CML (busulfan, IFN- $\alpha$ , or Ara-C). CML therapies other than imatinib were prohibited on-study. Exceptions included hydroxyurea for the treatment of elevated WBC count ( $>50 \times 10^9/L$ ); usage was limited to 2 weeks.

### Study design

Patients were treated within an international Novartis-sponsored protocol. Imatinib mesylate (formerly STI-571; Glivec in Europe, Gleevec in the United States; Novartis Pharmaceuticals, Basel, Switzerland), a 2-phenylamino pyrimidine compound was kindly supplied by Novartis pharmaceuticals. Patients received 400 mg of imatinib mesylate orally once a day.

Patients were monitored by real time quantitative - polymerase chain reaction (RQ-PCR) for *BCR-ABL* transcript levels in peripheral blood at diagnosis before commencing imatinib and at 3-month intervals thereafter for a follow up period (FUP) of 36 months. Patients were categorized according to molecular response criteria into: complete molecular response, major molecular response, suboptimal responders and resistant patients. The assessment of response was according to the European Leukemia Net criteria (ELN) [17].

The mutation screening was performed in patients who showed no optimal response to imatinib (patients with evidence of suboptimal response or resistance to imatinib). To test whether kinase domain mutations may be identified in responding patients, mutation screening was also performed in

some randomly selected patients who showed optimal response to imatinib (patients who achieved a complete molecular response or a major molecular response).

### Mutation analysis

The patients were screened for *BCR-ABL* gene mutations using allele specific oligonucleotide - polymerase chain reaction (ASO-PCR). To date, more than 100 different kinase domain mutations have been identified in imatinib - resistant CML patients. For ASO-PCR, we selected 4 different mutations on the basis of their detection frequency in mutation analyses in other literature. The mutations panel that was applied in this study included Q252H (a), Q252H (b), Y253H, Y253F mutations.

### Blood sampling

Two ml of peripheral venous blood from each patient were collected under a complete aseptic condition and added to EDTA vacutainer tube for complete blood count and DNA extraction.

#### 1. DNA extraction

Genomic DNA was extracted from peripheral blood according to the initial salting-out extraction method using Genra Puregene blood kit (QIAGEN, Hilden, Germany).

#### 2. ASO-PCR assay

An allele specific oligonucleotide - polymerase chain reaction (ASO-PCR) assay was established for the detection of 4 known mutations which were selected according to their frequency in imatinib-resistant CML patients. Mutated or wild-type sequences were specifically amplified in a PCR reaction performed on DNA to analyze the most frequently identified mutations in the kinase domain of the *BCR-ABL* gene (amino acids 220 to 498). The strong specificity of the assay was demonstrated for each mutation by detection of *ABL* sequence in DNA from clinically-declared healthy controls with no Philadelphia positive leukemia.

#### 3. DNA agarose gel electrophoresis

The amplified products from ASO-PCR were detected by electrophoresis on a 2% agarose gel. The resolved DNA bands are detected by staining the gels with ethidium bromide, followed by destaining with water and finally photographed under UV illumination with a digital camera.

### Statistical analysis

All analyses were performed using the statistical package for the social sciences (SPSS software 17; SPSS Inc., Chicago, USA). The chi-square test was used to determine the significance of the difference in frequency of mutations and resistance in patients with > 2-fold rise in *BCR-ABL* transcript levels in consecutive samples. Kaplan-Meier survival analysis, was used to test for differences between patients with mutations and patients without mutations in terms of

overall survival .Overall survival is calculated from the time the treatment began until death

### 3- Results

Twenty adult patients diagnosed with Philadelphia-positive (Ph<sup>+</sup>) chronic phase-chronic myeloid leukemia (CP-CML); including 12 males (60%) and 8 females (40%) were referred to the outpatient clinics of the hematology unit, Tanta University Hospital. The median age of patients at diagnosis was 42 years (range 18-72 years). Sixteen patients including 8 patients with primary resistance, 4 patients with secondary resistance and 4 suboptimal responders were analyzed for the presence of BCR-ABL gene mutations. Moreover, four patients' including 2 patients with complete molecular response, and 2 patients with major molecular response were randomly selected and analyzed for the presence of BCR-ABL gene mutation. The mean follow up period was 31.5 months. Twelve patients developed resistance whether primary or secondary. A mutation event was associated with primary resistance in 5 out of 8 patients (62.5%) who did not demonstrate at least one log reduction in *BCR-ABL/ABL* % ratio since the start of treatment. In addition, a mutation event was associated with secondary resistance in 1 out of 4 patients (25%) who demonstrated a rise in *BCR-ABL/ABL* % ratio of at least one log or more on serial testing. In contrast, no mutations have been detected in the remaining eight patients. The patient characteristics and outcome are illustrated in tables 1,2.

Four different *BCR-ABL* kinase domain mutations were detected in 6 out of 12 imatinib-resistant CML patients (Fig1). Mutations of the P-loop (amino acids 248-255) were the most common and were detected in the six patients, including one patient with Q252Ha, two patients with Q252Hb, one patient with Y253H, two patients with Y253F. The ASO-PCR products on ethidium bromide-stained agarose gel for mutant patients are illustrated in figures2,3.

The overall survival (OS) of mutation screened patients (n=20) were analyzed. The 2 years overall survival was 93% and 67% for patient with and without mutations respectively ( $P=0.001$ ). (Figure 4).

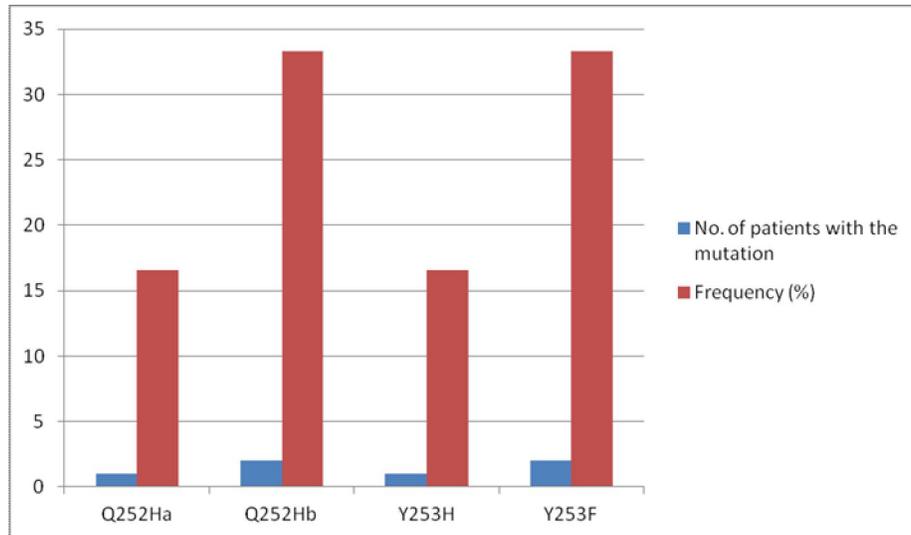
**Table 1: characteristics of imatinib-resistant patients**

	All patients	Resistant patients with mutations	Resistant patients without mutations	P value
No of patients	12	6	6	0.05>
Males	8	4	4	
Females	4	2	2	
Age (years)				0.36
Mean	45.9	48.8	43	
Median	46.5	48.5	44.5	
Range	29-65	29-65	30-52	
Duration of imatinib treatment(months)				
Median	24.5	23.5	24.5	0.64
Range	18-28	20-28	18-27	
Cytogenetic response of patients at mutation detection				
Partial cytogenetic response	2	0	2	0.29
Minor cytogenetic response	7	4	3	
No cytogenetic response	3	2	1	

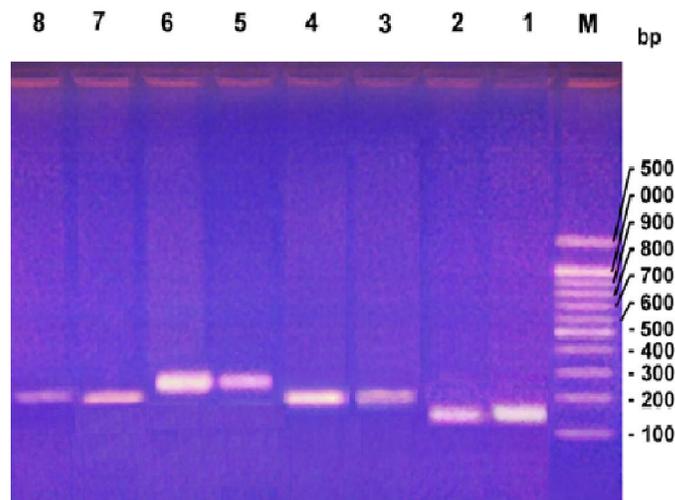
**Table 2: mutational status, and disease outcomes of imatinib-resistant patients**

UPN	Therapeutic response	Mutational status	Disease outcome
1	Primary resistance	Y253F (P)	Progression to AP, died.
2	Secondary resistance	NM	switch to SG-TKI, alive.
3	Primary resistance	Q252Hb (P)	Switch to SG-TKI, alive.
4	Secondary resistance	NM	Dose escalation, alive.
5	Primary resistance	Y253H (P)	Dose escalation, Progression to ABC, died.
6	Secondary resistance	Q252Hb (P)	Progressions to AP, dose escalation, switch to SG-TKI, alive.
7	Primary resistance	NM	Switch to SG-TKI, alive.
8	Primary resistance	NM	Progression to AP, switch to SG-TKI, alive.
9	Primary resistance	NM	Switch to SG-TKI, Progression to AP, died.
10	Primary resistance	Q252Ha (P)	Dose escalation, died.
11	Secondary resistance	NM	Dose escalation, alive.
12	Primary resistance	Y253F (P)	Progression to ABC, died.

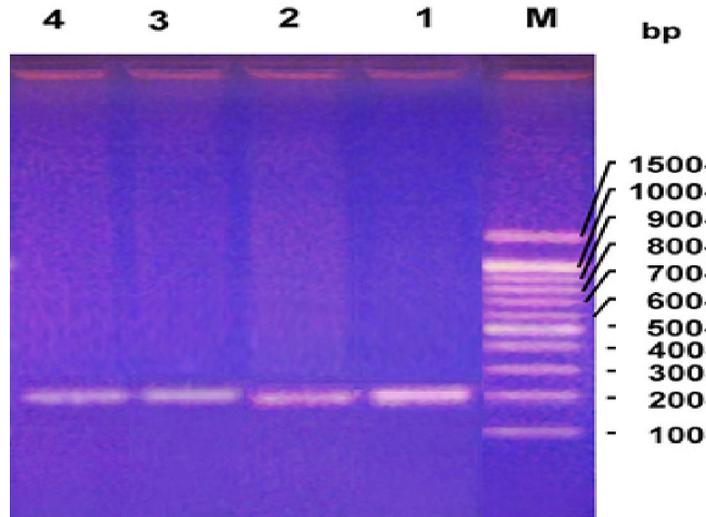
UPN, unique patient number; AP, accelerated phase; ABC, acute blast crisis; P, P-loop; SG-TKI, second generation - tyrosine kinase inhibitor; NM, no mutation.



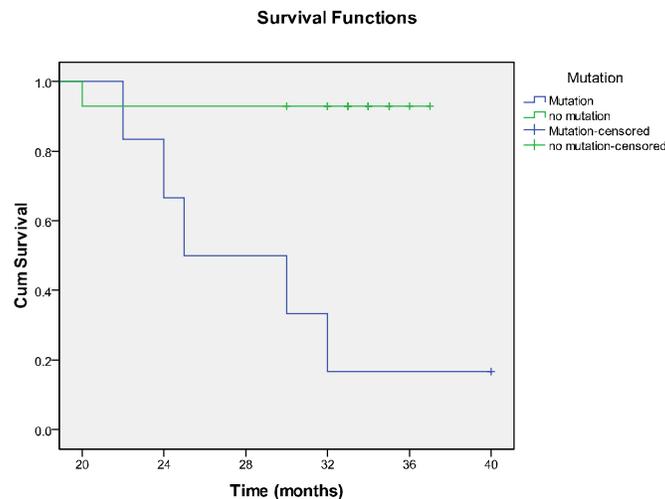
**Figure 1:** Frequency and types of *BCR-ABL* mutations in imatinib-resistant CML patients.



**Figure 2:** ASO-PCR products on ethidium bromide-stained agarose gel for patients' no.1, 2, 3 and 4. M: DNA marker (ladder; 100 bp). Lanes 1 & 2: DNA of patient no.1 amplified with wild type and mutant type primers, respectively, for Y253F mutation (198 bp). Lanes 3 & 4: DNA of patient no.3 amplified with wild type and mutant type primers, respectively, for Q252Hb mutation (200 bp). Lanes 5 & 6: DNA of patient no.5 amplified with wild type and mutant type primers, respectively, for Y253H mutation (199 bp). Lanes 7 & 8: DNA of patient no.6 amplified with wild type and mutant type primers, respectively, for Q252Hb mutation (200 bp).



**Figure 3:** ASO-PCR products on ethidium bromide-stained agarose gel for patients' no.5 and 6. M: DNA marker (ladder; 100 bp). Lanes 1 & 2: DNA of patient no.10 amplified with wild type and mutant type primers, respectively, for Q252Ha mutation (200 bp). Lanes 3 & 4: DNA of patient no.12 amplified with wild type and mutant type primers, respectively, for Y253F mutation (198 bp).



$\text{Log Rank}=11.59$   $p\text{-value}=0.001^*$

**Figure 4:** Overall survival (OS) of mutation screened patients.

#### 4- Discussion

Chronic myeloid leukemia (CML), which accounts for 15% of adult leukemias, can occur at any age, although the incidence of the disease increases with age [18]. Reciprocal translocation between chromosomes 9 and 22,  $t(9;22)(q34;q11)$  is reported to be the main factor contributing to CML. This unique chromosome arrangement, known as Philadelphia chromosome translocation, generates a BCR-ABL fusion gene that encodes a constitutively active tyrosine kinase protein. BCR-ABL fusion gene has now become the universally accepted molecular

signature and the transforming event in CML pathogenesis.

The mRNA molecules transcribed from BCRABL fusion gene usually contain one of the two BCR-ABL junctions designated as b2a2 (e13a2) and b3a2 (e14a2). However, both mRNAs translate into p210 Kda fusion protein that may up-regulate the tyrosine kinase activity. This results in transformed cells that have growth factor independent proliferation along with decreased apoptosis, defective adhesion, as well as genomic instability.[19]

Today, imatinib has become the standard of care for the treatment of CML and represents the established front-line therapy for nearly all patients with CML [18]. 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) [12]. Across the historical trials of imatinib, approximately 65–90% of chronic-phase (CP) CML patients experience CCyR at/by 12–24 months [20–25]. Resistance to imatinib treatment can also occur after patient 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 MCyR, 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 [26]. 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. The most common mutations with imatinib were T315I, G250E, M244V, M351T, and E255K/V. In most studies, more patients with secondary resistance developed mutations during imatinib

To date, the clinical impact of mutations has been assessed using low sensitivity techniques (Sanger sequencing). The presence of mutations at lower levels can be identified with more sensitive techniques, such as mass spectrometry or ultra-deep sequencing, 65, 66 but data are not yet sufficient to interpret the clinical relevance of the mutations detected by these more sensitive techniques. Mutations, which should not be confused with ABL1 polymorphisms, 67 are suggestive of genetic instability and increased risk of progression. More than 80 amino acid substitutions have been reported in association with resistance to imatinib. [15, 27]

Metaphase karyotyping may reveal additional clonal chromosomal abnormalities in Ph1 cells (CCA/Ph1), a situation referred to as clonal cytogenetic evolution. CCA/Ph1 defines TKI failure. CCA/Ph1 is associated with shorter OS on second-line imatinib (after rIFN $\alpha$  failure) but not second-line dasatinib or nilotinib. [16]. It is necessary to determine the optimal increase in *BCR-ABL* transcript levels that should trigger mutation testing [28]. The present study adds proof of the association between the rise in *BCR-ABL* transcript levels and the detection of kinase domain mutations in CML patients treated with imatinib. We chose 2-fold as our initial threshold. **Branford et al.**, [29] have previously found that a 2-fold increase in *BCR-ABL* transcript levels is predictive of mutation detection.

Twelve patients had such a rise, and 6 of these had detectable mutations. None of the 8 patients with stable or decreasing levels had any detectable mutation. Thus, there was a significantly higher incidence of mutations in those who had a more than 2-folds rise compared with those who had stable or decreasing *BCR-ABL* transcript levels. This finding indicates that screening patients for mutations when the *BCR-ABL* level remains stable or decreases may not be warranted. **Branford et al.**, reported that 61% of patients with a single rise of at least 2-fold in the *BCR-ABL* mRNA levels had detectable mutations, whereas only one mutation was detected among 158 cases with stable or decreasing *BCR-ABL* transcript levels. [29]. The present study showed that, every imatinib-treated CML patient who had a rise of > 2-fold in *BCR-ABL* transcript levels already had evidence of or subsequently developed evidence of imatinib resistance. Resistance occurred in 12/12 (100%) patients with a rise of > 2-fold in *BCR-ABL* transcript levels compared with (0%) patients with stable or decreasing *BCR-ABL* levels.

Concerning the association between the detection of *BCR-ABL* mutations and imatinib resistance, the findings of the present study revealed that virtually every CML patient treated with imatinib who had a *BCR-ABL* kinase domain mutation detected by ASO-PCR already had evidence of or subsequently developed evidence of imatinib resistance.

A mutation event was associated with primary resistance in 5/8 patients who did not demonstrate at least one log reduction in *BCR-ABL/ABL* % ratio since the start of treatment and with secondary resistance in 1/4 patients who demonstrated a rise in *BCR-ABL/ABL* % ratio of at least one log or more on serial testing. This finding indicates that *BCR-ABL* mutations were mostly seen in patients with primary intrinsic rather than secondary acquired imatinib resistance. A possible explanation is that, primary CML stem cells display instability of the *BCR-ABL* fusion gene. Thus, patients may possess leukemic stem cells with *BCR-ABL* kinase domain mutations before initiation of imatinib therapy and would likely be predisposed to development of resistance to imatinib [30]. Previously, it has been indicated that in many cases the mutant clone is present prior to imatinib therapy [31], even at diagnosis [32].

Previously, **Hochhaus et al.**, [33] reported *BCR-ABL* kinase domain mutations at a lower frequency (2 of 44 patients with relapsed or refractory disease). The differences in the frequency of mutation detection may be attributed to differences in the sensitivities of the techniques, differences in the time point of analysis, or differences between the patient populations in the studies. It might also be

due to a difference in the portion of patients in advanced phases. The portion of patients in advanced phase (including AP and BC) was between 21 and 33% of all patients in the previous studies while this portion in the present study was 6/12; including 4/12 in AP and 2/12 in BC. Therefore, when we consider the frequency of mutation to be higher in advanced phases than in CP, relatively large portion of patients in the advanced phases brought higher frequency of mutation in comparison with other studies. The Gruppo Italiano Malattie Ematologiche dell'Adulto (GIMEMA) co-operative group in Italy reported that the overall frequency of mutations evaluated in 297 resistant patients was 43%; the frequency did, however, range from 14% in the therapy-resistant patients who started imatinib in early chronic phase to 83% among patients with blast crisis [34].

Data of the present study indicate that among imatinib-resistant patients, overall survival seem to be affected significantly by the presence of mutations. These findings suggest that in patients with resistance to imatinib, the presence of a *BCR-ABL* point mutation is significantly associated with a greater likelihood of subsequent disease progression and shorter survival. Thus, the presence of *BCR-ABL* mutations may identify a subset of patients with particularly poor prognosis in terms of time to progression. These findings are in agreement with those of *Branford et al.*, [29] in that patients with mutations had inferior overall survival compared with un-mutated cases.

In conclusion, molecular monitoring of *BCR-ABL* transcript levels by RQ-PCR can identify degrees of molecular response that predict durable remission and in turn prolonged progression free-survival, as well as patterns of response that provide an early indication of relapse and acquisition of resistance. Moreover, this monitoring will facilitate comparisons of different imatinib-based treatment strategies. Mutation analysis may be initiated if the *BCR-ABL* transcript level measured by RQ-PCR rises by > 2-fold. Furthermore, this rule is applicable even when the *BCR-ABL* transcript levels are very low. A more rapid *BCR-ABL* transcripts doubling time may indicate that the patient is relapsing into an aggressive disease. There is the possibility that further mutations will be detected. This suggests that the range of mutations will increase and reinforces the requirement for mutation monitoring.

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