Identification of second generation tyrosine kinase inhibitors relevant mutations in a cohort of chronic myeloid leukemia patients treated in a single center in Romania

Identificarea mutațiilor relevante pentru inhibitorii de tirozin kinază de generația a doua, într-o cohortă de pacienți cu leucemie cronică mieloidă tratați într-un singur centru din România

Kinga Tatar[1*], Rodica Talmaci[2], Dumitru Jardan[2], Maria Damian[3], Adriana Coliță[2], Hortensia Ioniță[1], Coriu Daniel[2]

1. University of Medicine and Pharmacy "Victor Babes", Timisoara
2. University Of Medicine and Pharmacy "Carol Davila", Bucharest
3. Institute "Cantacuzino", Bucharest

Abstract

Mutations in the kinase domain (KD) of BCR-ABL are the most prevalent mechanism of acquired imatinib (IM) resistance in patients with chronic myeloid leukemia (CML), however there are large variances in the literature regarding the frequency of identified mutations in different cohort of patients and data originating from Romania are missing. Here we examine the frequency of tyrosine kinase mutations in the first cohort of Romanian CML patients, (all patients have been treated in Fundeni Clinical Institute). We used the technique of semi-nested polymerase chain reaction (PCR) for the amplification of the KD of BCR-ABL fusion gene, followed by direct sequencing (Sanger method).We also assessed the clinical relevance of the identified mutations and the proportion of patients in which mutational testing had led to a clinical decision. In our very heterogeneous cohort of patients only one fifth of patients had identifiable mutations and only 2 patients, representing 6.25 % of all tested patients and 28.5% from patients with identified mutations had a second generation tyrosine kinase inhibitor (SGI) clinically relevant mutations. In a recent cohort of patients with mutations from Adelaide, Australia, 166 of 386 (43%) had one or more SGI clinically relevant mutations. The technique of mutational testing of BCR-ABL kinase domain remains a highly valuable method for evaluating/predicting the therapeutic response of CML patients. Its value is significantly enhanced by the appropriate selection of the patients referred for this examination.

Keywords: *mutations, chronic myeloid leukemia, tyrosine kinase inhibitors, resistance*

Rezumat

Mutațiile la nivelul domeniului tirozin kinazei BCR-ABL reprezintă principalul mecanism implicat în apariția rezistenței dobândite la imatinib la pacienții cu leucemie mieloidă cronică. Cu toate acestea în literatura de specialitate există variații largi legate de frecvența cu care sunt raportate aceste mutații în diferite cohorte de

[*]**Corresponding author:** Coriu Daniel MD, Hematology Clinic, Fundeni Clinical Institute, Sos. Fundeni Nr. 258, sector 2, Bucharest, Romania. E-mail: daniel_coriu@yahoo.com

pacienți, iar datele provenind din Romania lipsesc. În lucrarea de față ne propunem să evaluăm frecvența apariției acestor mutații într-o cohortă de pacienții cu leucemie mieloidă cronică din România (toți pacienții provin dintr-un singur centru - Institutul Clinic Fundeni), utilizând tehnica semi-nested PCR pentru amplificarea domeniului kinazei genei de fuziune BCR-ABL, urmată de secvențiere directă (metoda Sanger). De asemenea ne-am propus să evaluăm relevanța clinică a mutațiilor identificate şi proporția pacienților la care testarea mutațiilor a determinat o decizie terapeutică. În cohorta noastră foarte heterogenă, o cincime din pacienți au prezentat mutații identificabile prin metoda utilizată. Dintre aceştia, doar 2 pacienți, reprezentând 6.25% din lotul testat, respectiv 28,5% din pacienții cu mutații identificate au avut mutații relevante pentru tirozin kinazele de generația a doua- mai exact în cazul acestor pacienții testarea a avut un rol determinant în conduita terapeutică ulterioară. Comparativ, într-un studiu recent al unei cohorte bine selecționate de pacienți din Adelaide, Australia, 43% (166 din 386) din mutațiile identificate, au prezentat relevanță din punctul de vedere al alegerii corecte a terapiei cu inhibitori de tirozin kinază de generația a doua. Tehnica testării mutațiilor la nivelul domeniului kinazei BCR-ABL este un procedeu extrem de valoros de evaluare/prezicere a răspunsului la terapie al pacienților cu leucemie mieloidă cronică, relevanța lui fiind sporită de o selecție atentă din partea clinicianului a cazurilor adresate în vederea acestei examinări.

Cuvinte cheie : *mutații, leucemie mieloidă cronică, inhibitori de tirozin kinază, rezistență*

Introduction

The current management of CML has been essentially transformed by the introduction of targeted therapy in the form of selective tyrosine kinase inhibitors (TKI) and patient outcome has dramatically improved to the extent that imatinib is nowadays generally considered as the first-line agent for nearly all patients presenting with CML, regardless of the phase of the disease. Impressive clinical responses are obtained in the majority of patients in chronic phase; however, not all patients experience an optimal response to IM, and furthermore, the clinical response in a number of patients will not be sustained. Since the introduction of IM many patients experienced drug resistance, leading to an extensive research with regards to the mechanisms underlying IM resistance, and novel molecules and therapeutic strategies have been evaluated in order to overcome resistance (1).

Although several mechanisms that lead to resistance were described, the molecular pathogenesis of resistance against kinase inhibitors in CML patients is best understood based on mutations within the ABL-kinase domain. Point mutations within the kinase domain will lead to suboptimal binding or complete blockade of IM from its binding site (2). There are nearly 100 described mutations of the fusion BCR-ABL tyrosine kinase. BCR-ABL mutations have been reported in patients with secondary resistance at a frequency ranging from 42% to 90% in different studies (3, 4).

Scientific background

The published incidence of mutations remains variable in different publications as a consequence of different methods of detection, nature of resistance, and disease phase examined. Mutations were first identified in 2001, in CML patients, in which restoration of BCR-ABL1 signal transduction while on IM therapy was associated with a T315I mutation (5). IM binds to the inactive conformation of BCR-ABL, leading to disruption of the adenosine triphosphate (ATP) binding site and blockade of the catalytic activity (6,7). In the presence of IM, BCR-ABL mutations that interfere with IM binding while still enabling ATP binding, or that alter the specific protein conformation required for IM binding, are selected (8-10). In the absence of IM, these mutations do not confer a growth advantage (11).

Threonine 315 forms a fundamental hydrogen bond with IM, disrupted by a single amino acid change with a bulkier isoleucine, which prevents IM localization within the ATP binding pocket by consequent stearic hindrance. The T315I mutation is one of the most common mutations arising in patients treated with IM; it

has been reported with frequencies ranging between 4 to 19% of resistant cases (12 -14) and is resistant to all ABL kinase inhibitors.

Although point mutations have been more frequently described and studied in the context of TKI resistance mostly in advanced-phase CML, they have also been found prior to initiation of TKI therapy (15), suggesting that pre-existing mutant clone do not acquire a survival advantage until subjected to a selective pressure of a TKI.

In addition, investigators have found no difference in mutational status in those patients who have relapsed. (16) The relevance of these observations remains unclear, specifically about whether certain mutations are responsible for disease progression or whether they occur as a consequence of the underlying genomic instability linked with advanced phase disease (17). Available data suggests that gain-of-function mutations may directly determine disease progression, whilst loss-of-function mutations are more often subject to selective pressure by IM (18, 19). Various mutations have considerably different capabilities to impact the transformation potency of BCR-ABL1, and in vitro studies have indicated relative transformation potencies of mutations from distinct sections of the kinase domain to be: Y253F > E255K (P-loop) > unmutated BCR-ABL1 ≥ T315In (IM binding site) > H396P (activation loop) > M351T (catalytic domain) (18).

A proportion of cases where relapse was reported after an initial response were associated with the emergence of newly acquired mutations. The T315I mutation was most commonly implicated, with a frequency of 36% (20). The inability to achieve a sustained cytogenetic response could in part occur as a consequence of the development of new therapy-resistant kinase domain mutations as patients are exposed to sequential TKIs, although some of the arising mutations were reported as having a relatively good in vitro sensitivity to the concurrent TKI (21).

In summary, the consequence of identifying a mutation remains unclear and seems relevant only according to the disease phase and response, with a greater impact inadvanced phase CML in which the mutated clone may be responsible for disease progression, but less certain in cases of on-going response to TKI therapy.

Apparently some BCR-ABL mutations do not determine resistance (17, 22, 23), however, at the moment of SGI initiation after IM failure, approximately 50% of the patients have detectable IM-resistant BCR-ABL mutations.

For CML patients starting a second line treatment with nilotinib or dasatinib after IM failure, clinical trials have demonstrated similar responses for patients with or without identified mutations, with the exception of T315I which is resistant to both drugs (24-32). This mutation demonstrates cross-resistance to IM, nilotinib, and dasatinib (33-35). However, a more detailed evaluation of responses to SGI therapy for individual mutations had lead to the identification of a limited number, other than T315I, that are less sensitive to either nilotinib or dasatinib (36-38). Furthermore, in vitro studies have identified mutations that confer a degree of insensitivity or resistance (39,40).

Although several mechanisms were described for the occurrence of resistance, according to a study performed by an Italian group 83% of the patients had a new mutation when they relapsed after an initial response (20). Since the introduction of new TKIs in the treatment of CML patients, new clinical data became available, allowing us to assess the accuracy of in vitro resistance studies in the clinical setting, but also their validity in the decision of appropriate therapeutic management. Recently, the clinical validation of in vitro sensitivity of different mutant clones has been demonstrated; these tests have proven to have a predictive role with regards to occurrence of response and long term outcome for patient treated with SGIs (36).

Patients and method

Based on the recent classification of mutations developed by the Adelaide group we retrospectively assessed the mutational testing results performed in one center in Romania in order to

identify the occurrence of SGI relevant mutations and their impact on therapeutic decision.

A number of 48 blood samples originating from 32 CML patients from Fundeni Clinical Institute's Hematology Clinic Bucharest have been assessed for BCR-ABL tyrosine kinase domain mutations. The technique used a semi-nested PCR for the amplification of the kinase domain of BCR-ABL fusion gene, followed by direct sequencing (Sanger method). This method allows identification of mutations when mutated clones have reached an abundance threshold of ~20-25%.

The research was performed between March 2008 and September 2009. Twelve patients had more than one mutational examination. In these patients epidemiological data at the date of first examination was considered.

The median age of subjects was 45 years, ranging between 21 and 69 years, 44% were females, 56% males.

Disease analysis

The CML patient population was heterogeneous with respect to stage of disease, type of response and therapy. The average duration of the disease was 23 months (limits 0-128 months).

Eighteen out of 32 (representing 56%) patients did not have any cytogenetic response at the moment of examination, the rest of included patients were in major cytogenetic response (MCyR).

Five out of 32 patients (19%) were in major molecular response (MMR) whilst the rest of patients had no molecular response on treatment.

Four patients were at diagnosis at the moment of mutational testing (before the commencement of tyrosine kinase inhibitor therapy); none of them had identifiable mutations at the time of diagnosis.

Treatment analysis

From the lot of 32 patients, 2 patients were receiving Hydroxiurea, 6 patients were on dasatinib, 1 patient was on nilotinib treatment and 21 patients were receiving IM in doses ranging from 300 mg to 800 mg. Four patients received 600 mg of IM, 13 patients were on 400 mg IM, 800 mg was given to 3 patients whilst 1 patient was on 300 mg due to poor tolerance. The remaining 2 patients have not been receiving any treatment prior to testing.

Table 1. Frequency of identified mutations according to disease stage

Disease stage	Number of patients	Patients with mutations
At diagnosis	4	0
Major molecular response	5	0
Major cytogenetic response	14	0
No cytogenetic response	18	7

Mutations of BCR-ABL domain were identified in 7 patients, 2 patients had 2 concomitant mutations. Frequency of mutations in literature is varying widely in different literature reports, depending on the characteristic of the analyzed patient cohorts. In our heterogeneous patient population, mutations were identified in 21.8% of patients in the overall tested patient population, but excluding patients at diagnosis (4 patients) and patients in MMR (5 patients) who have no indication for mutation testing the percentage rises to 30.4%. In the patient population without cytogenetic response, mutated clones were identified in 40% of patients. BCR-ABL mutations have been reported in patients with secondary resistance at a frequency ranging from 42% to 90% in different studies (5, 12).

Mutation M244V (sensitive to both nilotinib and dasatinib) was the only mutation identified in 2 different patients in our cohort. This is concordant with other cohorts of patients assessed in the literature (8).

Two out of the 7 patients with identified mutations (F359V and E255K) had SGI clinically relevant mutations as recently classified in literature (Adelaide group) (41). The significance of

Table 2. Identified mutations in the Romanian cohort

Patient	Mutation at first testing	Mutation at second testing	Mutation at 3rd testing	Affected region
Patient 1	L387M 60%	L387M 90% M244V 10%		Activation loop P loop –confers varying response to escalating the dose of imatinib, sensitive to both nilotinib and dasatinib
Patient 2	F359V 100%	F359V 100%		Catalytic domain, less sensitive to nilotinib
Patient 3	E459K 100%			C-terminal lobe Good sensitivity to both SGI
Patient 4	E255K 100%			P loop (less sensitive to nilotinib)
Patient 5	E450A 60% Q252H 50%	E450A 40% Q252H 10%	Q252H 100%	C-terminal lobe P loop(same sensitivity to dasatinib and nilotinib)
Patient 6	E450K 100%			C-terminal lobe- confers partial insensitivity to IM
Patient 7	M244V 100%			P loop- confers varying response to escalating the dose of IM, sensitive to both nilotinib and dasatinib

identifying the mutation F359V during IM treatment is that it provides to the clinician relevant information for choosing the second generation TKI. However in the particular case of our patient, the mutation was identified while the patient was under nilotinib treatment, probably as a consequence of the selective pressure of the drug, triggering switch to the alternative second generation TKI option, namely dasatinib. There are no data available for this patient before the commencement of nilotinib, although identifying this mutation earlier in the course of treatment, more specifically before the initiation of nilotinib treatment, would have allowed for a better choice of treatment and improved outcome for this particular patient. In case of E255K mutation, this mutation was identified before the choice of second generation therapy, and has lead to a clear-cut decision in the choice of the appropriate second generation TKI. To summarize (*Table 2*), in our cohort only 2 patients, representing 6.25 % of tested patients and 28.5% from patients with identified mutations had SGI clinically relevant mutations, whilst in a recent cohort from Adelaide, Australia in a cohort of patients with mutations, 166 of 386 (43%) had one or more SGI clinically relevant mutations (41).

Discussions

The important topic of the impact of BCR-ABL mutations on response after a change to SGI therapy was assessed in a recent article by the Adelaide group, led by Susan Branford, by an examination of the available clinical data. The mutation status may be an essential parameter in the therapeutic decisions

after IM failure or after failure of a SGI. In the above mentioned article the authors assessed the frequency of mutations conferring a degree of clinical insensitivity to SGIs that are detectable at the time of IM cessation. These are collectively referred to as SGI clinically relevant mutations. They also examined whether the disease phase influences their frequency and the occurrence of multiple mutations in IM-treated patients and the extent to which disease phase influences their detection (41).

The level of correlation between the impacts of mutations identified by *in vitro* assays versus clinical studies is still to be determined. At the same extent data is still needed to determine whether in vitro sensitivity represents a reliable parameter when assessing patient's probability to achieve positive response to a SGI. As most of the existing data suggests, in vitro sensitivity represents a useful parameter when we are considering IM dose increase.

As mentioned above, in our very heterogeneous cohort of patients only one fifth of patients had identifiable mutations. When it comes to clinical decisions in choosing a certain second generation TKI based on mutation testing results, in only 2 cases the result tests offered information that lead to a clear-cut decision.

The vast majority of mutations identified in different screens generally fall within four regions of the kinase domain, including, the ATP-binding loop (P-loop), contact site (e.g., T315 and F317), SH2 binding site (e.g., M351) and A-loop (42). Although this classification is well known and accepted in the scientific community, there are recent efforts in the international community to classify mutations according to their clinical relevance in the therapeutic decision. Thus, the available strategies to overcome resistance (IM dose escalation (43), alternative therapy with a SGI (33, 37) to which the mutant has documented sensitivity, withdrawing TKI therapy to allow the mutant clone to recede, as well as non-BCR-ABL1-dependent therapies [44]) would be implemented in a timely and structured manner. According to this new classification, mutations are divided in 4 groups. Currently, Class A indicates no compelling clinical evidence to suggest that the mutation would not respond to the inhibitor. In case of Class B mutations, *in vitro* assessment consistently indicates that the mutation may confer intermediate insensitivity/resistance to the inhibitor, or clinical evidence may be suggestive of reduced sensitivity. At this stage, the presence of these mutations should have no impact on clinical decisions and additional clinical assessment is required before an alternative inhibitor would be recommended.

Class C indicates the existence of compelling clinical evidence to recommend an alternative inhibitor, whilst Class D mutations are insensitive to SGI therapy. From the available studies, we now have a more clear understanding of the BCR-ABL mutations for which there is compelling clinical evidence that response could be compromised by treatment with one and/or another of the SGIs if present after IM failure.

Under the pressure of increasing doses of dasatinib or nilotinib, a certain number of emergent mutations were identifier by various resistance screens. These observations were in a great extent corresponding with the sensitivity of different mutated clones assessed in cell proliferation assays.

Mutations V299L at dasatinib contact residues appeared to play an important role in patient's response to this drug. Mutations at T315 and F317 residues were identified in 95% of all mutants recovered in 2 resistance screens. Among these, novel mutations F317V/I/S/C and T315A were detected; these mutations have not been previously reported in IM-treated patients (45,46). In one study, F317V and T315A were the most frequently reported mutations (41% and 30%, respectively) and had 40- to 90-fold reduced dasatinib sensitivity compared with unmutated BCR-ABL, demonstrating the second highest IC50 values after the well known T315I in an assessment performed by O'Hare *et al* (34).

Table 3. Second Generation TKI clinically relevant mutations

Mutation	Insensitive to dasatinib	Insensitive to nilotinib	Therapeutic relevance class
T315I	Yes	Yes	D
F317/L/I/C/V	Yes	No	C
V299L	Yes	No	C
E255K/V	No	Yes	C
F359V/C	No	Yes	C
Y253H	No	Yes	C

In the case of nilotinib resistance screens, mutation T315I represented 49% of all identified mutations.(45,46). Mutation Y253H, a common IM resistant mutation was also identified quite frequently and had the second highest IC50 after T 315I.

In all three mutational assays studied, E255K, Y253H, and T315I mutations were identified whilst, E255V and Q252H were found in 2 out of 3 screens. With the exception of T315I, all mutations were effectively suppressed by nilotinib concentrations of 2000 nM, which can be achieved *in vivo* by therapeutic nilotinib doses (25).

Since the introduction of the second generation TKIs, a number of mutation considered relevant for dasatinib or nilotinib response in the second or 3rd line setting were identified and were considered as involved in resistance.

In conclusion, from clinical point of view the SGI relevant mutations are T315I, F317L, V299L, Y253H, E255K/V, and F359V/C, the finding of which would influence the therapeutic decision (*Table 3)*.

These mutations are classified in *Table 3* as either class D (no role for SGI therapy) or class C (compelling clinical evidence to recommend an alternative inhibitor).

From the perspective of this recent data, few questions arise with regards to our patient cohort assessment. First of all besides research purposes, mutation testing of CML patients in usual clinical setting should be restricted to the European LeukemiaNet (ELN) recommendations (in occurrences of suboptimal response or failure; always required before changing to other TKIs or other therapies). Following these recommendations would ensure that mutational testing is performed in a cost-effective manner, offering in the same time a useful tool for clinical decisions. In our cohort, only 2 patients, representing 6.25 % of tested patients and 28.5% from patients with identified mutations had SGI clinically relevant mutations, whilst in a recent cohort of patients with mutations from Adelaide, Australia, 166 of 386 (43%) had one or more SGI clinically relevant mutations.

As a general conclusion of the mutational studies performed on the first Romanian cohort, the research team has successfully set up and validated a sensitive technique for detecting BCR-ABL tyrosine kinase mutations in CML patients and assessed a number of 48 samples originating from 32 patients.

However, there is an identified need to familiarize hematologists with the recent concept of SGI clinically relevant mutations for a more beneficial integration of mutation analysis in the everyday practice and streamline the needs for testing and the clinical relevance of the information provided.

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Abbreviations

ATP -adenosine triphosphate
CML -chronic myeloid leukemia
ELN -European LeukemiNet
IM -imatinib
KD -kinase domain
MCyR - major cytogenetic response
MMR - major molecular response
PCR -polymerase chain reaction
SGI -second generation tyrosine kinase inhibitor
TKI -tyrosine kinase inhibitors

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