Philadelphia-positive acute lymphoblastic leukemia patients already harbor *BCR-ABL* kinase domain mutations at low levels at the time of diagnosis

Simona Soverini,[1] Antonella Vitale,[2] Angela Poerio,[1] Alessandra Gnani,[1] Sabrina Colarossi,[1] Ilaria Iacobucci,[1] Giuseppe Cimino,[2] Loredana Elia,[2] Annalisa Lonetti,[1] Marco Vignetti,[2] Stefania Paolini,[1] Giovanna Meloni,[2] Valeria di Maio,[2] Cristina Papayannidis,[1] Marilina Amabile,[1] Anna Guarini,[2] Michele Baccarani,[1] Giovanni Martinelli,[1] and Robin Foà[2]

[1]Department of Hematology and Oncological Sciences "L. e A. Seràgnoli", University of Bologna, Bologna, Italy, and [2]Department of Hematology and Cellular Biotechnologies, "Sapienza" University of Rome, Rome, Italy

ABSTRACT

Background
In patients with Philadelphia-positive acute lymphoblastic leukemia, resistance to treatment with tyrosine kinase inhibitors is frequent and most often associated with the development of point mutations in the *BCR-ABL* kinase domain. We aimed to assess: (i) in how many patients *BCR-ABL* kinase domain mutations are already detectable at relatively low levels at the time of diagnosis, and (ii) whether mutation detection correlates with subsequent response to therapy.

Design and Methods
We retrospectively analyzed samples collected at diagnosis from 15 patients with Philadelphia-positive acute lymphoblastic leukemia who subsequently received tyrosine kinase inhibitor therapy (dasatinib) by cloning the *BCR-ABL* kinase domain in a bacterial vector and sequencing 200 independent clones per sample.

Results
Mutations at relatively low levels (2-4 clones out of 200) could be detected in all patients – eight who relapsed and seven who achieved persistent remission. Each patient had evidence of two to eight different mutations, the majority of which have never been reported in association with resistance to tyrosine kinase inhibitors. In two patients out of six who relapsed because of a mutation, the mutation (a T315I) was already detectable in a few clones at the time of diagnosis. On the other hand, a patient who was found to harbor an F317L mutation is in persistent remission on dasatinib.

Conclusions
Our results suggest that the *BCR-ABL* kinase domain is prone to randomly accumulate point mutations in Philadelphia-positive acute lymphoblastic leukemia, although the presence of these mutations in a relatively small leukemic subclone does not always preclude a primary response to tyrosine kinase inhibitors.

Key words: *BCR-ABL*, kinase domain mutations, acute lymphoblastic leukemia, outcome.

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Correspondence:
Giovanni Martinelli, MD,
S. Orsola-Malpighi Hospital,
Via Massarenti 9, 40138
Bologna, Italy.
E-mail: martg@tin.it

The online version of this article has a Supplementary Appendix.

Introduction

Incorporation of the tyrosine kinase inhibitor (TKI) imatinib mesylate into frontline treatment of patients with Philadelphia chromosome-positive (Ph+) acute lymphoblastic leukemia (ALL) has significantly improved the antileukemic efficacy of induction therapy.[1] Nevertheless, responses are often short-lived and relapse is frequently associated with selection of point mutations in the *BCR-ABL* kinase domain which impair inhibitor binding.[2] The rapid development of resistance and mutations in Ph+ ALL patients receiving imatinib supported the hypothesis that, at least in a proportion of patients, mutations might already be present prior to TKI treatment. Indeed, by using a sensitive cloning and sequencing strategy, Hofmann *et al.*[3] were able to retrospectively trace the mutations detected at relapse back to the samples archived prior to starting TKI therapy in two of four patients with advanced Ph+ ALL who had received imatinib after developing refractoriness or resistance to standard chemotherapy. A subsequent investigation[4] searched for pre-existing *BCR-ABL* kinase domain mutations in a cohort of newly diagnosed Ph+ ALL patients enrolled in a study of frontline imatinib-based therapy in the elderly. Nine of the 22 (41%) patients investigated were found to harbor mutations as assessed by denaturing-high performance liquid chromatography (D-HPLC) and sequencing, indicating that mutated clones responsible for subsequent relapse were already present at the time of diagnosis within the *BCR-ABL*-positive cell population.[4] Since the combination of these two techniques is known to have a lower detection limit of 10-15% (10 to 15 mutated molecules in a background of 100 wild-type molecules),[5] we wondered: (i) how many patients with newly diagnosed Ph+ ALL would turn out to harbor kinase domain mutations if we increased the sensitivity of the screening method, and (ii) whether this would allow the prediction of subsequent treatment failure.

To address these issues, we retrospectively analyzed a cohort of patients enrolled in a phase II study of dasatinib treatment of *de novo* Ph+ ALL (GIMEMA LAL1205) with known outcome, by cloning the *BCR-ABL* kinase domain and sequencing 200 independent clones per sample.

Design and Methods

Patients

This study was retrospectively conducted on bone marrow samples collected at the time of diagnosis from 15 patients enrolled in a phase II study of the treatment of adult *de novo* Ph+ ALL with dasatinib (GIMEMA LAL1205). Patients enrolled in the study were given dasatinib 70 mg *bid*. A steroid pre-phase was started 7 days prior to dasatinib administration, continued up to day +31, and then tapered. Dasatinib treatment was initially planned to last 12 weeks, with the possibility of extending this period at the clinician's discretion.

The characteristics of the 15 patients herein analyzed are summarized in Table 1. There were nine females and six males and the median age of these patients was 56 years. Eight patients were positive for $p190^{BCR-ABL}$ and seven patients were positive for $p210^{BCR-ABL}$. Eight patients developed resistance after the 12-week induction phase while still on TKI therapy (dasatinib in seven cases, imatinib in one), six of whom had evidence of a TKI-resistant mutation (Table 1). The remaining seven patients were in persistent remission 12 to 24 months after the start of dasatinib treatment (with molecular remission status at last follow-up detailed in Table 1).

The Ethics Committees of all participating Institutions gave their approval to this study, and specific informed consent was signed by all the patients, in accordance with the Declaration of Helsinki and its amendments.

Cytogenetics and minimal residual disease monitoring

Conventional chromosome G-banding analysis was used for cytogenetic analysis. *BCR-ABL* transcript levels were assessed by real-time reverse transcription (RT)-polymerase chain reaction (PCR) as previously described[6] at baseline and at +22, +43, +57 and +84 days, as per protocol. Minimal residual disease monitoring was continued at regular intervals thereafter, unless relapse occurred. Results were expressed as *BCR-ABL/ABL*% and molecular response was defined as an undetectable quantitative molecular response if the transcript was undetectable by real-time RT-PCR, a major molecular response if the *BCR-ABL/ABL*% value was lower than 0.12, and a minor molecular response if the *BCR-ABL/ABL*% value was between 0.12 and 1.00.

Conventional BCR-ABL *kinase domain mutation analysis*

All 15 patients had already been screened for the presence of *BCR-ABL* kinase domain mutations by nested RT-PCR followed by D-HPLC (WAVE 3500-HT; Transgenomic, Cramlington, UK) at the time of diagnosis, at regular intervals during therapy and again in the case of relapse, as per protocol. In D-HPLC-positive cases, bidirectional sequencing was then performed on an ABI PRISM 3730 (Applied Biosystems, Foster City, CA, USA) to characterize the precise nucleotide substitution(s). D-HPLC and sequencing analyses were performed as previously reported.[7,8]

Mutation analysis of diagnostic samples by cloning and sequencing

For cloning, a single fragment corresponding to the kinase domain region where the reported *BCR-ABL* mutations map (codons 244-486) was generated by nested RT-PCR using ProofStart DNA polymerase (Qiagen, Hilden). The first round of amplification, performed in order to increase the sensitivity of mutation detection by selecting only the translocated *ABL* allele, was conducted using the same primers and amplification conditions as above. A 1 μL aliquot of the first PCR product was then re-amplified using the following primers, Full_KD_Fwd, GTGTGTCCCCCAACTACGAC and Full_KD_Rev, CCTTTTCCACTTCGTCTGAG, and amplification conditions, initial denaturation step of 5 min at 95°C; amplification for 35 cycles (denaturation: 30 s at 95°C; annealing: 40 s at 58°C; extension: 1 min at 72°C); final extension for 7 min at 72°C. The kinase domain fragments were then cloned into a pCR2.1-TA vector (TOPO TA Cloning Kit; Invitrogen) according to the manufacturer's instructions. Two hundred independent clones per sample were harvested and the *BCR-ABL* kinase domain was sequenced. Precautions were taken to avoid contamination and false positive results. Bacteria were grown in multiple plates and only well isolated colonies were picked up. Mutations were confirmed by bidirectional sequencing. Mutations detected in single clones were discarded; mutations detected in two independent clones or more were accepted. For comparison, the kinase domain of the *ABL* gene, amplified with the same primers as above, was analyzed in parallel in three healthy individuals. In addition, the *BCR-ABL* kinase domain of patients n. 2, 5 and 8 (Table 1) was analyzed again in the sample collected at the time of relapse (30 independent clones were sequenced in these latter cases).

Results

Two groups of patients were included in our retrospective

analysis (Table 1): eight patients who relapsed while on TKI therapy and seven patients who were in persistent remission. In all 15 cases, D-HPLC-based mutation screening performed at diagnosis, as per protocol, had failed to identify any *BCR-ABL* kinase domain mutations (Table 1). Six patients in the first group had TKI-resistant mutations detectable by D-HPLC and direct sequencing at the time of relapse: five patients, who relapsed on dasatinib therapy, were positive for a T315I mutation; one patient, who relapsed on imatinib therapy (to which she was switched after the 12-week induction period because of dasatinib intolerance), was positive for an E255K mutation (Table 1). In the seven patients who were in persistent remission (molecular remission status at last follow-up detailed in Table 1), no *BCR-ABL* kinase domain mutation had ever been detected by D-HPLC during routine monitoring from diagnosis up to the time of last follow-up (*data not shown*).

Our cloning and sequencing approach retrospectively applied to the samples stored at the time of diagnosis, before the start of dasatinib therapy, showed evidence of *BCR-ABL* kinase domain mutations in all 15 patients, irrespective of their subsequent outcome on TKI therapy (Table 1). Mutations were detected in two to four out of 200 independent clones sequenced, confirming that they were present at low levels, below the lower detection limits of the techniques routinely used for mutation screening of patients. The same cloning and sequencing approach was also applied to the analysis of the kinase domain of normal *ABL* in three healthy individuals. In two of these three patients, occasional single mutated clones were detected; however, the same nucleotide change was never present in two independent clones. For this reason, mutations detected in only one clone in the patients analyzed were discarded, to rule out potential sequence artifacts introduced during the cloning procedure.

Each patient had evidence of two to eight different mutations (detailed in Table 1). The median number of mutations per patient was the same (n=4) in those who relapsed (n=8) and in those who achieved a persistent remission (n=7); in addition, it was also the same (n=4) in those harboring $p190^{BCR-ABL}$ (n=8) and in those harboring $p210^{BCR-ABL}$ (n=7). Cytogenetic data at diagnosis and at relapse (when available) are detailed in *Online Supplementary Table S1*. Patients who had a complex karyotype at diagnosis, showing cytogenetic abnormalities additional to the Philadelphia chromosome, did not seem to harbor a higher number of mutations with respect to the others – although investigation of such a correlation is hampered by the unavailability of cytogenetic data for 4/15 patients, which made the two groups too small for any appropriate comparison.

Overall, 61 different point mutations were detected: 40 (66%) were missense mutations, 17 (28%) were silent and 4

Table 1. Patients' characteristics, status at last follow-up evaluation and mutations detected by direct sequencing and by cloning and sequencing of 200 independent clones per sample.

N.	Sex	Age	*BCR-ABL* type	Status (time from the start of dasatinib, mo)	Mutations at diagnosis (D-HPLC)	Mutations at diagnosis (cloning and seq)	Mutation at relapse (D-HPLC and direct seq)	TKI at relapse
1	M	54	p190	Relapse (10)	WT	L266P (2/200); A337A (3/200)	T315I	DAS
2	M	56	p210	Relapse (5)	WT	V289A (2/200); V335V (4/200); K378E (2/200); L411P (3/200)	T315I	DAS
3	M	56	p210	Relapse (6)	WT	L298P (3/200); R307Q (2/200); W405C (3/200)	WT	DAS
4	F	49	p190	Relapse (6)	WT	E275E (2/200); E281K (4/200); T319A (2/200); N336D (3/200)	WT	DAS
5	F	74	p210	Relapse (8)	WT	G250R (3/200); I293T(2/200); V304I (3/200); D363D (2/200); R367stop (2/200); K378E (2/200)	T315I	DAS
6	F	58	p210	Relapse (7)	WT	K245R (2/200); L327L (3/200)	E255K	IM
7	F	61	p210	Relapse (3)	WT	I293T (2/200); V304I (4/200); T315I (2/200); C346stop (2/200); W405C (3/200); P439L (3/200)	T315I	DAS
8	F	30	p210	Relapse (6)	WT	Y264C (2/200); T315I (2/200); A344T (2/200); L451L (2/200)	T315I	DAS
9	F	58	p190	Remission [MMR] (15)	WT	Q252stop (2/200); L384P (3/200); T392I (2/200); A407A (3/200); N479S (2/200)	N.A.	N.A.
10	F	34	p190	Remission [UQMR] (16)	WT	K245R (2/200); A288T (3/200); R460R (2/200)	N.A.	N.A.
11	M	52	p190	Remission [MMR] (12)	WT	G254E (2/200); Y326Y (2/200); E334E (3/200); A487V (3/200)	N.A.	N.A.
12	F	73	p210	Remission [MMR] (22)	WT	E352K (3/200); A380A (2/200); T389T (3/200); Q477stop (2/200)	N.A.	N.A.
13	M	72	p190	Remission [MMR] (24)	WT	G249S (3/200); Y253Y (3/200); Y257H (4/200);E292G (3/200); F317L (3/200); Y342Y (2/200); Y353Y (3/200); H396P (2/200)	N.A.	N.A.
14	F	36	p190	Remission [mMR] (19)	WT	E352G (2/200); A366A (3/200)	N.A.	N.A.
15	M	50	p190	Remission [mMR] (15)	WT	E286E (2/200); I360T (2/200); I403T (3/200); N479S (2/200)	N.A.	N.A.

The number of clones positive for each specific mutation is indicated in parentheses. mo, months; D-HPLC, denaturing-high performance liquid chromatography; seq, sequencing; WT, wild-type; N.A., not applicable; DAS, dasatinib; IM, imatinib; MMR, major molecular response; UQMR, undetectable quantitative molecular response; mMR, minor molecular response (see Design and Methods *for definitions of molecular response).*

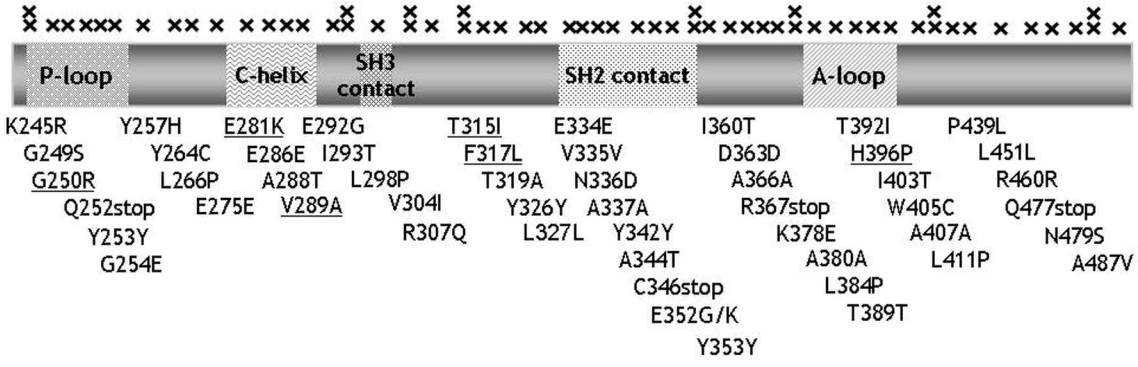

Figure 1. Distribution of the mutations with respect to the main regions of the *BCR-ABL* kinase domain. The exact position and amino acid substitution are detailed below the horizontal bar. Mutations that have been reported in association with TKI resistance are underlined. The relative frequency of each mutation is indicated as the number of crosses over the bar. The K245R, I293T, V304I, T315I, E352K, K378E, W405C and N479S mutations were detected in two patients; the remaining ones were detected in one patient each. The kinase domain is not drawn in scale.

(6%) were nonsense mutations. A map of the mutations found in our study is depicted in Figure 1. Mutations were randomly and uniformly distributed all over the kinase domains and no particular mutation hotspot could be identified. Only five of the 61 (8%) mutations we found have been reported in TKI-resistant patients (namely, E281K, V289A, T315I, F317L, and H396P); the remaining 56 mutations have never been associated with resistance to any TKI in published studies.[9] In patients n. 7 and 8, the T315I mutation that was responsible for dasatinib resistance, and was identified by D-HPLC and direct sequencing at relapse, turned out to have been already present at diagnosis (in two and three clones, respectively) (Table 1).

In patients n. 2, 5 and 8, cloning and sequencing of 30 independent clones was also performed on the samples collected at the time of relapse. The results are shown in Table 2. In patient n. 2, all the 22 clones positive for mutations carried a T315I. Likewise, in patient n. 8, all the 17 mutated clones had a T315I. More interestingly, in patient n. 5, 12 clones were positive for the T315I only, four clones were positive for both the T315I and the Y293T mutations, and four clones were positive for both the V304I and T315I mutations. The Y293T and V304I mutations were already detectable, in a much smaller percentage of clones, at the time of diagnosis.

Table 2. Mutations detected by cloning and sequencing of 30 independent clones per sample in relapse samples from patients n. 2, 5 and 8.

Pt #	Mutations at relapse (cloning and seq)	Mutation at relapse (D-HPLC and direct seq)
2	T315I (22/30)	T315I
5	T315I (12/30); Y293T+T315I (4/30); V304I+T315I (4/30)	T315I
8	T315I (17/30)	T315I

The number of positive clones is indicated in parentheses. seq, sequencing; D-HPLC, denaturing-high performance liquid chromatography.

Discussion

Previous observations[3,4] had indicated that *BCR-ABL* kinase domain mutations are already present at the time of diagnosis in a proportion of Ph+ ALL patients. Our study confirms and extends these observations. Our results, although derived from a relatively small cohort of patients, suggest that increasing the sensitivity of the detection method may allow mutations to be identified in virtually all patients with Ph+ ALL.

However, the great majority of the mutations found in our screening have never been reported in association with TKI resistance;[9] hence it can be concluded that they are unlikely to be clinically relevant, that is, to drive drug resistance. Most of such small Ph+ mutated subclones are destined to fade away under the pressure of TKI therapy since they do not have any selective advantage. The same analysis (although on a smaller number of clones) performed on the samples collected at the time of relapse from patients n. 2, 5 and 8 (Table 2) showed that 'critical' mutations, such as the T315I, become predominant as resistance develops. Small mutated subclones that are apparently clinically 'innocuous' – like those harboring the V304I and the Y293T mutations – may outgrow as a sort of 'bystander effect', only when their survival and proliferation is driven by the secondary acquisition of the pan-resistant T315I mutation (patient n. 5, Table 2). This case was particularly interesting; the most likely explanation for this case is that the T315I mutation was independently acquired by (i) the original V304I-positive clone; (ii) the original Y293T-positive clone; and (iii) a third, originally unmutated, Ph+ clone.

What biological significance could these relatively small (1-2% of bone marrow cells) mutant clones have? It can be hypothesized that (potentially irrelevant) *BCR-ABL* kinase domain mutations arise in small, competing subclones whose survival is driven by yet unknown additional abnormalities. Another possible explanation is that (at least some) mutations may have functional consequences on *BCR-ABL* resulting in a slightly increased growth advantage of the clone – until a TKI is administered.

In two patients (n. 7 and 8, Table 1) of the six who subse-

quently relapsed on a TKI because of a kinase domain mutation, the same T315I identified by direct sequencing at the time of relapse to dasatinib was already detectable at low levels at diagnosis. In the remaining four patients, however, it was not possible to trace the mutation detected at relapse (T315I in three cases, E255K in one case) back to the pretreatment sample, either because it had not yet occurred or because our detection method was not sensitive enough to highlight it. One patient (n. 13, Table 1) was found to harbor an F317L mutation at diagnosis (2/200 clones sequenced). Reports from us and others[10-13] suggest that the F317L mutation may confer resistance in patients treated with dasatinib; nevertheless, this patient achieved and maintained a major molecular remission. How can this be explained? The cellular IC_{50} of dasatinib for the F317L-*BCR-ABL* mutant has been assessed in three different *in vitro* studies,[14-16] and has been reported to be 4- to 13-fold higher than that of unmutated *BCR-ABL* – which would actually predict for a decreased sensitivity to dasatinib. However, an IC_{50} value derived after incubation of a TKI with a murine cell line engineered to express a mutated form of *BCR-ABL* may not always mirror the actual sensitivity of patients harboring the same mutation *in vivo*. Humans constitute a more complex and heterogeneous system, and *in vivo* there are additional factors influencing drug sensitivity and resistance, which may make different contributions in different patients (e.g., variability in the extent to which the drug is metabolized or transported). Accordingly, a recently published case report[17] describes a patient with imatinib-resistant, F317L-positive chronic phase chronic myeloid leukemia who achieved a stable major molecular response on dasatinib. Another possible explanation for the lack of F317L selection in our patient is that the hierarchical level at which the mutations arise may make the difference. Very low levels of TKI-resistant mutations have also been detected prior to the start of imatinib in the setting of patients with advanced phase chronic myeloid leukemia, but in some cases the mutations did not outgrow and did not lead to treatment failure.[18] The authors of the study hypothesized that this phenomenon could be due to a limited self-renewal capacity of the cell clones harboring the low level mutations, and warned against high-sensitivity mutation screening of patients before the start of TKI treatment. This might also be the case for Ph+ ALL, although analyses of larger series of patients are needed to evaluate this hypothesis.

There seemed to be no correlation between the number of low level mutations, intended as a theoretical measure of the degree of genetic instability acting in each patient, and the likelihood of relapse, since the median number of mutations per patient was the same in those who relapsed and in those who achieved and maintained a remission. We also examined the cytogenetic data (when available) of the patients. No association was identified between a higher number of low level mutations and a complex karyotype at diagnosis (i.e., the presence of cytogenetic abnormalities additional to the Philadelphia chromosome). However, cytogenetic data were unavailable for some patients, hampering attempts to find such an association.

Sequencing of hundreds of clones is a cumbersome and expensive way to increase the sensitivity of mutation detection, hence it cannot be applied in a large number of patients. Another drawback of such cloning and sequencing approaches is the risk of obtaining false positive results. The risk of contamination is indeed high, but every possible measure was taken in order to counteract it. Artifacts may also be generated during the amplification of the kinase domain and the early steps of bacterial propagation. On the other hand, alternative strategies that have been set up to increase the sensitivity of *BCR-ABL* mutation detection are almost exclusively PCR-based,[18-24] hence mutation-specific. These techniques do have lower detection limits, but only allow the identification of a limited number of candidate mutations. Furthermore, they share with cloning and sequencing a similar risk of producing false positive results because of amplification errors. Indeed, higher sensitivity in mutation detection is generally achieved at the expense of 'reliability', in that every time there is an amplification step of any kind, the false positive rate inexorably increases. With this *caveat* in mind, we reasoned that cloning and sequencing was anyway the best choice in an attempt to conjugate higher sensitivity with the possibility of performing a comprehensive screening of the entire kinase domain for nucleotide substitutions. In order to circumvent the problem of false positive results, we accepted only those mutations detected in two or more independent and well-separated colonies and discarded the occasional single mutants we found. Coming back to patient n. 8, it is interesting to note that two of the mutations we found in very few clones at diagnosis (Table 1) were confirmed at the time of relapse (Table 2). Next-generation sequencers will soon allow high throughput and high sensitivity mutation screening of the kinase domain. With the 454 GS FLX (Roche Diagnostics) instrument, for example, it is possible to perform massive parallel sequencing of individual amplicons up to 400 bp-long. Three amplicons would fully cover the *ABL* kinase domain, and a 100,000x coverage would allow mutations to be detected with a lower detection limit of 0.01%.

Although we cannot fully exclude that some among the many mutations we found were detected by chance, what seems to emerge from our current results is that the *BCR-ABL* kinase domain of Ph+ ALL patients is genetically unstable and starts randomly accumulating point mutations from the time of diagnosis, although not all of these mutations affect the response over time to TKI therapy. Thus, from a clinical point of view, prospective screening for low level mutations at diagnosis seems worthless, since results cannot be used to reliably anticipate the emergence of drug resistance. TKI treatment should rapidly result in the selective elimination of innocuous and/or short-lived subclones harboring low level mutations, and in enrichment of clinically relevant ones. Most patients have an initial response and few relapse before 3 months, so performing a mutation screening after the first 3 months of TKI therapy might prove much more 'informative' in identifying those patients who are likely to relapse because of an emerging low-level mutated clone. This is worth exploring in future studies, and it may prove to be highly clinically relevant once the emerging next-generation sequencing technologies allowing for more straightforward, high-sensitivity mutation screening become available for routine use.

Authorship and Disclosures

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