Mutations that confer resistance to therapy

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The t(9;22)(q34;q11) chromosomal translocation gives rise to the Philadelphia chromosome (Ph), whose product is the BCR-ABL fusion protein (1). This constitutively activated tyrosine kinase represents the pathogenic substrate of chronic myelogenous leukemia (CML) and Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ ALL). The development of imatinib, a small-molecule inhibitor of BCR-ABL at its ATP-binding site, provided a new, causative treatment option for Ph+ leukemia.[2,3] A drawback of imatinib therapy presented itself in emerging resistance towards imatinib, found to be caused by several molecular resistance mechanisms, in particular by point mutations in the kinase domain of BCR-ABL that prevent imatinib but not ATP, from binding to the enzyme.

Frequency of primary and secondary resistance

The occurrence of primary hematologic resistance in early chronic phase CML treated with imatinib or second generation TKI, such as dasatinib or nilotinib, is a rare event. Primary resistance is defined as the failure to achieve a certain degree of initial response. According to the treatment recommendations from the European LeukemiaNet (ELN), primary imatinib resistance exists if a patient fails to achieve: a complete hematologic response (CHR) by 3 months, any cytogenetic response (CyR) by 6 months, a partial CyR (PCyR) by 12 months, or a complete CyR (CCyR) by 18 months on imatinib.[4] In the current ELN recommendations, failure to achieve a major molecular response (MMR) at 18 months is not yet defined as primary resistance but as a suboptimal response. However, patients who fail to achieve a MMR at 18 months have an increased risk of developing imatinib resistance. Hematologic imatinib failures in early chronic phase occur in less than 5% of cases.[5,6] In contrast, primary cytogenetic failures are more common and occur in 3-18% at 6 months,[3,6] 15-27% at 12 months,[6,7] and 23-49% at 18 months in CML patients treated with imatinib.[6,8] Primary treatment failure occurs less frequently with the more potent TKI dasatinib and nilotinib.[9,10] Since the frequency of primary resistance depends on recommended arbitrary endpoints, which should be achieved during therapy, the numbers for primary resistance may increase also for the second-generation inhibitors in the future, as these endpoints will certainly be modified over time.

Secondary resistance is defined as a loss of a previously achieved hematologic, cytogenetic, or molecular response despite continued TKI treatment. In early chronic phase CML patients, secondary resistance is an infrequent event. In the IRIS study, early chronic phase CML patients were treated with imatinib and only approximately 4% of patients per year had a progression event. Importantly, the annual rates of secondary resistance or death in the IRIS study continuously decreased from the second (7.5%) to the sixth year (0.4%) so that the risk to develop secondary resistance to TKI peaks in the first years of treatment.[3] The situation is different in advanced phase CML, such as accelerated phase (AP), blast crisis (BC), or BCR-ABL positive ALL in which primary and secondary resistance are observed much more frequently. In advanced phase CML, primary hematologic failure was reported in 18-30% of patients with AP and in 60% of patients with BC. After 4 years, resistance to imatinib had emerged in 45-70% of patients in accelerated phase and up to 90% of patients in blast crisis CML.[2,11-14]

Mechanisms of resistance

The mechanisms of TKI resistance were first studied in cell culture-based systems. BCR-ABL positive cells were incubated at suboptimal concentrations of imatinib for longer periods of time and some clones developed a moderate imatinib resistance.[15-17] It could be demonstrated that some of these clones acquired amplification of the *BCR-ABL* gene or overexpression of the multidrug-resistance membrane associated transporter protein MDR-1.[15-17] However, in these first *in vitro* studies in which gradually increasing concentrations of the inhibitor were applied, no mutations in the BCR-ABL kinase domain could be identified. Clinical studies in which resistance to imatinib primarily occurred in advanced-phase CML and Ph+ ALL prompted intensive investigations to identify mechanisms of imatinib resistance in primary patient samples. In 2001, it was shown for the first time by Sawyer's group that 3 out of 11 imatinib resistant patients with advanced CML or Ph+ ALL displayed amplification of the *BCR-ABL*

gene.[18] In addition, this group was able to identify a mutation in the *BCR-ABL* kinase domain in 6 out of 9 imatinib resistant patients. This mutation leads to an amino acid exchange at position 315 from threonine to isoleucine (Figure 1).[18] The larger amino acid isoleucine instead of threonine at this position blocks binding of imatinib while still allowing the kinase to utilize ATP,[19] resulting in strong resistance against the drug.[18] Subsequently, a large number of additional *BCR-ABL* mutations were identified in imatinib resistant patients leading to variable degrees of resistance.[20-25] The location of the most abundant BCR-ABL point mutations in the kinase domain relative to the imatinib binding site is shown in Figure 2. As illustrated, most mutations are scattered around the imatinib-binding site interfering with drug binding. Besides BCR-ABL amplification and BCR-ABL kinase domain mutations, cytogenetic abnormalities in addition to the Philadelphia chromosome were noted in imatinib resistant patients,[25] indicating that clonal evolution of an imatinib resistant leukemic subclone had occurred. BCR-ABL amplification, BCR-ABL kinase domain mutations, upregulation of drug transporters or additional BCR-ABL independent mutations may all alone or in combination occur in TKI resistant patients. In the following, this article will focus on BCR-ABL mutations as resistance mechanism, which can be identified in approximately 50% of cases of TKI resistance in early chronic phase CML and in the majority of cases in advanced phase CML and PH+ ALL.

Resistance mutations identified for imatinib

The first imatinib resistance mutation, which was discovered, leads to an exchange of threonine at position 315 to isoleucine[18] (Figure 1). Interestingly, a crystal structure analysis of the *ABL* kinase domain already published one year before this resistance mutation was identified in a patient, predicted threonine 315 to be a critical position (gatekeeper position) required for imatinib binding to *ABL*.[19] The T315I mutation mediates a strong imatinib resistance *in vitro* while the kinase activity of BCR-ABL is preserved or even enhanced.[18,26] To date, more than 90 different imatinib resistance mutations have been described affecting more than 55 amino acid residues in BCR-ABL.[7,18,20-24,27-30] The great majority of the almost 100 reported resistance mutations can only be detected in very few patients. However, some mutations are more common, among them mutations at the amino acid residues G250, Y253, E255, T315, M351, F359, and H396. They account for approximately 70% of all mutations identified in resistant patients. Patients developing imatinib resistance mutations have a lower progression free and overall survival.[31,32]

In chronic phase CML, mutations are the most frequent resistance mechanism overall and can be detected in about 40-59% of cases of secondary resistance.[33-37] Mutations are less frequent in cases of primary resistance in chronic phase CML and were reported in 24% of cases.[37,38] In contrast, the vast majority of patients with CML blast crisis and Ph+ ALL and imatinib resistance display resistance mutations.[39,40]

Most mutations identified in imatinib resistant patients are located within the *ABL* kinase domain. They lead to structural changes of the kinase domain so that imatinib is no longer able to prevent ATP from binding to the ATP pocket while at the same time, the kinase activity is retained. Based on the mechanism of resistance conferred by these mutations, they can be classified into two distinct groups:

1. Imatinib-contact positions, such as Y253, T315, and F317. This class of mutations affects amino acids, which are directly involved in binding of the drug. Mutations at these positions thus directly impede drug binding.
2. Mutations that destabilize the inactive conformation of BCR-ABL. These mutations include exchanges at positions located within the activation loop, such as H396 and M388, or mutations of SH2-contact positions, such as M351. This second class of mutations most likely shifts the equilibrium of the kinase from

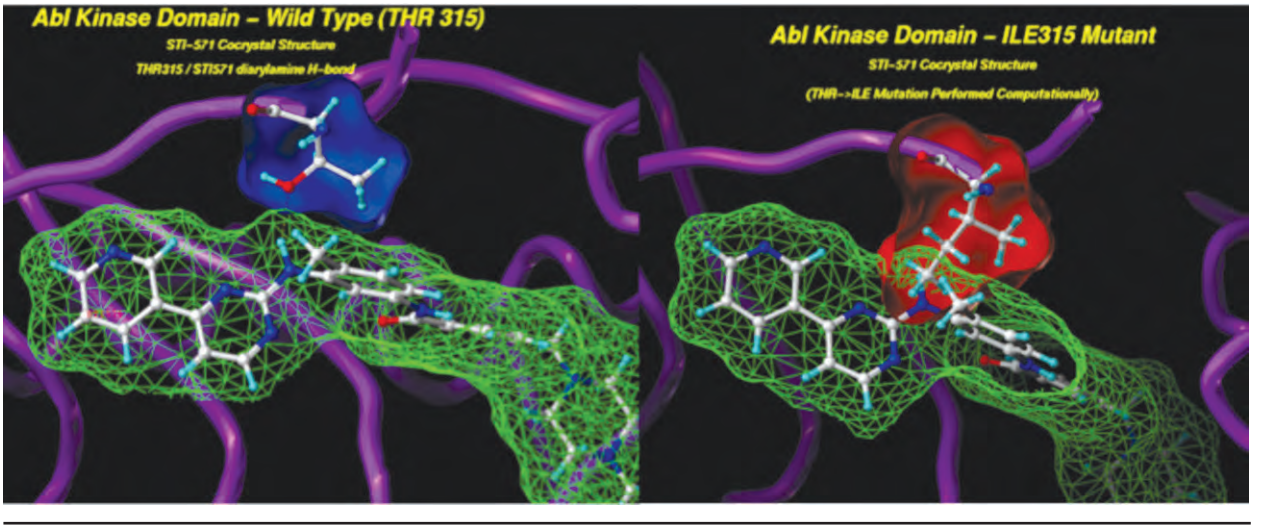

Figure 1. The position of threonine 315 (marked in blue, left) in Bcr-Abl relative to bound imatinib is depicted. Exchange of the compact amino acid isoleucine at position 315 to the bulkier amino acid isoleucine (marked in red on the right) leads to a strong steric hindrance. Picture is a courtesy of Darren R. Veach, MSKCC,NY.

an inactive towards an active state. Since imatinib most efficiently binds to the inactive conformation of the kinase with the activation loop in a closed position,[19] access of imatinib to the ATP-binding site is impaired.[20,24,41]

Besides the mutations in the kinase domain described above, in rare cases, imatinib resistance mutations outside of the kinase domain have been described.[42] These mutations are localized in the linker, SH2, SH3, and Cap domains adjacent to the kinase domain and were first identified in an *in vitro* resistance screen[43] and subsequently in few cases of imatinib resistant patients in a study performing sequence analysis outside of the kinase domain in imatinib resistant patients.[42] These domains have been reported to inhibit the kinase domain[44] and thus mutations in this region can enhance kinase activity. T212R was identified in an imatinib resistant patient and also mediated tyrosine kinase inhibitor resistance *in vitro*. Although mutations outside the kinase domain seem to be very rare, they may be responsible for resistance in patients in which a typical mutation in the kinase domain cannot be detected.

Mutations directly affecting imatinib binding (class I mutations) usually lead to strong imatinib resistance in most cases. Examples are T315I, Y253H, and E255V.[18,20] In contrast, mutations, which stabilize the inactive conformation (class II mutations), such as H396P, often lead to moderate imatinib resistance only.[20,24,41] Therefore, the type of mutation can affect the therapeutic management in case of imatinib resistance. Increasing the dose of imatinib may be sufficient to overcome resistance due to a moderately class II resistant BCR-ABL mutation but will not be sufficient to block a strong class I resistance mutation. Therefore, TKI IC_{50} values for different resistance mutations determined *in vitro*, as shown in Table 1, might be of help in choosing an effective second line therapy.

Resistance mutations have also been grouped based upon the region of the kinase domain in which the mutation occurred. Frequently affected regions include single amino acid residues, which directly interact with the drug, such as the "gatekeeper" position T315. Other domains frequently affected by mutations include the activation loop (A-loop), the ATP phosphate-binding loop (P-loop), and the C-Helix. However, in contrast to the categorization in class I and class II mutations described above, the mere position of an exchange does not allow to estimate the degree of resistance. As an example, the class of P-loop mutations include both moderate (G250A, Q252H, E255D), as well as strong imatinib resistance mutations (G250E, Y253H, E255V).[45]

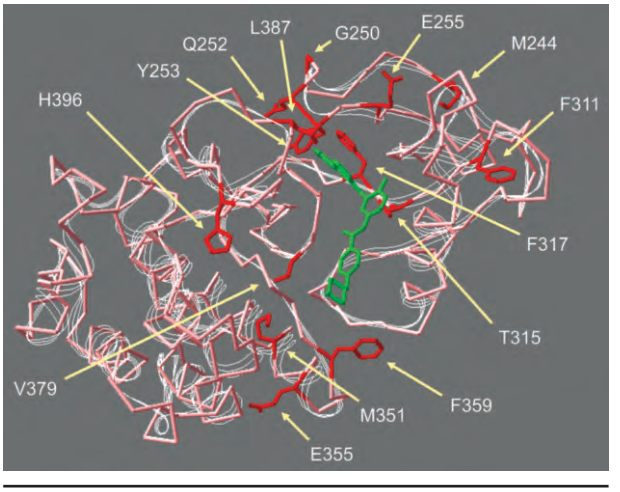

Figure 2. The crystal structure of the BCR-ABL kinase domain is shown in purple, imatinib in green and common imatinib resistance mutation are highlighted in red and marked by an arrow. Many mutations affect amino acid residues directly involved in binding oft the drug (class I mutations) whereas other positions are not in direct contact with imatinib and modulate the structure oft the binding pocket (class II mutations).

Mutations associated with second-generation inhibitors

Most mutations described above have been identified and characterized in the setting of imatinib resistance. The second-generation tyrosine kinase inhibitors dasa-

Table 1. Cellular IC_{50}-values of frequently observed imatinib resistance mutations for imatinib, nilotinib, and dasatinib. IC_{50}-values are based on studies performed in cell lines, which express BCR-ABL mutations that were identified in patients with CML or Ph+ ALL and resistance to imatinib cited in the manuscript.

	Imatinib		Nilotinib		Dasatinib	
	IC_{50} [μM]	factor IC_{50} wt	IC_{50} [nM]	factor IC_{50} wt	IC_{50} [nM]	factor IC_{50} wt
Wild-type	0.4	-	25	-	1	-
P-loop						
M244V	2.3	5.8	67	2.7	1.3	1.3
L248V	1.5	3.8	102	4.2	NR	-
G250A	1.3	3.3	65	2.6	NR	-
G250E	3.9	7.5	145	5.8	1.8	1.8
Q252H	1.2	3	67	2.7	3.4	3.4
Y253F	9	22.5	125	5	1.4	1.4
Y253H	>10	>25	700	28	1.3	1.3
E255K	10	25	566	23	5.6	5.6
E255V	>10	>25	681	27	11	11
C-helix						
D276G	1.5	3.8	69	2.8	NR	-
F311L	1.3	3.25	23	1	1.3	1.3
T315I	>10	>25	>10.000	>400	>5.000	>5.000
T315S	3.8	9.5	NR	-	NR	-
F317L	1.5	3.8	80	3.2	7.4	7.4
SH2-contact						
D325N	1.5	3.8	25	1	NR	-
S348L	0.7	1.4	26	1	NR	-
M351T	1.3	3.25	33	1.3	1.1	1.1
E355G	0.4	1	47	1.9	NR	-
F359C	1.2	3	291	12	NR	-
F359V	1.2	3	161	6.4	2.2	2.2
A-loop						
L387F	1.1	2.8	39	1.6	NR	-
L387M	1	2.5	49	2	2	2
H396P	2.5	6.25	41	1.6	0.6	1
H396R	1.75	4.4	41	1.6	1.3	1.3

tinib and nilotinib are active in imatinib resistant disease, including patients with BCR-ABL kinase mutations with the notable exception of T315I.[46,47] Given first line in chronic phase CML, both compounds compare favorably to imatinib with respect to response rates at a follow-up of 24 months.[10,48] *In vitro* studies suggest a narrowed, but partially overlapping spectrum of resistance mutations with the novel inhibitors compared with imatinib (Table 1).[49-53] As clinical data and follow up times with second-generation inhibitors in first and second line therapy mature, a specific pattern of resistance mutations in patients has emerged that is associated with resistance to dasatinib and nilotinib.

Clinical studies demonstrated that mutations identified *in vitro* (Q252H, E255K/V, V299L, F317L, and T315I for dasatinib; Y253H/F, E255K/V, F311I, T315I, and F359C/V for nilotinib) were associated with less favorable response rates, and also emerged at the time of disease progression receiving second line treatment.[54-56] The mutations F317L/I/C/V, V299L, T315A, and T315I for dasatinib, and the mutations Y253H, E255K/V, F359V/C, and T315I for nilotinib have been classified as SGI (second generation inhibitor) clinically relevant mutations by most investigators.[57] This set of mutations also emerged as newly acquired mutations with dasatinib or nilotinib as 2nd or 3rd TKI, and in patients receiving nilotinib or dasatinib as first line therapy.[9,10,58] Interestingly, studies investigating nilotinib or dasatinib in the second line setting could demonstrate that patients already harboring imatinb resistance mutations at the start of second line therapy had a greater risk of developing additional mutations.[55,58] This indicates that there is a subset of patients with a larger risk to develop resistant mutations due to enhanced genetic instability of the BCR-ABL oncogene.

Resistance mutations occurring during sequentially TKI therapy

Many patients are treated with a sequence of three or more Abl TKIs. The frequency of resistance mutations increases with sequential TKI therapy and was found in one study in 83% of cases.[58] Clinical data suggest that this therapeutic approach is associated with the emergence of specific mutational patterns, like selection for the pan-resistant T315I mutation and acquisition of additional, drug-specific mutations that in some cases emerge in the form of compound mutations, *i.e.*, several mutations on the same BCR-ABL transcript, or multiple mutations, *i.e.*, several mutations in different disease clones in one patient.[55,58-60] It has been suggested that compound mutations modulate the response to inhibitors in unexpected ways, thereby making prediction of response more complex.[57] Multiple mutations emerging in patients during sequential TKI therapy might be compound mutations in sequentially resistant cell clones, making sequential monotherapy a much less effective choice for these patients. A different study identified a poor risk subgroup of imatinib resistant CML patients with multiple mutations detected by sensitive mutation analysis, but not by conventional sequencing.[61] The presence of multiple mutations adversely affected response to second line nilotinib or dasatinib and favored the emergence of new mutations as detected by standard sequencing, with 10 out of 25 cases harboring more than one mutation. Therapeutic strategies minimizing the occurrence of resistance may consist in upfront combination therapy of several TKIs, combination therapy with interferon or first line therapy with nilotinib or dasatinib.

Cell-based *in vitro* assays may help to study the potential resistance mutation pattern emerging after differently ordered sequential TKI treatments and to study the impact of compound mutations on drug sensitivity. A recent study aimed at depicting the clinical approach of sequential inhibitor therapy *in vitro*, using a modified, cell-based screening model (Bauer *et al.*, in preparation). The results revealed significant differences in response characteristics and evolution of individual mutations depending on the order and concentration of nilotinib and dasatinib sequentially administered in identical replicates of imatinib resistant cell lines. Sequential TKI monotherapy *in vitro* was more often associated with step-wise acquisition of double and triple mutations compared with acquisition of the pan-resistant T315I mutation. The composition of compound mutations depended on inhibitor type and order of application. This growth/resistance behavior *in vitro* is reminiscent of the development of multidrug-resistant bacterial strains and is compatible with a "fertile ground" process with step-by-step acquisition of additional mutations in one disease clone.

Which second line therapy should be selected in patients with resistance mutations?

Correlation of mutational status and clinical outcome indicate that the *in vitro* sensitivity of a certain mutation towards a second-generation inhibitor predicts for a greater chance to achieve a CCyR after imatinib failure.[56,62,63] Another study found such a correlation for some but not all mutations.[54] In addition, recently a paper correlated clinical trial data with published *in vitro* IC_{50} values, which had been adjusted to the peak plasma levels achievable with nilotinib and dasatinib.[64] This study found only a poor correlation of adjusted IC_{50} values and clinical responses for many mutations, indicating that mutation analysis not in all cases can predict treatment outcome. This is probably due to the fact that in addition to the type of mutation, drug influx and efflux and individual pharmacogenetics also play an important role for the response to a second line TKI. Thus, treatment choice in the second line setting cannot be based solely on IC_{50} values determined *in vitro*. On the other hand, a small subset of mutations clearly predict for clinical outcome in the second line setting.[57] It is clear that presence of T315I is associated with lack of response to nilotinib and dasatinib. In addition, mutations at F317 and V299 are associated with a poorer response to dasatinib, whereas mutations at Y253, E255, and F359 are associated with a lower response to nilotinib.[57] Therefore, in these few selected cases, second line therapy should be based on the sensitivity of the mutations and chosen accordingly. In the vast majority of cases, however, second line therapy can be selected according to pre-existing comorbidities or personal preference.

T315I

T315I stands out among the resistance mutations, as BCR-ABLT315I is the only mutation, which does not respond to any of the currently approved TKIs (Table 1). This has led to an intensive search for third generation inhibitors which either are able to block the ATP binding pocket despite the presence of the bulkier isoleucine at position 315, such as ponatinib or allosteric inhibitors which do not block the ATP pocket (DCC-2036).[65-67] Ponatinib has produced impressive responses in T315I mutated patients in a phase I/II trial.[68] Although recruitment in the ponatinib trials is completed, this drug can be obtained in many European countries within a compassionate use program. DCC-2036 is currently under investigation in a phase I trial. In addition, other drugs have shown activity in T315I mutated patients, such as the plant alkaloid omeacetaxine in a phase II trial.[69-73] The action of this drug is not yet known and it displays significant myelotoxicities. Since approved drugs for T315I mutated patients are not available at this time, allogeneic stem cell transplantation should be considered to improve the prognosis of these patients. If this is not an option, these patients whenever possible should be treated within a clinical trial with compounds with documented activity against T315I mutated Abl.

Mutational analysis

An ELN consensus paper recommends mutational analysis in any patient with CP-CML with suboptimal response or failure to respond to imatinib according to the ELN criteria, or after loss of a previously achieved CHR, CCyR, or MMR. In addition, mutational analysis is recommended at any time prior to changing therapy.[4]

Different methods are currently used for the detection of *BCR-ABL* kinase domain mutations, and their sensitivity ranges from lower than 0.01 to 20%. DHPLC (denaturing high-performance liquid chromatography)-based methods are utilized in many laboratories since they offer the advantage of high throughput capacity screening for multiple mutations at one time.[74-76] Sensitivity is in the range of 0.1 to 10%. Mutations detected by HPLC are confirmed by conventional direct sequencing. The sensitivity of conventional direct sequencing is in the range of 20%.[28] Both methods may not pick up mutations only present in a subset of cells. Whether these low level mutations contribute to disease prognosis is a matter of debate. Several investigators found level mutation not correlated with event-free or overall survival or with response to TKI.[23,24,77-80] Thus, the presence of a resistance mutation at low level does not mean that the affected disease clone will be selected in the presence of TKI.

Highly sensitive methods include allele-specific (ASO)-PCR, DHPLC/wave technology, SSCP, PCR-RFLP, or next generation sequencing technologies. However, a recent comparison of these non-standardized techniques in different laboratories showed a great variability in the detection of low-level mutations in a given sample.[81] Therefore, direct sequencing seems sufficiently sensitive to detect clinically significant mutant leukemic subpopulations, and in the moment still can be considered as the technology of choice. Detection of low-level mutant disease clones in a patient without evidence for disease progression may have prognostic impact in the future but cannot be recommended routinely at the moment.[61,82]

Resistance mutations in other malignancies

CML is not only a model disease for the development of TKI but also a pacemaker disease to explore mechanisms of TKI resistance. In this context, similar mutations conferring resistance to kinase inhibitors were also identified in several other malignancies. Imatinib resistance mutations were identified in FIP1L1-PDGFRα in patients with hypereosinophilic syndrome.[45,83] and in cKit in patients with gastrointestinal stromal tumors (GIST).[84,85] In addition, a resistance mutation in the kinase domain of FLT3-ITD in an AML patient treated with the kinase inhibitor PKC412 has been described (86). Similarly, in patients with non-small cell lung cancer (NSCL) treated with the kinase inhibitor gefitinib, an exchange of threonine at position 790 to methionine in the *epidermal growth factor receptor* (EGFR) was reported.[87,88] Mutations in the EGFR receptor even seem to account for the majority of cases of secondary resistance in gefitinib and erlotinib treated NSCLC patients.[89] Interestingly, the pattern and the resistance mechanisms of mutations identified in other oncogenic kinases and malignancies remarkably resemble the situation found in TKI resistant CML patients. T790M found in TKI resistant NSCLC patients, cKit/T670I found in TKI resistant GIST, and also FIP1L1-PDFGRα/T674I found in TKI resistant patients with hypereosinophilic syndrome are all homologous to the position T315 in the Abl kinase domain. Thus, many lessons learned in the past concerning resistance mutations in CML might be extrapolated to other malignancies.

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