

Contribution of ABL Kinase Domain Mutations to Imatinib Resistance in Different Subsets of Philadelphia-Positive Patients: By the GIMEMA Working Party on Chronic Myeloid Leukemia

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on behalf of the GIMEMA Working Party on Chronic Myeloid Leukemia

Abstract Purpose: ABL kinase domain mutations have been implicated in the resistance to the BCR-ABL inhibitor imatinib mesylate of Philadelphia-positive (Ph+) leukemia patients.

Experimental Design: Using denaturing high-performance liquid chromatography and sequencing, we screened for ABL kinase domain mutations in 370 Ph+ patients with evidence of hematologic or cytogenetic resistance to imatinib.

Results: Mutations were found in 127 of 297 (43%) evaluable patients. Mutations were found in 27% of chronic-phase patients (14% treated with imatinib frontline; 31% treated with imatinib post-IFN failure), 52% of accelerated-phase patients, 75% of myeloid blast crisis patients, and 83% of lymphoid blast crisis/Ph+ acute lymphoblastic leukemia (ALL) patients. Mutations were associated in 30% of patients with primary resistance (44% hematologic and 28% cytogenetic) and in 57% of patients with acquired resistance (23% patients who lost cytogenetic response; 55% patients who lost hematologic response; and 87% patients who progressed to accelerated phase/blast crisis). P-loop and T315I mutations were particularly frequent in advanced-phase chronic myeloid leukemia and Ph+ ALL patients, and often accompanied progression from chronic phase to accelerated phase/blast crisis.

Conclusions: We conclude that (a) amino acid substitutions at seven residues (M244V, G250E, Y253F/H, E255K/V, T315I, M351T, and F359V) account for 85% of all resistance-associated mutations; (b) the search for mutations is important both in case of imatinib failure and in case of loss of response at the hematologic or cytogenetic level; (c) advanced-phase chronic myeloid leukemia and Ph+ ALL patients have a higher likelihood of developing imatinib-resistant mutations; and (d) the presence of either P-loop or T315I mutations in imatinib-treated patients should warn the clinician to reconsider the therapeutic strategy.

Imatinib mesylate (1–5) is a potent and selective inhibitor of the oncogenic BCR-ABL tyrosine kinase, which is deregulated in as many as 95% of chronic myeloid leukemia (CML) patients and in ~20% of adult Philadelphia-positive (Ph+) acute lymphoblastic leukemia (ALL) patients. Despite its striking efficacy, however, resistance is observed in a proportion of patients, especially those with Ph+ ALL or advanced-stage CML.

Through the contribution of several research groups, the past 4 years have brought us considerable knowledge on the molecular mechanisms underlying resistance to imatinib (reviewed in ref. 6). Reactivation of BCR-ABL tyrosine kinase activity within the leukemic clone is most commonly associated with the emergence of point mutations in the ABL kinase domain that impair imatinib binding without affecting ATP

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Received 6/23/06; revised 8/24/06; accepted 10/5/06.

Grant support: European LeukemiaNet, Associazione Italiana Leucemie, Associazione Italiana per la Ricerca sul Cancro, Fondazione Del Monte di Bologna e Ravenna, Fondo Istituzionale per la Ricerca di Base, and Programma di Rilevante Interesse Nazionale.

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doi:10.1158/1078-0432.CCR-06-1516

binding or kinase activity (7, 8). To date, >40 different amino acid substitutions have been reported (9–19). Most of these amino acid substitutions have already been characterized in terms of the extent to which they abrogate sensitivity to imatinib (10, 11, 13, 20). However, very few studies have been published on large and homogeneous series of resistant patients, and this has made it difficult to establish to what extent mutations account for, or at least contribute to, resistance in its different clinical manifestations or in distinct disease categories.

To shed further light on this issue, we have collected and analyzed, for the presence of ABL kinase domain mutation, samples from 370 CML or Ph+ ALL patients treated with imatinib at multiple centers of the GIMEMA Working Party on CML, who had clinical evidence of hematologic or cytogenetic resistance.

Patients and Methods

Patients and definitions. Between January 2004 and November 2005, bone marrow and/or peripheral blood samples from 370 CML or Ph+ ALL patients at the time of first evidence of resistance to imatinib (according to the definitions given below) were collected from multiple centers of the GIMEMA Working Party on CML and analyzed at our institution for the presence of ABL kinase domain mutations. All of the patients were receiving imatinib at standard doses of 400 to 600 mg/d. Informed consent for participation to this study was provided according to the Declaration of Helsinki. Chronic phase, accelerated phase, and blast crisis were defined as in recent studies (2–5, 21). Clinical features of lymphoid blast crisis resembled Ph+ ALL; therefore, these diseases were analyzed together. Primary hematologic resistance was defined as failure to achieve and sustain any hematologic response for at least 4 weeks during the first 3 months of imatinib therapy. Primary cytogenetic resistance was defined as failure to achieve and sustain a complete cytogenetic response (100% Ph⁻ metaphases, based on the evaluation of a minimum of 20 marrow cells) for at least 4 weeks during the first 12 months of imatinib therapy. Acquired resistance was defined as loss of complete cytogenetic response, loss of hematologic response, or progression to accelerated phase or blast crisis.

Sensitivity and reliability of mutation detection is very dependent on the quality and integrity of RNA (17). Given that no bedside RNA stabilization was done before shipment of blood or bone marrow to our institution, samples were first of all assessed for the level of BCR-ABL and ABL transcripts (22, 23) and were subjected to mutation screening only if the RNA obtained from the sample contained a measurable level of BCR-ABL transcript and if the ABL control gene level indicated a nondegraded RNA. Samples from 73 (20%) patients were therefore discarded because of inadequate RNA quality, which was influenced neither by patient clinical features nor by type of resistance, but depended only on shipment conditions and time to delivery. Two hundred and ninety-seven patients were therefore evaluable for

the aims of this study. Median age at imatinib start was 49 years (range, 17–70 years). Median time between diagnosis and imatinib start was 32 months (range, 0–160 months). Median duration of imatinib was 25 months (range, 4–42 months). Classification of patients in terms of disease phase and type of resistance is shown in Table 1.

RNA extraction and reverse transcription-PCR. Total cellular RNA was extracted from leukocytes and reverse transcribed as previously reported (19, 24).

Denaturing-high performance liquid chromatography analysis. Scanning of the ABL kinase domain for the presence of mutations was done as previously reported, with minor modifications (19, 24). Briefly, after a first amplification of a fragment spanning both the BCR-ABL breakpoint and the ABL kinase domain, three overlapping amplicons covering the kinase domain (amino acids 206–335, 262–421, and 371–524) were generated by nested PCR and were screened for the presence of sequence variations by denaturing high-performance liquid chromatography (DHPLC; WAVE 3500-HT; Transgenomic, Cramlington, United Kingdom). Sensitivity of the assay ranged between 5% and 10% (data not shown). To ensure that mutations present in ≥90% of BCR-ABL⁻positive cells could not escape DHPLC detection, a mixture of wild-type and patient PCR products in a 1:1 ratio was also run for all samples studied.

Direct sequencing. Direct sequencing of DHPLC-positive cases was done on an ABI PRISM 3730 (Applied Biosystems, Foster City, CA) as previously reported (19, 24). The sensitivity of the method was 20% to 25% (data not shown).

Statistical analysis. Fisher's exact test was used to test for differences in mutation frequency among categories of patients. Analyses were done using the SPSS software (SPSS, Inc., Chicago, IL).

Results

DHPLC and sequence analyses for ABL kinase domain mutations. DHPLC analysis showed evidence of one or more sequence variations in 127 of 297 (43%) patients. Subsequent direct sequencing failed to detect any nucleotide substitution in 18 patients (4 chronic-phase patients treated with imatinib frontline, 12 chronic-phase patients post-IFN failure, 1 accelerated-phase patient, and 1 Ph+ ALL); however, in all cases, the presence of a mutation became evident when sequence analysis was repeated on a second sample taken after 1 to 2 months from DHPLC analysis. In eight patients (two Ph+ ALL, two myeloid blast crisis, two lymphoid blast crisis, one accelerated phase, and one chronic phase post-IFN failure), multiple mutations simultaneously occurred so that, overall, 135 mutations were detected. Mutations mapped to 17 codons, the most frequent ones being E255K/V (21 patients, 17%), Y253F/H (17 patients, 13%), T315I (15 patients, 12%), M351T (14 patients, 11%), F359V/I (14 patients, 11%), M244V (13 patients, 10%), and G250E (13 patients, 10%). Distribution and relative frequency of kinase domain mutations found in

Table 1. Classification of the patient population under study

	All	Primary resistance (%)	Acquired resistance (%)
Total no.	297	152 (51)	145 (49)
Chronic phase	198	116 (59)	82 (41)
Imatinib frontline	44	28 (64)	16 (36)
Imatinib after α -IFN failure	154	107 (69)	47 (31)
Accelerated phase	21	5 (24)	16 (76)
myeloid blast crisis	32	5 (16)	27 (84)
lymphoid blast crisis/Ph+ ALL	46	7 (15)	39 (85)

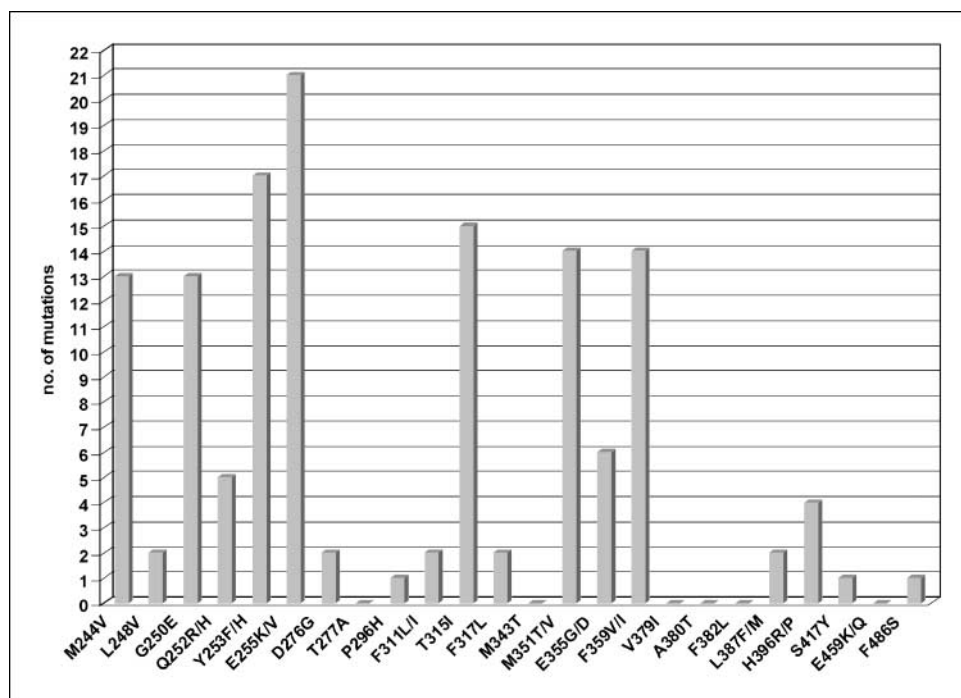


Fig. 1. Distribution and relative frequency of ABL kinase domain mutations found in our study.

our study are shown in Fig. 1. Mutations falling within the P-loop region (codons 248-255) were found in 58 patients (46% of all mutated patients).

Frequency and distribution of mutations according to disease phase at the time of analysis. Mutations were found in 54 of 198 (27%) first-chronic-phase patients [6 of 44 (14%) treated with imatinib frontline; 48 of 154 (31%) treated with imatinib after α -IFN failure], 11 of 21 (52%) accelerated-phase patients, 24 of 32 (75%) myeloid blast crisis patients, and 38 of 46 (83%) lymphoid blast crisis/Ph+ ALL patients (chronic phase versus accelerated phase, $P = 0.02$; accelerated phase versus blast crisis, $P = 0.02$; chronic phase versus blast crisis, $P < 0.0001$; Table 2). When we examined the position and relative frequency of mutations by disease phase, we noticed a trend toward the preferential association of P-loop and T315I mutations and advanced stages of disease (Fig. 2).

Frequency and distribution of mutations according to type of resistance. Mutations were associated in 45 of 152 (30%) patients with primary resistance (8 of 18 hematologic and 37 of 134 cytogenetic) and in 82 of 145 (57%) patients with acquired resistance (12 of 52 patients who lost complete cytogenetic response, 18 of 33 patients who lost hematologic response, and 52 of 60 patients who progressed to accelerated phase/blast crisis; primary versus acquired, $P < 0.0001$; Table 3). Primary or acquired resistance did not seem to significantly differ in terms of type or relative frequency of mutations responsible for the resistance (Fig. 3); however, 40 of 52 mutated patients who had progressed to accelerated phase/blast crisis harbored P-loop or T315I mutations (data not shown).

Discussion

The aim of the present study was to investigate the presence of ABL kinase domain mutations in a large series of Ph+, imatinib-resistant cases. This was done to assess the extent to

which mutations account for, or at least contribute to, resistance in its different clinical manifestations or in distinct disease categories. Of 297 CML and Ph+ ALL patients with adequate RNA quality for BCR-ABL amplification and DHPLC/sequencing analyses, 127 (43%) showed evidence of one or more kinase domain mutations. Such an incidence is somewhat lower than expected, but this is mainly due to the high degree of variability in mutation frequencies we observed among different subsets of patients. Indeed, when we separately considered the mutation frequencies by disease phase (Table 2), we observed that the contribution of kinase domain mutations to the resistant phenotype was much lower in chronic-phase patients than in accelerated-phase and blast crisis patients, with lymphoid blast crisis and Ph+ ALL patients having the greatest likelihood of harboring one, or even multiple, mutations. Similarly, mutation incidence in patients with primary resistance was much lower than in patients with acquired resistance (Table 3). The relatively low overall frequency of mutation in our series with respect to other published studies may therefore be explained by the predominance of chronic-phase patients (67%) as well as by the relatively high number of patients with primary resistance (51%).

The difference in mutation incidence between disease phases leads to several considerations. On one hand, it points toward advanced-phase CML and Ph+ ALL cases as patients at high risk of emergence of resistance-associated mutant clones. There are currently no published data supporting the evidence that a systematic screening allowing for an early detection of emergent kinase domain mutations is more beneficial than examining ABL sequences only in case overt resistance to imatinib is observed. However, our data suggest that, at least in the setting of advanced-phase CML or Ph+ ALL patients, it might be worth assessing—ideally in the context of a prospective study—whether a regular mutation monitoring may assist clinicians in treatment optimization. On the other hand, the evidence

Table 2. Frequency of mutations according to disease phase

	All	Mutated (%)
Total no.	297	127 (43)
Chronic phase	198	54 (27)
Imatinib frontline	44	6 (14)
Imatinib after α -IFN failure	154	48 (31)
Accelerated phase	21	11 (52)
Myeloid blast crisis	32	24 (75)
Lymphoid blast crisis/Ph+ ALL	46	38 (83)

that mutations in chronic-phase patients account for approximately a quarter of resistant cases only highlights the need to find out which is the actual predominant mechanism(s) of resistance acting in this setting, which now gathers the overwhelming majority of CML patients on imatinib therapy. BCR-ABL gene amplification and additional chromosomal aberrations are also known to be associated with imatinib resistance (9, 11); however, they seem to play a role mainly in advanced CML phases. Point mutations in BCR or ABL regions other than the kinase domain have been hypothesized based on the results of an *in vitro* saturation mutagenesis screening for mutations conferring resistance to imatinib (25) and on the assumption that any amino acid substitution favoring the active conformation of BCR-ABL (to which imatinib is unable to bind) may confer resistance; this, however, have not been described in patients as yet. Overexpression of drug transporters, such as hOCT1, has recently been reported in some chronic-phase patients with primary cytogenetic resistance (26) and is an issue that deserves further elucidation. Among chronic-phase patients, however, mutation incidence in those who had received imatinib after α -IFN failure was approxi-

mately twice as high as in those who had received imatinib as first-line therapy (31% versus 14%). Such an intriguing difference between early and late chronic phase supports the hypothesis that mutations tend to accumulate during the natural course of the disease as a result of a progressively increasing genetic instability and are therefore a feature of CML clinical deterioration and not necessarily a phenomenon observed only against a background of imatinib exposure. This would fit with the recent observation that kinase domain mutations may be detected in a substantial fraction of imatinib-naïve patients with advanced-phase CML (27).

Although there were 17 codons affected by mutations and the relative frequencies of each single amino acid substitution were consequently low, we observed that P-loop and T315I mutations were more recurrently found in advanced-stage CML and Ph+ ALL patients (Fig. 2). Even more importantly, in most cases, there seemed to be a close association between the emergence of these mutant clones and progression of patients from chronic phase to accelerated phase or blast crisis. The P-loop (amino acids 248-256) is a highly conserved region responsible for ATP phosphate binding (28, 29). Amino acid 315 is the so-called gatekeeper residue—the hydroxyl group of threonine 315 forms a hydrogen bond with imatinib, and the side chain also sterically controls the binding of the inhibitor to the ATP-binding site. Substitution of threonine with a bulkier and more hydrophobic isoleucine abolishes the hydrogen bond and determines a steric clash that renders the active site inaccessible not only to imatinib but also to most second-generation inhibitors (30). Among several mutants, G250E, Q252H, Y253F/H, E255K/V, and T315I displayed the highest IC₅₀ values in biochemical and cellular assays (20). However, the virtually complete insensitivity to imatinib conferred by such mutations may not be the only explanation for such a particularly aggressive leukemic phenotype. It has actually been hypothesized that the above-mentioned mutants may be

Fig. 2. Position and relative frequency of mutations according to disease phase. CP, chronic phase; AP, accelerated phase; myBC, myeloid blast crisis; lyBC, lymphoid blast crisis.

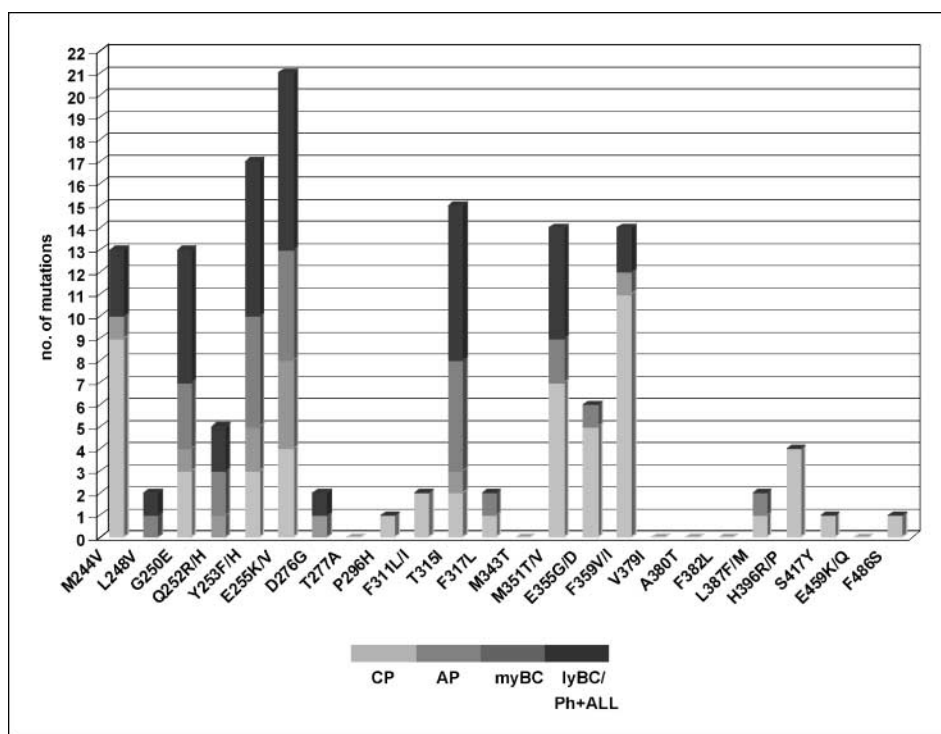


Table 3. Frequency of mutations according to type of resistance

	All	Mutated (%)
Total no.	297	127 (43)
Primary resistance	152	45 (30)
Hematologic	18	8 (44)
Cytogenetic	134	37 (28)
Acquired resistance	145	82 (57)
Loss of CCR	52	12 (23)
Loss of HR	33	18 (55)
Progression to AP/BC	60	52 (87)

Abbreviations: HR, hematologic response; CCR, complete cytogenetic response.

gain-of-function forms, which are characterized by a greater transforming potency with respect to other mutant forms or to wild-type BCR-ABL at least under the selective pressure of imatinib (14, 31).

Although it is rather well established that mutations are the main cause of resistance in relapsing patients, few and contrasting data are currently available about the incidence of mutations in patients with primary resistance to imatinib (11, 15). Some authors even hypothesized that resistance mechanisms other than ABL kinase domain mutations may underlie lack of response to imatinib. To the best of our knowledge, ours is the first study to investigate the contribution of ABL kinase domain mutations to primary resistance in a large series of patients. We show here that mutations can also be found in patients with primary resistance, although at a much lower frequency, and that there is no difference between primary and acquired resistance in terms of the identity of the amino acid substitutions that are responsible. Nevertheless,

some 70% of patients with primary resistance do not have evidence of kinase domain mutations. Again, because the contribution of kinase domain mutations to resistance in this setting of patients is modest, additional work is needed to find out whether as yet unidentified mechanism(s) exists.

According to recently published guidelines on CML management (21), mutation analysis of patients treated with imatinib is suggested in case there is evidence of inadequate response or any sign of loss of response. The knowledge of whether a kinase domain mutation is present, as well as of the type of mutation, may contribute to a timely and rational therapeutic management, especially now that the armamentarium against CML and Ph+ ALL is about to include second-generation inhibitors like dasatinib (BMS-354825) and nilotinib (AMN-107; refs. 32, 33). For those patients harboring mutations that are known to confer only moderate resistance to imatinib, dose escalation may be beneficial. For those who have evidence of mutations conferring total insensitivity to imatinib, allogeneic transplant, if feasible, or alternative inhibitors have to be considered. Our data support the notions that (a) mutation analysis should be done both in case of imatinib failure and in case of loss of hematologic or cytogenetic response; (b) the subsets of advanced-phase CML and Ph+ ALL patients are to be considered high-risk groups; and (c) the occurrence of P-loop or T315I mutations in patients treated with imatinib should trigger a rational reconsideration of the therapeutic strategy.

Appendix A. GIMEMA Working Party on Chronic Myeloid Leukemia

The following members of the GIMEMA Working Party on CML actively participated in this study: M. Lazzarino and S. Merante (Pavia); D. Ferrero and C. Della Casa (Turin);

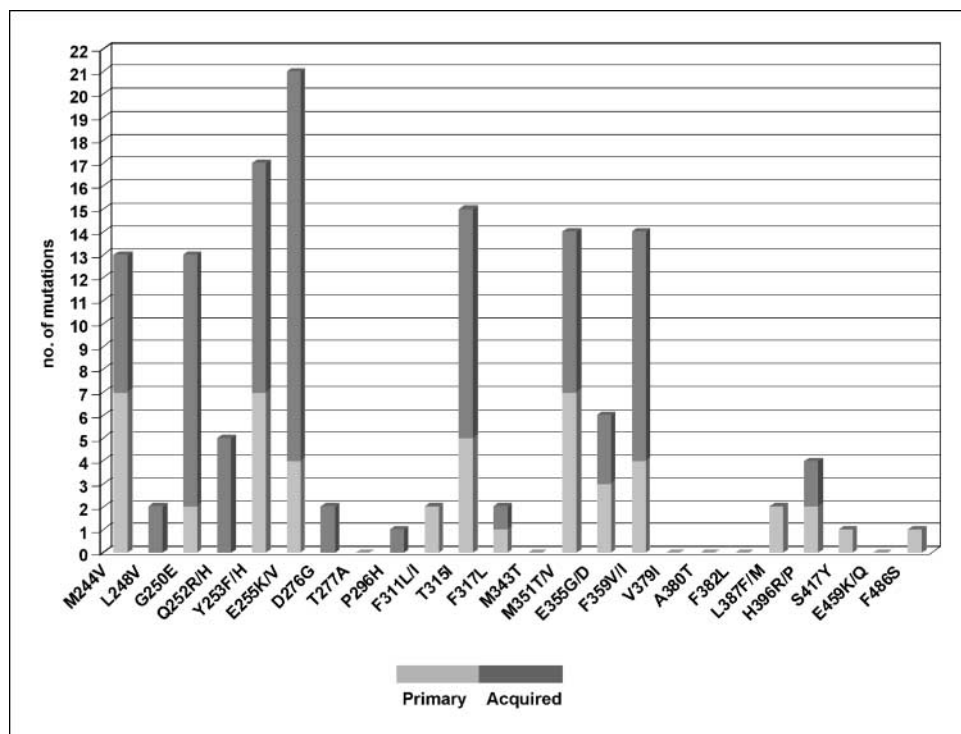


Fig. 3. Position and relative frequency of mutations according to type of resistance.

A. Bacigalupo and A. Dominietto (Genoa); S. Amadori and A. Cantonetti (Rome); R. Fanin (Udine); M. Michieli and M. Rupolo (Aviano); V. Liso and G. Specchia (Bari); G. Alimena e M. Breccia (Rome); D. Cilloni, G. Rege-Cambrin, E. Gottardi, and M. Fava (Orbassano); G. Visani and G. Nicolini (Pesaro); F. Lauria and M. Bocchia (Siena); T. Barbui and U. Giussani (Bergamo); E. Angelucci and E. Usala (Cagliari); E. Curioni (Milan); V. Abbadessa (Palermo); R. Marasca and G. Leonardi (Modena); G. Rossi and A. Capucci (Brescia); E. Morra and E. Pungolino (Milan); A. Peta and F. Iuliano (Catanzaro); S. Mirto, S. Tringali, and D. Turri (Palermo); P. Leoni and S. Rupoli (Ancona); A. Bosi and V. Santini (Florence); A. Liberati and E. Donti (Perugia); A. Zaccaria, E. Zuffa, and B. Giannini (Ravenna); M. Candela and G. Danieli (Ancona); S. Morandi and C. Bergonzi (Cremona);

E. Gallo and P. Pregno (Turin); G. Fioritoni (Pescara); A. Tabilio, C. Mecucci, and D. Falzetti (Perugia); A. Gallamini and M.A. Pistone (Cuneo); A. De Blasio (Latina); G. Pizzolo, A. Ambrosetti and V. Meneghini (Verona); F. Nobile, M. Martino, and E. Oliva (Reggio Calabria); F. Rodighiero and A. D'Emilio (Vicenza); G. Semenzato and L. Trentin (Padua); L. Cavanna, D. Vallisa, and E. Trabacchi (Piacenza); Dr. Giroto and Dr. D'Ardia (Ivrea); and L. Gugliotta and P. Avanzino (Reggio Emilia).

Acknowledgments

We thank Dr. Vilma Mantovani, Daniela Bastia, and Marinella Cenci (Center for Applied Biomedical Research, Bologna, Italy) for their contribution to DHPLC analysis, and Katia Vecchi for her valuable assistance.

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Contribution of ABL Kinase Domain Mutations to Imatinib Resistance in Different Subsets of Philadelphia-Positive Patients: By the GIMEMA Working Party on Chronic Myeloid Leukemia

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Clin Cancer Res 2006;12:7374-7379.

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