

LETTER TO THE EDITOR

An activating KRAS mutation in imatinib-resistant chronic myeloid leukemia

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Mutations in the kinase domain of BCR-ABL that impair drug binding are the best characterized mechanism of imatinib resistance in patients with chronic myeloid leukemia (CML) or Philadelphia chromosome (Ph)-positive acute lymphoblastic leukemia.¹ Some patients without kinase domain mutation-based resistance exhibit increased BCR-ABL expression.² However, in a sizable proportion of patients, neither of these mechanisms is demonstrable.³ Causes of imatinib resistance implicated in such patients include insufficient intracellular drug levels due to increased efflux or reduced influx or sequestration of imatinib through increased plasma protein binding.² Further, some kinase domain mutation-negative patients appear to have truly BCR-ABL-independent imatinib resistance, for example, through the activation of SRC family kinases or adaptive granulocyte-macrophage colony-stimulating factor secretion.^{4,5} A myeloproliferative disease with features of CML can be induced by a variety of genetic lesions, including activating mutations of *RAS*, *PTPN11*, *JAK2* and receptor tyrosine kinases such as *FLT3*. We therefore hypothesized that patients with acquired imatinib resistance but no evidence of BCR-ABL mutations or gene amplification may have activating mutations in such imatinib-insensitive pathways or that they may have acquired resistance mutations in the imatinib targets KIT and platelet-derived growth factor receptor α/β .

We identified a total of 17 imatinib-resistant CML patients (nine males and eight females) with a median age of 57 years (range: 34–69 years) (Table 1) who had no evidence of a BCR-ABL mutation on standard diagnostic sequencing. At the time of the study, cytogenetic analysis revealed a median of 40% (range: 10–100%) Ph-positive metaphases, fluorescence *in situ* hybridization showed a median of 43% (range: 1.5–98.5%) BCR-ABL-positive interphases, and quantitative PCR showed a BCR-ABL/G6PD median ratio of 2.5% (range: 0.52–210%). Out of the 17 patients, 10 never achieved a complete cytogenetic response before this study and seven had lost this level of response at the time of sampling. Sequence analysis of BCR-ABL covering the Cap, SH3, SH2 and kinase domains of ABL (exons 1–9) confirmed the absence of mutations. Mutation screening of KRAS (exons 3–5), NRAS (exons 2–5), PTPN11 (exons 3, 4, 8 and 13), KIT (entire coding region), FLT3 (exons 14 and 20), JAK2 (exon 14), PDGFR α (exons 12, 14 and 18) and PDGFR β (exons 11 and 17) revealed several previously described polymorphisms, but no novel mutations were detected. One of 17 patients (5.8%) harbored a missense mutation (C→T) in exon 3 of KRAS leading to replacement of threonine 58 with isoleucine (T58I). Approximately 30% of amplicons were mutant, as confirmed by bidirectional sequencing of three independently amplified PCR products.

The patient found to harbor the T58I KRAS mutation was a 62-year-old man with an 8-year history of chronic phase CML. He received initial therapy with interferon- α and cytarabine. In 2001, he was started on imatinib 400 mg daily and achieved a minor cytogenetic response that he subsequently lost, probably

due to the appearance of a clone with a second copy of the Ph chromosome (Table 2). As a result, he was enrolled in a study combining imatinib 800 mg daily with arsenic trioxide. He achieved a transient major cytogenetic response. Upon relapse, he was switched to dasatinib. The T58I mutation was absent while the patient was responding to 800 mg of imatinib, but it became detectable before relapse and remained detectable (at approximately 30%) in 5 subsequent samples collected over a period of 18 months (Table 2). During this time, the patient achieved a second major cytogenetic response on dasatinib. Immediately before relapse on dasatinib, the T58I allele became undetectable simultaneously with a rapid increase of the clone with the additional copy of Ph. None of the samples tested were positive for BCR-ABL kinase mutations at any time.

T58I is an activating mutation of KRAS that has been previously reported in patients with Noonan syndrome who developed juvenile myelomonocytic leukemia.⁷ KRAS mutations are associated with various other human cancers including colorectal cancers, though the specific T58I exchange has not been observed in these studies.⁸ To assess the functional relevance of T58I in BCR-ABL-positive cells, we introduced KRAS T58I, KRAS G12D (a strongly activating mutation) and wild-type KRAS into 32D cells expressing BCR-ABL (32Dp210^{BCR-ABL}) or parental 32D cells. Consistent with previous studies,⁹ neither wild-type KRAS nor any of the mutants rendered parental 32D cells interleukin-3 independent (Figure 1a). To test whether the KRAS T58I mutant may affect imatinib or dasatinib sensitivity, KRAS T58I-expressing 32Dp210^{BCR-ABL} cells were grown in the absence of interleukin-3 with graded concentrations of imatinib or dasatinib. Proliferation assays revealed a reduction in sensitivity to both imatinib and dasatinib in 32Dp210^{BCR-ABL} cells co-expressing wild-type or mutant KRAS. The effect was strongest with G12D, intermediate with T58I and weakest with wild-type KRAS. Notably, 15% of 32Dp210^{BCR-ABL} cells co-expressing KRAS T58I retained viability at 10 μ M imatinib (Figure 1b) or 100 nM dasatinib (Figure 1c) over 3 days in culture as compared to 0% of 32Dp210^{BCR-ABL} cells expressing wild-type KRAS. Consistent with this, there was less apoptosis in 32Dp210^{BCR-ABL} cells co-expressing KRAS T58I (85%) as compared to those co-expressing wild-type KRAS (100%) at intermediate concentrations of imatinib (1.25 μ M) (Figure 1d) or dasatinib (10 nM) (Figure 1e) after 2 days in culture. Differences were less pronounced at higher concentrations of either drug. No effect was observed in 32D parental cells expressing wild-type or mutant KRAS (data not shown). Notably, our demonstration of KRAS T58I in a patient with imatinib resistance is analogous to findings by Lievre *et al.*,¹⁰ who identified other types of KRAS mutations in association with resistance to cetuximab, a drug targeting epidermal growth factor receptor in colorectal cancer. Thus, escape from the effects of tyrosine kinase inhibitor therapy by the activation of alternative pathways can occur in the setting of solid tumors as well as leukemia. Evidently, alternative therapies are required to treat this kind of resistance. In the case of RAS mutations, this could include Farnesyl transferase inhibitors or agents targeting the mitogen-activated protein kinase pathway.¹¹

Table 1 Characteristics of the patient cohort

| Patient | Age (years)/sex | Time on imatinib (months) | Imatinib response (at study onset) | Cytogenetics (% Ph+) | Bone marrow FISH (% BCR-ABL+) | RT-PCR (%) |
|---------|-----------------|---------------------------|------------------------------------|----------------------|-------------------------------|------------|
| 1 | 62/M | 46 | Relapse | 35 | 14.5 | 0.98 |
| 2 | 69/M | 65 | Primary resistance | 85 | 64 | 16 |
| 3 | 55/M | 60 | Primary resistance | 55 | 49 | 0.55 |
| 4 | 63/F | 76 | Primary resistance | ND | ND | 9.2 |
| 5 | 35/F | ND | Primary resistance | 100 | 87 | 1.4 |
| 6 | 69/M | 48 | Relapse | 90 | 76.5 | 5.3 |
| 7 | 45/M | 40 | Relapse | 40 | 37 | 0.52 |
| 8 | 53/F | 52 | Relapse | 10 | 1.5 | 0.1 |
| 9 | 58/F | 69 | Primary resistance | 35 | 32 | 3.2 |
| 10 | 54/F | 12 | Suboptimal response | 30 | 24.5 | 1.7 |
| 11 | 68/F | 44 | Primary resistance | 85 | 88 | 7.1 |
| 12 | 51/F | 70 | Relapse | 35 | 35.5 | 1.6 |
| 13 | 34/F | 11 | Primary resistance | 100 | ND | ND |
| 14 | 57/M | 19 | Relapse | 100 | 98.5 | 210 |
| 15 | 68/M | 11 | Relapse | ND | 85 | 88 |
| 16 | 76/M | 16 | Primary resistance | 100 | 74.5 | 6.4 |
| 17 | 61/M | 11 | Suboptimal response | 20 | 15 | 1.8 |

Abbreviations: ND, not determined; RT-PCR; reverse transcriptase PCR.

Primary resistance was defined as the failure to achieve a major cytogenetic response (MCyR, <35% Ph+ metaphases) after at least 12 months of imatinib therapy. Suboptimal response was defined as 1–35% Ph-positive metaphases after 12 months of imatinib treatment. Relapse was defined by a loss of MCyR or CCyR at the time of the study. Fluorescence *in situ* hybridization (FISH) was performed on unseparated bone marrow interphase cells using the Vysis *BCR/ABL* dual fusion probe. *BCR-ABL* was measured in peripheral blood, normalized for expression of *G6PD*, and expressed in percent (*BCR-ABL/G6PD*), as described.⁶

Table 2 Follow-up summary of patient with *KRAS* T58I mutation

| Sample date | Treatment before cytogenetic evaluation (mg/day) | Cytogenetics (% Ph+) | Bone marrow FISH (% BCR-ABL+) | <i>KRAS</i> T58I allele (%) | <i>BCR-ABL</i> kinase domain |
|-----------------------------|--------------------------------------------------|----------------------|-------------------------------|-----------------------------|------------------------------|
| February 2002 | Imatinib 400 | 65 | 41.5 | 0 | Native |
| December 2002 ^a | Imatinib 400 | 100 | 78 | 0 | Native |
| December 2003 ^a | Imatinib 600–800+ Arsenic trioxide | 20 | 31.5 | 0 | Native |
| October 2004 ^a | Imatinib 800 + Arsenic trioxide | 35 | 14.5 | 31.8 | Native |
| June 2005 ^a | Imatinib 800 | 85 | 62.5 | ND | Native |
| October 2005 ^a | Dasatinib 140 | ND | 23 | 28.8 | Native |
| December 2005 ^a | Dasatinib 100 | 30 | 24.5 | ND | Native |
| March 2006 ^a | Dasatinib 80 | ND | 13.5 | 37.4 | Native |
| April 2006 ^a | Dasatinib 80 | 8 | 10 | ND | Native |
| September 2006 ^a | Dasatinib 80 | 15 | 24 | 34.9 | Native |
| December 2006 ^a | Dasatinib 80 | 33 | 29 | 33.1 | Native |
| February 2007 ^a | Dasatinib 80 | 20 | 51 | 0 | Native |
| May 2007 ^a | Dasatinib 80 | 95 | 60.5 | 0 | Native |

ND: Not determined owing to poor sample quality.

^aThe patient samples where the Ph+ cells were double Ph+ due to an extra copy of a derivative of chromosome 22 of *BCR-ABL*. Data shown here by cytogenetics (metaphases) and fluorescence *in situ* hybridization (FISH) (interphases) represent total *BCR-ABL*-positive clones.

The transient appearance of a cell clone with an activating *KRAS* mutation could have two explanations. One possibility is that the *KRAS* mutant clone may have arisen independently of the CML clone and may be comparable to the Ph-negative but cytogenetically abnormal clones seen in some 5–10% of CML patients treated with imatinib, which are frequently transient.¹² Alternatively, as suggested by the temporal correlation with cytogenetic relapse, it is possible that the *KRAS* T58I mutant conferred partial resistance to imatinib, which promoted relapse and prevented the extinction of the clone on subsequent dasatinib therapy. Consistent with this, we found that co-expression of *KRAS* T58I and *BCR-ABL* in 32D cells reduced imatinib and dasatinib sensitivity, though not to the same degree as the strongly activating *KRAS* G12D mutation. Ultimately, a second clone, cytogenetically

characterized by a second copy of the Ph chromosome, became dominant and suppressed the *KRAS* T58I clone below the detection threshold of direct sequencing. Distinguishing between these two possibilities would require single cell or colony assays, but unfortunately no suitable archived material is available.

Overall, our data show that activating RAS mutations may contribute to imatinib resistance in some CML patients who relapse with native *BCR-ABL*, although these mutations are apparently not a common mechanism of drug resistance in CML. In contrast, we found no evidence of mutations in *PTPN11*, *JAK2*, *FLT3* or resistance mutations in the activation loops of *KIT* or *PDGFR α/β* in any of the 17 patients. Given that the cohort under study is small, however, it remains possible that such mutations would be detected in a larger

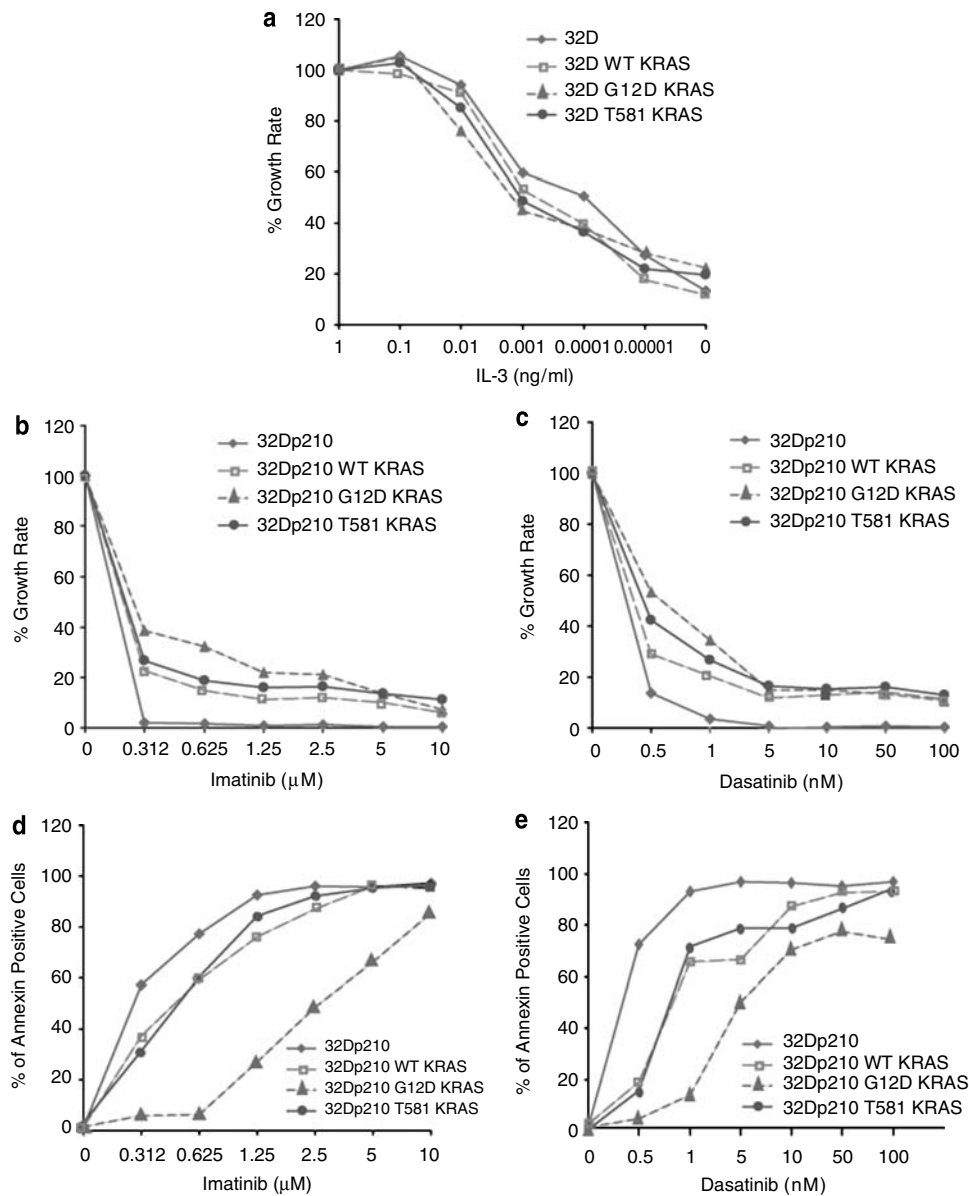


Figure 1 The *KRAS* T581 mutation is activating and confers increased resistance to imatinib and dasatinib in *BCR-ABL*-positive cells. (a) T581, G12D and wild-type (WT) *KRAS* were stably expressed in 32D cells and interleukin-3 independence was tested. (b and c) *BCR-ABL*-positive 32D cells stably expressing mutant or WT *KRAS* were tested for sensitivity to imatinib and dasatinib in cell proliferation assays and (d and e) for apoptosis in response to imatinib and dasatinib. Values shown are the mean value from two independent experiments performed in triplicate.

group of patients. Additionally, the proportion of *BCR-ABL*-positive interphases was <50% in 7/17 samples. As direct sequencing was used for screening of *KIT*, *KRAS*, *NRAS* and *JAK2*, mutations present in the *BCR-ABL*-positive cells may have been missed, given that a mutant allele must be present at >20–25% to be detectable by this technique. This is less likely for mutations of *PTPN11*, *FLT3* and *PDGFR α/β* , which were analyzed by D-HPLC with a sensitivity of approximately 10%. Irrespective of this, our data suggest that *BCR-ABL* mutation-negative resistance is not due to a frequent or universal mutation in one of the genes under study. Thus, in the majority of patients, other yet unknown mechanisms must account for resistance, and elucidating these mechanisms will be crucial for developing new therapeutic approaches.¹¹

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