KRAS and BRAF Mutations Predict Primary Resistance to Imatinib in Gastrointestinal Stromal Tumors

Claudia Miranda, Martina Nucifora, Francesca Molinari, Elena Conca, Maria Chiara Anania, Andrea Bordoni, Piercarlo Saletti, Luca Mazzucchelli, Silvana Pilotti, Marco A. Pierotti, Elena Tamborini, Angela Greco, and Milo Frattini

Abstract

**Purpose:** Gastrointestinal stromal tumors (GIST) are characterized by gain-of-function mutations in KIT/PDGFRA genes leading to a constitutive receptor activation which is well counteracted by imatinib. However, cases in which imatinib as first-line treatment has no effects are reported (primary resistance). Our purpose is to investigate alterations in downstream effectors, not reported so far in mutated GIST, possibly explaining the primary resistance to targeted treatments.

**Experimental Design:** Two independent naive GIST cohorts have been analyzed for KIT, PDGFRA, KRAS, and BRAF mutations by direct sequencing. Cell lines expressing a constitutively activated and imatinib-responding KIT, alone or in combination with activated KRAS and BRAF, were produced and treated with imatinib. KIT receptor and its downstream effectors were analyzed by direct Western blotting.

**Results:** In naive GISTs carrying activating mutations in KIT or PDGFRA a concomitant activating mutation was detected in KRAS (5%) or BRAF (about 2%) genes. In vitro experiments showed that imatinib was able to switch off the mutated receptor KIT but not the downstream signaling triggered by RAS–RAF effectors.

**Conclusions:** These data suggest the activation of mitogen—activated protein kinase pathway as a possible novel mechanism of primary resistance to imatinib in GISTs and could explain the survival curves obtained from several clinical studies where 2% to 4% of patients with GIST treated with imatinib, despite carrying KIT-sensitive mutations, do not respond to the treatment.

Introduction

Gastrointestinal stromal tumors (GIST) are the most common mesenchymal tumors of the gastrointestinal tract. This uncommitted term, formally referred to tumors showing smooth muscle differentiation, identifies after 1998 the most representative example of “simple sarcomas” (1), in which a single receptor tyrosine kinase (RTK) mutation plays a crucial role in dictating both pathogenesis and predictivity. Hirota and colleagues (2) in fact showed for the first time that a significant subset of GISTs harbored mutations in the RTK KIT gene. Subsequently, in 2003, the gene encoding for the homologous receptor PDGFRA was showed to be mutually exclusively mutated in these tumors (3). Currently, we know that KIT alterations (principally deletions, point mutations, and insertions) affect exons 11 and 9 and rarely exons 13 and 17. Cumulatively, KIT alterations are carried by approximately 70% to 80% of GISTs. PDGFRA mutations, deletions, and point mutations in exons 18, 12, and 14 are present in about 5% to 10%. The rate of GISTs carrying wild-type KIT and PDGFRA genes accounts for 10% to 20% of cases (4). As result of KIT and PDGFRA mutations, these tumors harbor constitutively activated KIT and/or PDGFRA receptors which, in turn, upregulate 2 main signal pathways, where the RAS-RAF-MEK-ERK and the PI3K-AKT-mTOR transducer protein kinases are involved.

It is widely reported that GIST respond well to imatinib (5), a selective tyrosine kinase inhibitor able to interfere...
Translational Relevance

This study reports for the first time the presence of activating mutations in KRAS and BRAF genes in a small percentage of gastrointestinal stromal tumors (GIST) carrying concomitant activating mutations in KIT or PDGFRA receptors. In vitro experiments in cell lines coexpressing an imatinib-responder KIT mutant and constitutively activated KRAS and BRAF proteins, showed that imatinib treatment was able to switch off KIT and its downstream signaling but not extracellular signal—regulated kinase (ERK)1/2 activation driven by the mutated KRAS and BRAF. These data suggest the activation of mitogen—activated protein kinase pathway as a possible novel mechanism of primary resistance to imatinib in GIST. Interestingly, our findings could explain the survival curves obtained from several clinical studies of patients with GIST treated with imatinib showing that about 2% to 4% of cases, despite carrying KIT-sensitive mutations, do not respond to the treatment.

with the activation of KIT and PDGFR receptors by competing with ATP in the ATP-binding pocket. Several clinical studies have been conducted in metastatic patients showing clinical response in 80% to 85% of the cases, that after a median of 2 years might become resistant to the treatment mainly due to the presence of secondary point mutations (6,7).

Generally, patients carrying KIT exon 11 mutations respond much better to targeted treatment than tumors carrying exon 9 mutations. Furthermore, a low response to imatinib has been observed in patients with wild-type KIT or PDGFRA receptors (7,8).

This low imatinib sensitivity has been related to an intrinsic conformational characteristic of the wild-type KIT and PDGFRA receptors whose ATP pocket show a lower affinity for imatinib, as supported by molecular modeling studies (9). Clinically, it has been defined as primary resistance and it is so defined when a continuous tumor growth is observed despite imatinib administration throughout the first 6 months of treatment. Recently, a further kind of resistance has been reported to be the presence of mutations in downstream effectors, such as BRAF, detected in a small percentage of nonmutated GISTs (10–12). To our knowledge, no mutations in the KRAS gene have been reported in GISTs wild-type for KIT and PDGFRA genes.

Our study reports for the first time the presence of KRAS and BRAF mutations in patients with GIST carrying also concomitant mutations in KIT and PDGFRA genes. By conducting biochemical and biologic studies in an in vitro model, we showed that cells expressing an imatinib-sensitive KIT mutant no longer respond to imatinib if KRAS or BRAF mutants are introduced. This suggests a possible novel mechanism of primary resistance to imatinib in GISTs that involves the activation of one of the downstream pathways.

Materials and Methods

Patients’ characteristics

Using the Ticino Cancer Registry database, 74 consecutive patients affected by GIST and diagnosed in Ticino from 1999 to 2008 were included in the study. Seventy one cases (96% of the entire cohort) were both microscopically confirmed and positive for CD117 expression (clone A4502, DakoCytomation; 1:50 dilution); 2 patients (2.7%) were microscopically confirmed and had a low expression of KIT; one patient (1.3%) was only histologically confirmed because no slides were available for immunohistochemical staining confirmation. Eight cases (10.6%) were detected incidentally as a secondary finding during investigations and surgical procedures for other causes.

Patients’ characteristics

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 MATERIALS AND METHODS

Molecular analysis

Formalin-fixed, paraffin-embedded tumor blocks were reviewed for quality and tumor content, and for each case a single representative tumor block, containing at least 70% of tumoral cells, was selected. Genomic DNA was extracted by the QIAamp Mini Kit (Qiagen) according to the manufacturer’s instructions.

KIT (exons 9, 11, 13, and 17), PDGFRA (exons 12, 14, and 18), BRAF (exons 11 and 15), and KRAS (exons 2 and 3) genes were analyzed by Direct Sequencing on 3130 (Applied Biosystems or 3500 DX Genetic Analyzer (Applied Biosystems Life Technologies), according to the literature (14–17). Each sequence reaction was carried out at least twice, starting from independent PCR reactions. In each case, the detected mutation was confirmed in the sequence as sense and antisense strands.

All the procedures in the laboratories of Locarno (Switzerland) and Milan (Italy) are registered in external quality control audits, and the laboratory of Milan participated to quality controls for GIST molecular analyses (14).

The analyses of KIT and PDGFRA mutations of patients 1 to 43 in the Swiss cohort have already been reported (17).
Expression vectors

KITΔ559 plasmid, carrying KIT cDNA harboring deletion of codon 559 has been previously described (18). KRAS cDNA carrying G to V mutation at codon 12 (GGT→GtT, G12V) was RT-PCR amplified from human bronchial epithelial cells (HBEC) cells transduced with pBABE vector carrying KRASG12V (19) using the following primers: forward (containing KRAS Kozak consensus sequences), GCCCGATATGCCTAGCATAAAATGACTGAAATATACTTTG and reverse, GCCGGATCCGCGCTTACATAATTACACATTT. EcoRV and NotI restriction sites were included in the primers (underlined). PCR product was digested with EcoRV and NotI restriction enzymes and inserted into pCDNA3.1Hygro vector carrying compatible ends. KRASG12D (GGT→GtT) and G12AG13D (GGT→GcT and GGC→GaC) mutants have been obtained by site-directed mutagenesis of KRASG12V, by the QuickChange II Site-Directed Mutagenesis Kit (Stratagene) following manufacturer’s instructions, using the following primers: 5'-TGGTAGTTGGAGCTGATGGCGTAGGCAAGAG-3' and 5'-CTCTTGCCTACGCCATCAGC-TCCAACTACCA-3' for G12D mutation; and 5'-GTGGTAGTTGGAGCTGCTGACGTAGGCAAGAGTGCC-3' and 5'-GGCACTCTTGGCTACGTACGTCAGCAGCTCCAACTACACC-3' for G12AG13D mutations. The presence of the mutations and the absence of undesired mutations were verified by DNA sequences for all constructs. pMCEF-BRAF V600E construct was kindly provided by Dr. R. Marais (Institute of Cancer Research, London, United Kingdom).

Cell cultures and transfections

HEK293T cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented by 10% fetal calf serum, in 5% CO2 humidified atmosphere, and transfected by Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. KITΔ559 cell line, derived from NIH3T3 cells stably expressing KITΔ559 mutant (18) was grown in DMEM supplemented by 5% fetal calf serum and G418 (400 μg/mL), in 10% CO2 humidified atmosphere, and transfected by calcium phosphate procedure as previously described (20). Two weeks after transfection, hygromycin-resistant colonies were isolated and propagated in the presence of both hygromycin (40 μg/mL; Roche) and G418 (400 μg/mL; Gibco). HEK293T and NIH3T3 derived cells were treated with 5 μmol/L imatinib for 20 and 48 hours, respectively. UO126 (Calbiochem, EMD Chemicals) treatment was carried out at 10 μmol/L. Photographs were taken at 10× magnification with a digital camera system coupled to microscope (LEICA, DMIRB, Leica Microsystems GmbH).

Western blot analysis

Transfected HEK293T and NIH3T3 cells were harvested following overnight serum starvation. Cell lysates were produced in radioimmunoprecipitation assay buffer (RIPA)-modified buffer (20 mmol/L Tris-HCl pH 7.4; 150 mmol/L NaCl; 5 mmol/L EDTA; and 1% Nonidet P-40) supplemented with the Complete Mini EDTA-Free Protease Inhibitor Cocktail (Roche), 1 mmol/L Na3VO4 and 1 mmol/L phenylmethylsulfonylfluoride. Protein samples (50 μg) were boiled in NuPAGE LDS sample buffer (Invitrogen) and separated on NuPAGE Novex 4% to 12% Bis-Tris gels (Invitrogen) in MOPS running buffer, then

Table 1 Characteristics of patients with GIST

<table>
<thead>
<tr>
<th>The Swiss cohort</th>
<th>Patients (N = 74), n (%)</th>
</tr>
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<tbody>
<tr>
<td>ICP patient characteristic</td>
<td>Age &gt;65</td>
</tr>
<tr>
<td>Age &gt;65</td>
<td>43 (58)</td>
</tr>
<tr>
<td>Age ≤65</td>
<td>31 (42)</td>
</tr>
<tr>
<td>Gender</td>
<td>Male 43 (58)</td>
</tr>
<tr>
<td>Gender</td>
<td>Female 31 (42)</td>
</tr>
<tr>
<td>Tumor location</td>
<td>Stomach 43 (58)</td>
</tr>
<tr>
<td>Tumor location</td>
<td>Small bowel 19 (26)</td>
</tr>
<tr>
<td>Tumor location</td>
<td>Others 12 (16)</td>
</tr>
<tr>
<td>Tumor size</td>
<td>&lt;2 19 (26)</td>
</tr>
<tr>
<td>Tumor size</td>
<td>2 ≤ 5 20 (27)</td>
</tr>
<tr>
<td>Tumor size</td>
<td>5 &lt; 10 18 (24)</td>
</tr>
<tr>
<td>Tumor size</td>
<td>&gt;10 13 (18)</td>
</tr>
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<td>Tumor size</td>
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<tr>
<td>Risk of malignancies</td>
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<tr>
<td>Risk of malignancies</td>
<td>Low 19 (26)</td>
</tr>
<tr>
<td>Risk of malignancies</td>
<td>Intermediate 11 (15)</td>
</tr>
<tr>
<td>Risk of malignancies</td>
<td>High 29 (39)</td>
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<table>
<thead>
<tr>
<th>The Italian cohort</th>
<th>Patients (N = 53), n (%)</th>
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<tbody>
<tr>
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<td>Age &gt;65</td>
</tr>
<tr>
<td>Age &gt;65</td>
<td>28 (53)</td>
</tr>
<tr>
<td>Age ≤65</td>
<td>25 (47)</td>
</tr>
<tr>
<td>Gender</td>
<td>Male 29 (55)</td>
</tr>
<tr>
<td>Gender</td>
<td>Female 24 (45)</td>
</tr>
<tr>
<td>Tumor location</td>
<td>Stomach 15 (28)</td>
</tr>
<tr>
<td>Tumor location</td>
<td>Small bowel 33 (63)</td>
</tr>
<tr>
<td>Tumor location</td>
<td>Others 5 (9)</td>
</tr>
<tr>
<td>Tumor size</td>
<td>&lt;2 1 (2)</td>
</tr>
<tr>
<td>Tumor size</td>
<td>2 ≤ 5 19 (36)</td>
</tr>
<tr>
<td>Tumor size</td>
<td>5 &lt; 10 20 (38)</td>
</tr>
<tr>
<td>Tumor size</td>
<td>&gt;10 6 (11)</td>
</tr>
<tr>
<td>Tumor size</td>
<td>unknown 7 (13)</td>
</tr>
<tr>
<td>Risk of malignancies</td>
<td>Very low 1 (2)</td>
</tr>
<tr>
<td>Risk of malignancies</td>
<td>Low 9 (17)</td>
</tr>
<tr>
<td>Risk of malignancies</td>
<td>Intermediate 8 (15)</td>
</tr>
<tr>
<td>Risk of malignancies</td>
<td>High 28 (53)</td>
</tr>
<tr>
<td>Risk of malignancies</td>
<td>Not valuable 7 (13)</td>
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</table>
transferred onto nitrocellulose filters and immunoblotted with the appropriated antibodies.

Anti-c-KIT (H1300) and anti-BRAF (F7) antibodies were purchased from Santa Cruz Biotechnology; anti-panRAS (Ab3) was from Calbiochem; and anti-vinculin, anti- phospho-ERK1/2, and anti-ERK1/2 antibodies were purchased from Sigma-Aldrich. The anti-phospho-KIT, phospho-AKT (Ser473; D9E) antibodies were from Cell Signal Technology. The immunoreactive bands were visualized using horseradish peroxidase–conjugated secondary antibodies (Sigma-Aldrich) and enhanced chemiluminescence (GE Healthcare).

Results and Discussion

Molecular analysis of KIT, PDGFRA, BRAF, and KRAS Swiss GIST cases. The molecular analyses were conducted on 60 of 74 patients with GIST for whom the DNA was available. All the results about the mutational analysis are reported in Table 2; The Italian cohort and Supplementary Table S1.

KIT mutations were identified in 34 of 60 patients (57%) and included deletions, insertions, duplications, and point mutations. The majority of mutations occurred in exon 11 (31 cases, 52%); 2 mutations in exon 9 (S476I and a duplication of codons 502–503; no. 37; no. 32) and 1 in exon 13 (K642E; no. 45) were observed, no mutations were identified in exon 17.

The PDGFRA gene was analyzed in 57 patients, because for 3 patients the material was not sufficient for this further analysis. Five patients (9%) showed a point mutation in PDGFRA gene; we identified 2 point mutations in exon 12 (P581S and V561D) and 3 in exon 18 (the D842V change in all cases). In addition, we found 5 silent mutations, 3 occurring in exon 12 (P557P in 2 cases and I565I in another) and 2 in exon 18 (both V824V). In one case (no. 4), a concomitant alteration was found both in KIT and in PDGFRA genes: a complex deletion in KIT exon 11 (Δ553–556 and W557R) and a P581S in PDGFRA exon 12. However, as mentioned in a previous publication (17), the P581S amino acid substitution has never been reported in literature and no functional data are available. Therefor, its putative role in PDGFRA activation remains unknown.

Overall, a point mutation in either KIT or PDGFRA genes was found in 38 cases (63%). These data indicate that the observed KIT and PDGFRA mutational rates are lower when compared with data present in the literature (4, 21–23). The discrepancies are not to be ascribed to technical methods, as for every sample a pathologist provided a selection of tumoral cells, following the procedures suggested by Van Krieken and colleagues (24) to obtain at least 70% of tumoral DNA avoiding normal cells through macrodissection. Furthermore, mutational analyses were conducted using widely accepted protocols (15, 16). It is therefore possible that the lower percentage of mutated GISTs (deriving, however, from 60 patients only) in the South of Switzerland (62% versus about 80% in the world) could be mainly due to the cohort characteristics. These data reported in the present work are in line with a previous report (17) and represent the first Swiss population–based study defined from a molecular point of view, that can reflect a distinctive feature of the Ticino population. This assumption is supported by the results obtained in the laboratory, where mutational rates in colon and lung cancer observed in patients from Ticino were different from those reported in literature (Frattini M; personal communication), thus confirming the epidemiologic origin of these differences.

Because KRAS and BRAF genes play a fundamental role in tumorigenesis (25–27) of several tumor types and they are 2 of the genes most deregulated among cancers (27), we decided to investigate their mutational status in the whole cohort of GISTs, thus including the mutated and the wild-type cases.

Mutations in codons 12 and 13 of KRAS, never identified before in GIST, have been detected in 3 of 60 patients (5%). In more detail, we identified one mutation in codon 12 (G12D: GGT→GaT; no. 5), one in codon 13 (G13D: GGC→GaC; no. 59), and a concomitant mutation in both codons (G12A/G13D: GGT→GCT and GGC→GaC; no. 21).

Interestingly, the 2 patients carrying the G12D and the G12A/G13D mutations were characterized by a concomitant deletion in exon 11 of KIT, Δ570–576, and Δ579, respectively. The patient carrying the G13D mutation showed a concomitant point mutation in exon 18 of PDGFRA gene (D842V). Patients with KRAS mutations were wild-type for BRAF gene, thus confirming the mutual

Table 2. GIST cases showing concomitant KIT/PDGFRA and KRAS/BRAF mutations

<table>
<thead>
<tr>
<th>Center</th>
<th>n</th>
<th>Site</th>
<th>Risk</th>
<th>CD117</th>
<th>KIT mutations</th>
<th>PDGFRA mutations</th>
<th>KRAS mutations</th>
<th>BRAF mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Locarno</td>
<td>5</td>
<td>Stomach</td>
<td>High</td>
<td>+</td>
<td>Δ570–576</td>
<td>WT</td>
<td>G12D</td>
<td>WT</td>
</tr>
<tr>
<td>Locarno</td>
<td>21</td>
<td>Small bowel</td>
<td>Intermediate</td>
<td>+</td>
<td>Δ579</td>
<td>WT</td>
<td>G12A/G13D</td>
<td>WT</td>
</tr>
<tr>
<td>Locarno</td>
<td>59</td>
<td>Stomach</td>
<td>Low</td>
<td>+</td>
<td>WT</td>
<td>D842V</td>
<td>G13D</td>
<td>WT</td>
</tr>
<tr>
<td>Milan</td>
<td>7</td>
<td>Small bowel</td>
<td>High</td>
<td>+</td>
<td>Δ555–558</td>
<td>WT</td>
<td>V600E</td>
<td></td>
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</table>

Abbreviations: WT, wild-type; IHC, immunohistochemistry.

aThe reporter numeration refers to the numbers reported in Supplementary Tables S1 and S2.
The presence of mutations in RTK genes and downstream proteins. Transiently transfected BRAF and KRAS mutants. Western blot analysis of HEK293T cells induced by expression of KIT, ERK1/2, and AKT phosphorylation of KIT, ERK1/2, and AKT, and BRAF protein levels are shown as control (Fig. 1A).

Biologic effects of concomitant KIT and KRAS or BRAF mutations

None of the patients carrying concomitant mutations of KIT and KRAS or BRAF genes were treated with imatinib. They underwent surgical eradication of the tumor; none of them had a metastatic disease, and they were classified as disease-free subjects at the last follow-up. For this reason, we explored in vitro the biologic consequences of the concomitant presence of KIT and KRAS or BRAF mutations detected in patients with GIST, with the final aim of investigating the contribution of KRAS and BRAF mutations to imatinib response. Similar studies were not conducted in the case of the patient carrying concomitant PDGFRAD842V and KRAS mutations, as the identified PDGFRAD842V mutation is insensitive to imatinib (8).

No cell culture established from patients with GIST carrying KIT and KRAS or BRAF mutations are available. Therefore, we constructed in vitro models consisting of cells transfected with both KRAS or BRAF and KITΔ559 mutants. The latter is constitutively activated and sensitive to imatinib (9), similarly to KITΔ559 and Δ570–576 mutations (30, 31) detected in KRAS-mutated patients (nos. 5 and 21 of the Swiss cohort) as well as the deletion Δ555–558 observed in the tumor mutated in BRAF (no. 7 of the Italian cohort).

KITΔ559 and BRAFV600E, alone or in combination, were transiently transfected in HEK293 cells. Phosphorylation of KIT, extracellular signal—regulated kinase (ERK)1/2, and AKT were investigated by Western blotting; KIT, ERK1/2, and AKT phosphorylation of KIT, ERK1/2, and AKT, and BRAF protein levels are shown as control (Fig. 1A).
In cells expressing the constitutively phosphorylated KITΔ559 protein ERK1/2 phosphorylation was below the detection level, whereas an increase in AKT phosphorylation was observed. Imatinib treatment strongly reduced KIT phosphorylation, and this resulted in the abrogation of AKT phosphorylation. Expression of BRAFV600E strongly induced ERK1/2 phosphorylation, which was not affected by imatinib treatment; no effect of BRAFV600E on AKT activation was observed. In cells coexpressing KITΔ559 and BRAFV600E, ERK1/2 phosphorylation was comparable with that induced by BRAFV600E alone. The inhibition of KITΔ559 by imatinib caused a strong decrease of AKT phosphorylation; in contrast, ERK1/2 phosphorylation was not affected. These data indicate that in cells coexpressing KITΔ559 and BRAFV600E, imatinib abrogates the KITΔ559-triggered AKT phosphorylation; however, it is not capable of affecting ERK1/2 phosphorylation, which is driven by BRAFV600E.

A similar approach was undertaken to investigate the effect of concomitant expression of KIT and KRAS mutants. Expression vectors carrying KRASG12D and KRASG12A/G13D mutants were transiently transfected in HEK293T cells, alone or in combination with KITΔ559 construct. Western blot analysis is shown in Fig. 1B. Expression of the constitutively phosphorylated KITΔ559 was associated to ERK1/2 phosphorylation below the detection level and to an increase in AKT phosphorylation. Cells expressing KRASG12D or KRASG12A/G13D mutants displayed ERK1/2 phosphorylation, which resulted in increase in the presence of KITΔ559, thus unveiling the capacity of KIT to trigger ERK1/2 activation. Similarly, activation of AKT was observed in cells expressing KRAS mutants, with a further increase in the presence of KITΔ559. The effect of imatinib on signaling pathways was then investigated. As expected, in KITΔ559-transfected cells, inhibition of KIT by imatinib resulted in the abrogation of AKT phosphorylation. No effect of imatinib was observed on AKT and ERK1/2 phosphorylation induced by the expression of KRASG12D and KRASG12A/G13D mutants alone, indicating that imatinib does not affect the signaling promoted by KRAS oncogenes. In keeping with this sample, in cases concomitantly expressing KITΔ559 and KRAS mutants, imatinib strongly reduced KIT phosphorylation; however, phosphorylation levels of ERK1/2 and AKT were only partially affected, as they were similar to those induced by expression of KRAS mutants alone. Our data indicate a synergism of KIT and KRAS mutants with respect to the activation of ERK1/2 and AKT. Imatinib treatment abrogates KIT phosphorylation and the related fraction of ERK1/2 and AKT activation. However, in the presence of KIT inhibition by imatinib, both pathways remain active, being triggered by KRAS oncogenes.

To further analyze the interplay between KIT and KRAS oncogenes, we investigated the biologic consequences of concomitant stable expression of KIT and KRAS mutants in the NIH3T3 cellular system, which represents a useful model for studying in vitro oncogene activity. The

Figure 2. Effect of imatinib and UO126 treatment on signaling and cell morphology of NIH3T3 cells coexpressing KITΔ559 and KRAS mutants. A, Western blot analysis of NIH3T3 cells stably expressing KITΔ559 and KRASG12A/G13D onco-
genes, the level of AKT phosphorylation was comparable with that observed in the presence of KITΔ559 only, and it was abrogated by imatinib. ERK1/2 phosphorylation was...
significantly increased, it was unaffected by imatinib but was completely reduced by treatment with the MAP/ERK kinase (MEK) inhibitor U0126. Both AKT and ERK1/2 pathways were abrogated by concomitant treatment with imatinib and U0126. Morphologic observation of cells used for these analyses is reported in Fig. 2B. Cells expressing KITΔ559 alone or in combination with KRASG12A/G13D displayed the typical NIH3T3-transformed phenotype, marked by loss of contact inhibition and spindle-shaped morphology. In cells expressing KITΔ559, imatinib treatment caused phenotype modification, resulting in cells that were flatter and more adherent, similar to naive NIH3T3 cells. On the contrary, imatinib had no effect on the transformed morphology of cells expressing both KITΔ559 and KRASG12A/G13D, suggesting that both oncogenes contribute to transformation. In these cells reversion to flat phenotype was observed when signaling triggered by both oncogenes were abrogated by simultaneous treatment with imatinib and U0126.

As mentioned earlier, the lack of information of patients’ response to imatinib does not allow to compare our in vitro results in an in vivo setting. Nevertheless our data, showing a full biochemical and cellular response in the presence of both KIT and MEK inhibitors, suggest that patients with GIST carrying concomitant KIT and KRAS or BRAF mutations could benefit of combinatorial therapy targeting pathways triggered by the 2 oncogenes.

In conclusion, the present work shows for the first time the occurrence of KRAS mutation in GISTs and the concomitant presence of KRAS or BRAF and KIT or PDGFRα mutations. Biologic and biochemical studies conducted in in vitro models suggested that KRAS and BRAF mutations may affect the response to imatinib of KIT imatinib-sensitive mutations, thus proposing a new molecular mechanism of primary resistance to targeted therapy in GIST. Recently, it has been reported that also PI3KCA mutations are present in mutated GISTs, thus reinforcing the role of downstream signaling in imatinib resistance (32). It is also worth mentioning that other alternative mechanisms could be present in imatinib-resistant cases possibly related to pharmacokinetic variability linked to the individual metabolic trait or alterations in the transporter enzymes (9). Interestingly, a critical revision of the survival curves obtained from the several clinical studies of patients with GIST treated with imatinib indicated that a percentage of cases, despite carrying KIT exon 11 mutations, do not respond to the treatment. It would be interesting to analyze these patients in the light of KRAS and BRAF mutations to verify what is hypothesized here. Moreover, the introduction of KRAS and BRAF mutational analysis in clinic diagnostic settings of patients with GIST, to better tailor the treatments, should be encouraged.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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