Predictive Biomarkers and Personalized Medicine

Clinical Cancer Research

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

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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. *Clin Cancer Res; 18(6); 1769–76. ©2012 AACR.*

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

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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-responding 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 cohort included 43 men and 31 women, with a median age of 65 years old (Table 1, The Swiss cohort).

The tumors were localized differently: 58% in the stomach, 26% in the small bowel, more rarely in the colon, rectum, or retroperitoneoum (16%). Median tumor size was 6.0 cm (range, 0.2–17 cm). GISTs were classified with the risk stratification system proposed by Fletcher and colleagues as very low (20%), low (26%), intermediate (15%), and high (39%) risk of malignancy (13; Table 1; The Swiss cohort).

A second cohort of GISTs, consisting of 53 tumors, randomly selected among the *KIT*/*PDGFRA* already molecularly analyzed cases for diagnostic and therapeutic purposes, was studied at the Istituto Nazionale Tumori (INT) of Milan for *KRAS* and *BRAF* mutations. There were 29 men and 24 women, with a median age of 62 years. Fifteen tumors were localized in the stomach (28%), 33 in the small bowel (63%), and 5 (9%) in the rectum or peritoneum. Risk stratifications (13) was as follow: 2% very low, 17% low, 15% intermediate, 53% high, and 13% not valuable (because they were metastatic lesions; Table 1; The Italian cohort).

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).

Table 1 Characteristics of patients with GIST

The Swiss cohort

ICP patient characteristic	Patients (N = 74), *n* (%)
Age	
>65	43 (58)
≤65	31 (42)
Gender	
Male	43 (58)
Female	31 (42)
Tumor location	
Stomach	43 (58)
Small bowel	19 (26)
Others	12 (16)
Tumor size	
<2	19 (26)
2 ≤ 5	20 (27)
5 < 10	18 (24)
≥10	13 (18)
unknown	4 (5)
Risk of malignancies	
Very low	15 (20)
Low	19 (26)
Intermediate	11 (15)
High	29 (39)

The Italian cohort

INT patient characteristic	Patients (N = 53), *n* (%)
Age	
>65	28 (53)
<65	25 (47)
Gender	
Male	29 (55)
Female	24 (45)
Tumor location	
Stomach	15 (28)
Small bowel	33 (63)
Others	5 (9)
Tumor size	
<2	1 (2)
2 < 5	19 (36)
5 < 10	20 (38)
>10	6 (11)
Unknown	7 (13)
Risk of malignancies	
Very low	1 (2)
Low	9 (17)
Intermediate	8 (15)
High	28 (53)
Not valuable	7 (13)

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), GCCCGATATCGCCTGCTGAAAATGACTGAATATAAACTTG and reverse, CGCGGATCCGCGTTACATAATTACACACTTTG. *Eco*RV and *Not*I restriction sites were included in the primers (underlined). PCR product was digested with *Eco*RV and *Not*I restriction enzymes and inserted into pCDNA3.1Hygro(−) vector carrying compatible ends. KRASG12D (GGT→GaT) and G12AG13D (GGT→GcT and GGC→GaC) mutants have been obtained by site-directed mutagenesis of $KRAS^{G12V}$, by the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) following manufacturer's instructions, using the following primers: 5′-TGGTAGTTGGAGCTGATGGCGTAGGCAAGAG-3′ and 5′-CTCTTGCCTACGCCATCAGCTCCAACTACCA-3′ for G12D mutation; and 5′-GTGGTAGTTGGAGCTGCTGACGTAGGCAAGAGTGCC-3′ and 5′-GGCACTCTTGCCTACGTCAGCAGCTCCAACTACCAC-3′ for G12AG13D mutations. The presence of the mutations and the absence of undesired mutations were verified by DNA sequences for all constructs. $pMCEF\text{-}BRAF^{V600E}$ 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% CO_2 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% calf serum and G418 (400 μg/mL), in 10% CO_2 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 Na_3VO_4 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

transferred onto nitrocellulose filters and immunoblotted with the appropriated antibodies.

Anti-c-KIT (H300) 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 the literature and no functional data are available. Therefore, 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

Center	*n*[a]	Site	Risk	CD117 IHC	*KIT* mutations	*PDGFRA* mutations	*KRAS* mutations	*BRAF* mutations
Locarno	5	Stomach	High	+	Δ570–576	WT	G12D	WT
Locarno	21	Small bowel	Intermediate	+	Δ579	WT	G12A/G13D	WT
Locarno	59	Stomach	Low	+	WT	D842V	G13D	WT
Milan	7	Small bowel	High	+	Δ555–558	WT	WT	V600E

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

[a]The reporter numeration refers to the numbers reported in Supplementary Tables S1 and S2.

exclusivity of mutations in these 2 genes, as already reported in other tumor types (28).

One BRAF mutations has been detected in a patient (no. 58) wild-type for *KIT* and *PDGFRA* genes, showing frequency consistent with literature data (10–12).

Italian GIST cases. To validate the observation derived from the earlier described Swiss case material, an independent group of 53 cases (Supplementary Table S2), randomly selected from amongst approximately 500 GISTs all already characterized for KIT and PDGFRA mutations and present at INT of Milan, was investigated further for the presence of mutations in *KRAS* and *BRAF* genes. The criteria used in the selection of this group of cases were principally the size, that is, a comparable number of cases, and the pathology, that is, the GIST histotype. In this group of tumors, the cases mutated in *KIT* represented the 79.3% (42 cases), the ones in *PDGFRA* represented 9.4% (5 cases), and those wild-type represented 11.3% (6 cases), in line with the literature data. The Institute in Milan represents the referral centre for GIST treatment in Italy and collects cases from the whole Italy reflecting the *KIT* and *PDGFRA* mutational spectrum of a population of a wide geographic region. It has to be noted that the elevated percentage of high risk cases, both for histotype and for anatomic localization (principally small bowel), mirrors the selection of pathologic second revision cases generally related to patients with the worst prognosis (Table 1).

The sequencing analysis of the downstream transducers in the Italian group revealed, interestingly, a *BRAF* mutation (GTG→GaG) causing the V600E amino acid substitution in 1 (Table 2, case no. 7) of the 53 cases (about 2%). This tumor showed a simultaneous *KIT* exon 11 mutation corresponding to a deletion of 4 amino acids (ΔV555–K558). A very similar deletion (ΔE554–K558) has been reported in a patient who responded to imatinib but developed acquired resistance to the drug after a median period of 20.2 months (29).

Cumulatively, these reported data, showing the concomitant presence of mutations in RTK genes and downstream in *BRAF* or *KRAS* genes, support the hypothesis of the involvement of the mitogen-activated protein kinase pathway in GIST development and suggest an interplay between the signals induced by the oncogenes. However, a more precise frequency of mutated GIST cases carrying activating mutations also in downstream effectors has to be better detected analyzing a higher number of cases such as all the patients enrolled into the phase II and III clinical trials for which the follow-up is present.

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 *PDGFRA* 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 KITΔ579 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 $BRAF^{V600E}$, 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, AKT, and BRAF protein levels are shown as control (Fig. 1A).

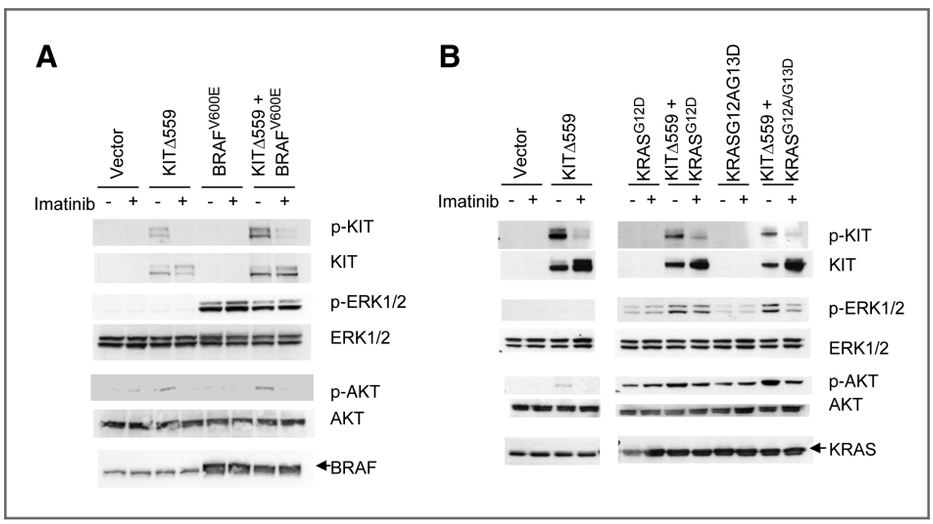

Figure 1. Effect of imatinib on ERK½ and AKT phosphorylation induced by expression of KIT and BRAF and KRAS mutants. Western blot analysis of HEK293T cells transiently transfected with KITΔ559 and BRAF (A) or KRAS (B) mutants alone or in combination, treated (+) or not (–) with 5 μmol/L imatinib. Arrows indicate transfected BRAF and KRAS proteins.

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 $BRAF^{V600E}$ strongly induced ERK1/2 phosphorylation, which was not affected by imatinib treatment; no effect of $BRAF^{V600E}$ on AKT activation was observed. In cells coexpressing KITΔ559 and $BRAF^{V600E}$ mutants ERK1/2 phosphorylation was comparable with that induced by $BRAF^{V600E}$ 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 $BRAF^{V600E}$, imatinib abrogates the KITΔ559-triggered AKT phosphorylation; however, it is not capable of affecting ERK1/2 phosphorylation, which is driven by $BRAF^{V600E}$.

A similar approach was undertaken to investigate the effect of concomitant expression of KIT and KRAS mutants. Expression vectors carrying $KRAS^{G12D}$ and $KRAS^{G12A/G13D}$ 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 $KRAS^{G12D}$ or $KRAS^{G12A/G13D}$ 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 $KRAS^{G12D}$ and $KRAS^{G12A/G13D}$ mutants alone, indicating that imatinib does not affect the signaling promoted by *KRAS* oncogenes. In keeping with this issue, in samples 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

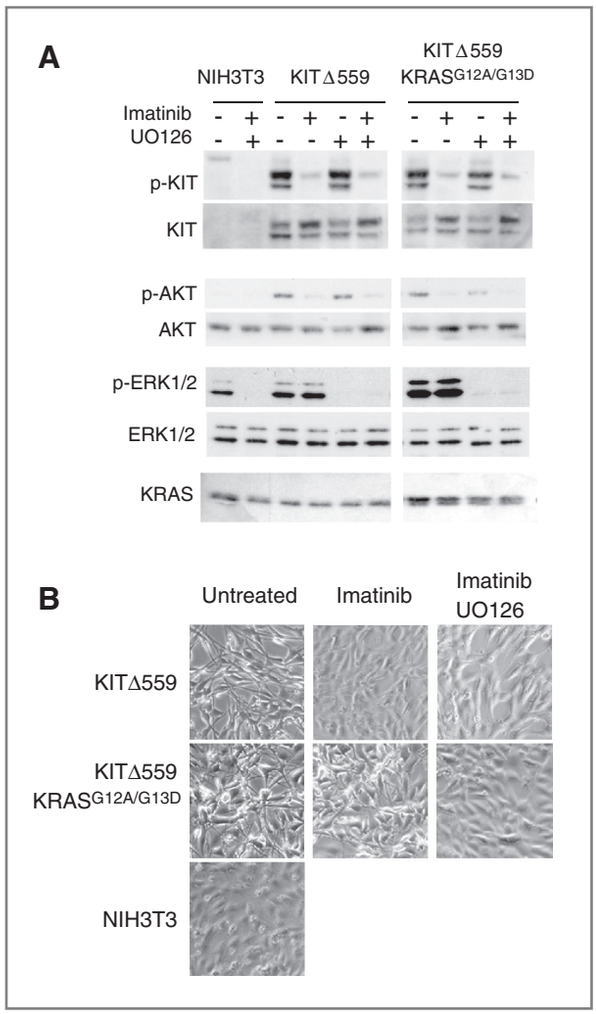

Figure 2. Effect of imatinib and UO126 treatment on signaling and cell morphology of NIH3T3 cells coexpressing KITΔ559 and KRAS mutants. A, Western blot analysis of NIH3T3 cells stably expressing KITΔ559 alone or in combination and KRAS mutants untreated or treated with imatinib (5 μmol/L, 48 hours), UO126 (10 μmol/L), or combination of the 2. B, phase contrast images of NIH3T3 cells stably expressing KITΔ559 alone or in combination, and KRAS mutants treated or not with imatinib (5 μmol/L, 48 hours), UO126 (10 μmol/L), or combination of the two. NIH3T3 morphology is shown as control. Photographs were taken at 10 × magnification with a digital camera system coupled with microscope (LEICA, DMIRB, Leica Microsystems GmbH).

NIH3T3-derived KITΔ559 cell line, exogenously expressing KITΔ559 mutant (18) was transfected with $KRAS^{G12A/G13D}$ construct, thus producing NIH3T3 clones stably expressing KIT and KRAS mutants. As shown in Fig. 2A, in cells expressing KITΔ559 oncogene a significant increase of AKT, with respect to naive NIH3T3 cells, was observed, and it was abrogated by imatinib treatment. ERK1/2 phosphorylation was only slightly increased and not affected by imatinib. In cells expressing both KITΔ559 and $KRAS^{G12A/G13D}$ oncogenes, 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 UO126. Both AKT and ERK1/2 pathways were abrogated by concomitant treatment with imatinib and UO126. Morphologic observation of cells used for these analyses is reported in Fig. 2B. Cells expressing KITΔ559 alone or in combination with $KRAS^{G12A/G13D}$ 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 $KRAS^{G12A/G13D}$, 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 UO126.

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 *PDGFRA* 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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