

Novel V600E BRAF Mutations in Imatinib-Naive and Imatinib-Resistant Gastrointestinal Stromal Tumors

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BRAF and *NRAS* are commonly mutated in cancer and represent the most frequent genetic events in malignant melanoma. More recently, a subset of melanomas was shown to overexpress KIT and harbor *KIT* mutations. Although most gastrointestinal stromal tumors (GISTs) exhibit activating mutations in either *KIT* or *PDGFRA*, about 10% of the cases lack mutations in these genes. It is our hypothesis following the melanoma model that mutations in *BRAF* or *NRAS* may play a role in wild-type GIST pathogenesis. Alterations in RAS/MEK/ERK pathway may also be involved in development of imatinib resistance in GIST, particularly in tumors lacking secondary *KIT* or *PDGFRA* mutations. Imatinib-naive wild-type GISTs from 61 patients, including 15 children and 28 imatinib-resistant tumors without secondary *KIT* mutations were analyzed. Screening for hot spots mutations in *BRAF* (exons 11 and 15) and *NRAS* (exons 2 and 3) was performed. A *BRAF* exon 15 V600E was identified in 3 of 61 GIST patients, who shared similar clinical features, being 49- to 55-years-old females and having their tumors located in the small bowel. The tumors were strongly KIT immunoreactive and had a high risk of malignancy. An identical V600E *BRAF* mutation was also identified in one of 28 imatinib resistant GIST lacking a defined mechanism of drug resistance. In conclusion, we identified a primary *BRAF* V600E mutations in 7% of adult GIST patients, lacking *KIT/PDGFRA* mutations. The *BRAF*-mutated GISTs show predilection for small bowel location and high risk of malignancy. A secondary V600E *BRAF* mutation could represent an alternative mechanism of imatinib resistance. Kinase inhibitors targeting *BRAF* may be effective therapeutic options in this molecular GIST subset. © 2008 Wiley-Liss, Inc.

INTRODUCTION

Gastrointestinal stromal tumors (GIST) are the most common mesenchymal tumors of the gastrointestinal tract, located mostly in the stomach and small bowel. GISTs express KIT and are thought to arise from a KIT-positive interstitial cell of Cajal (ICC), the pacemaker cells of the GI tract. Although most GISTs show activating mutations in either *KIT* or *PDGFRA*, 10–15% of cases have a wild-type genotype for these two oncogenes (Hirota et al., 1998; Rubin et al., 2001; Heinrich et al., 2003a,b). Imatinib mesylate (STI571, GleevecTM, Novartis Pharmaceuticals, Basel, Switzerland), a selective tyrosine kinase inhibitor targeting KIT and PDGFRA, is the frontline therapy for metastatic and unresectable GIST patients showing clinical responses in 80% of cases (Demetri et al., 2002). Despite a high rate of response in patients with *KIT* exon 11 mutated GISTs, the failure rate is significantly higher in patients with a wild type genotype, suggesting an alternative activated pathway not targeted by imatinib therapy (Heinrich et al., 2003a; Debiec-Rychter et al., 2004).

Activating *KIT* mutations, similar to those described in GISTs, have been reported recently in a subset of acral and mucosal malignant melanomas (Willmore-Payne et al., 2005; Antonescu et al., 2007). These mutations cluster within the juxtamembrane domain of KIT and occur in melanomas overexpressing the KIT protein. In contrast, most cutaneous melanomas harbor mutations in either *BRAF* or *NRAS* (Cruz et al., 2003; Michaloglou et al., 2007). *BRAF* mutations are detected in more than half of melanoma cases and their incidence is dependent to UV light exposure, being most common in melanomas arising in skin intermittently

Supported by: ACS MRSG CCE-106841, P01CA47179, Life Raft Group, GIST Cancer Research Fund, Shuman Family Fund for GIST Research, CA102613, CA102774, HL/DK55748.

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Received 7 February 2008; Accepted 1 June 2008

DOI 10.1002/gcc.20589

Published online 9 July 2008 in Wiley InterScience (www.interscience.wiley.com).

exposed to the sun (Curtin et al., 2005). The vast majority of *BRAF* mutations in melanomas are V600E missense mutations identified in a dominant hot spot, within the kinase domain (Michaloglou et al., 2007). *BRAF* is a member of the RAF family of serine/threonine protein kinases that are important effectors of RAS activation and is involved in the RAS-RAF-ERK signaling pathway, which connects extracellular signals to transcriptional regulation. *BRAF* mutations are seen in 7% of all cancers. Apart from melanoma, *BRAF* mutations are also implicated in the pathogenesis of certain epithelial malignancies, such as papillary thyroid carcinoma, colorectal carcinoma, as well as in some benign/pre-neoplastic lesions, such as melanocytic nevi and serrated colonic polyps (Kebebew et al., 2007; Michaloglou et al., 2007; Minoo et al., 2007).

Our hypothesis is that activating *BRAF* and/or *NRAS* mutations may play a role in the pathogenesis of GISTs lacking an identifiable mechanism of KIT or PDGFRA activation. We further postulate that the acquisition of secondary *BRAF* or *NRAS* mutations may be involved in the mechanism of imatinib resistance in GIST.

MATERIALS AND METHODS

Patient Selection and Clinicopathologic Features

Patients with a diagnosis of GIST were identified from the Memorial Sloan-Kettering Cancer Center sarcoma database. Patient demographics, treatment data, and follow up information were obtained from chart review. The pathologic diagnosis was confirmed using standard H&E staining and immunoreactivity for CD117 (DAKO Corp., Carpinteria, California; 1:500) on formalin fixed paraffin embedded tissue. In case no. 4 other immunohistochemical studies were performed, including antibodies for PDGFRA (LabVision; 1:500), desmin (DAKO, 1:50) and myogenin (Ventana Medical Systems, Inc, Tucson, Arizona; prediluted). In addition, the protein expression status for p16 and PTEN was examined by immunohistochemistry using prediluted antibodies from Ventana Medical Systems, Inc, Tucson, Arizona. Pediatric and adult wild-type GISTs from imatinib-naïve patients were included in the study. Also selected for analysis were imatinib resistant GISTs lacking a defined mechanism of resistance, such as the presence of a secondary mutation in *KIT*/*PDGFRA*. The study was approved by the Institutional Review Board.

KIT/*PDGFRA*, *BRAF*, and *NRAS* Genotyping

Genomic DNA was isolated either from snap-frozen tumor tissue in 48 samples or from paraffin-embedded tissue in 41, as described previously (Antonescu et al., 2003). Adequate DNA for mutational analysis was obtained in all 89 samples which were then tested for the known sites of *KIT* (exons 9, 11, 13, 14, and 17) and *PDGFRA* (exons 12, 14, and 18) mutations. Primer sequences and annealing temperatures were as described (Antonescu et al., 2003, Agaram et al., 2007). Direct sequencing of PCR products was performed for all exons tested and each ABI sequence was compared to the NCBI human *KIT* and *PDGFRA* gene sequences.

BRAF exons 11 and 15 and *NRAS* exons 2 and 3 were screened for mutations by Denaturing High Pressure Liquid Chromatography (DHPLC) using the WAVE System (Transgenomic, Inc., Omaha, Nebraska). The elution temperature for each amplicon was calculated using the WAVEMAKER™ software. The PCR products of cases with DHPLC profiles indicating mutations were further confirmed by direct sequencing in half of the wild-type sequences as well as all mutated samples.

One case (case no. 4) was also subjected to FISH analysis for the presence of *KIT* or *PDGFRA* gene copy alterations, using paraffin sections from pre- and post-imatinib samples. The *KIT* probes used were two overlapping BAC clones: CTD-3180G20 and RP11-722F21 (Invitrogen), labeled by nick-translation with Spectrum Green (Vysis, Abbott Laboratories, Illinois). The *PDGFRA* probe used included 2 BAC clones that spanned about 290 kb around the gene: RP11-117E8 and RP11-231C18. A chromosome 4 centromeric probe labeled with Spectrum Orange (CEP 4, Vysis) was used as reference.

RESULTS

Eighty-nine samples from 87 patients were selected for the molecular analysis. From the untreated group, there were 61 wild-type GIST patients, including 15 children (≤ 18 years of age), 5 young adults (>18 , ≤ 30 years), and 41 adults. The remaining 26 patients were selected from the imatinib resistance group which lacked secondary mutations in *KIT* or *PDGFRA*.

BRAF^{V600E} Mutations are Present in a Subset of Small Bowel *KIT*/*PDGFRA* Wild-Type GISTs

Three (4%) of 61 *KIT*/*PDGFRA* wild-type GISTs showed a *BRAF* exon 15 V600E mutation, while

TABLE 1. Clinical and Pathologic Findings in BRAF mutated GIST patients

	Age/Sex	Primary Tumor Size (cm)	Primary Tumor Site	MF/50 HPF	Stage at presentation	CD117	PTEN	P16	LFU/mo
1 ^a	52/F	10	SB	90	Periton Mets	P	P	N	DOD/18
2	55/F	10	SB	5	Primary	P	NA	NA	NED/9
3	49/F	9	SB	50	Primary	P	P	P	NED/13
4 ^b	66/M	20	Stomach	68	Primary	N	N	N	NED/32

F, female; M, male; cm, centimeters; SB, small bowel; periton mets, peritoneal metastases; MF, mitotic figures; HPF, high power fields; P, positive; N, negative; DOD, dead of disease; NED, no evidence of disease; AWD, alive with disease; NA, not available, LFU, last follow-up; mo, months.

^aGenotyping on normal tissue as well.

^bImatinib resistant GIST.

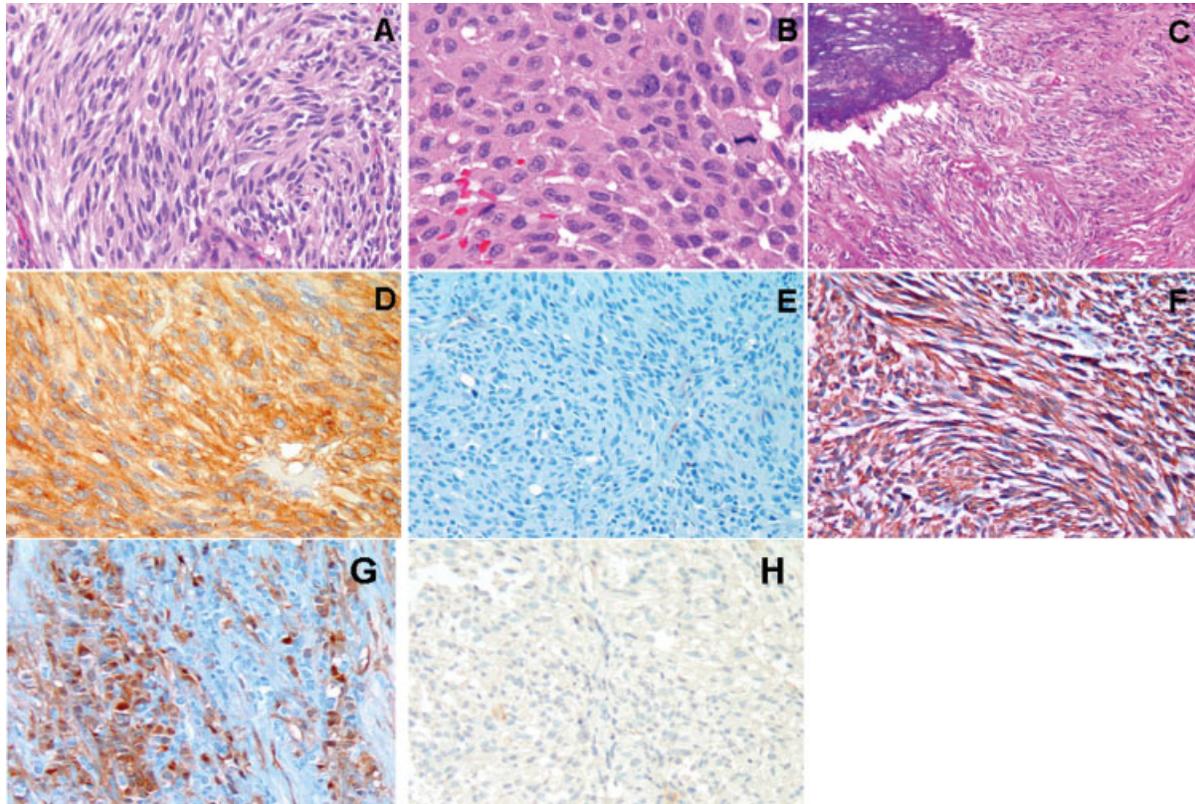


Figure 1. Pathologic findings in BRAF mutated GIST, showing a predominantly monomorphic spindle cell morphology (A: patient no. 1, HE, $\times 200$), with only focal epithelioid features and high mitotic activity (B: patient no. 1, HE $\times 400$); one tumor showed focal areas of dystrophic calcification (C: patient 4, HE, $\times 100$). Tumors were diffusely immunore-

active for CD117 (D: patient no. 2, $\times 200$). Additional findings included: loss of P16 protein expression while PTEN expression was maintained (E, F: patient no. 1; $\times 200$), as compared to patient no. 3 who had maintained P16 protein expression (G, $\times 200$) and to patient no. 4 who lost PTEN (H, $\times 200$).

none were detected in *BRAF* exon 11. In one of these three *BRAF* mutation positive patients, normal tissue was available for molecular analysis, which did not display the V600E, thus excluding the possibility of a germline mutation. The three patients with *BRAF*-mutated GISTs shared similar clinical findings, being middle-aged females (range of 49–55 years) with tumors located in the small bowel (Table 1). Microscopically, the three tumors had a predominantly spindle cell morphology and in two cases there was a small epithelioid compo-

nent as well (Figs. 1A and 1B). In one case the tumor showed areas of sclerosis and dystrophic calcification (Fig. 1C). Tumors were diffusely immunoreactive for CD117 (Fig. 1D). There were no distinctive histological features that would distinguish this subset from the more commonly *KIT*-mutated tumors. The mitotic count ranged from 5 to 90 mitoses/50 high power fields (HPFs). These three patients were considered to have a high risk of malignancy GIST either based on tumor size (≥ 10 cm), high mitotic count (≥ 10 MF/50 HPFs)

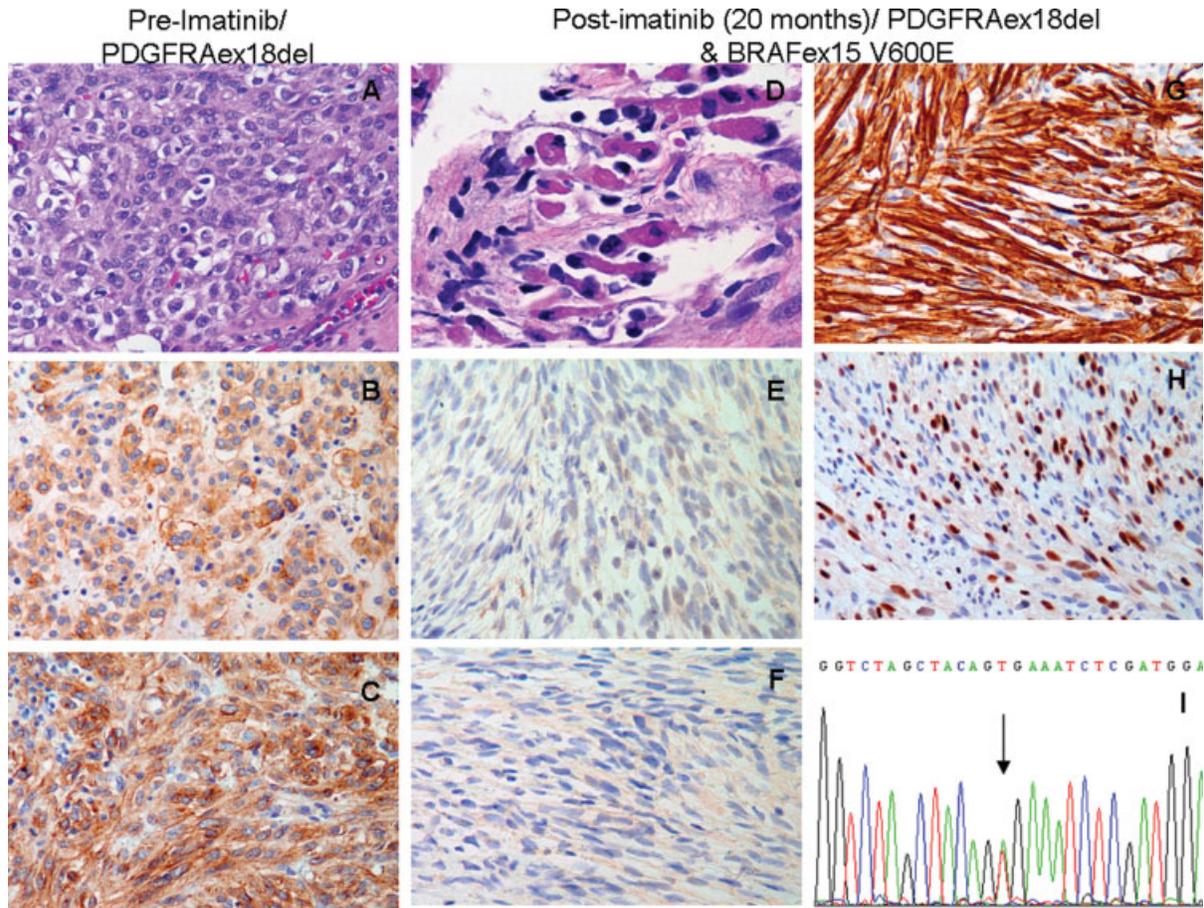


Figure 2. Pre-imatinib pathologic findings revealed a high risk primary gastric GIST with epithelioid morphology (A, HE, $\times 200$) and diffuse reactivity for CD117 (B, $\times 200$) and PDGFRA (C, $\times 200$). The post-imatinib resection for peritoneal metastasis showed foci of viable spindle cells with abundant eosinophilic cytoplasm and strap cells, resembling an embryonal rhabdomyosarcoma phenotype (D, HE,

$\times 400$), which was negative for CD117, PDGFRA, (E,F, $\times 200$), but strongly positive for skeletal muscle markers (G, desmin, $\times 200$; H, myogenin, $\times 200$). The mutation analysis of this sample revealed in addition to a PDGFRA exon 18 deletion, a BRAF exon 15 V600E substitution (I, ABI sequence).

or both. One patient (Patient no. 1), who developed peritoneal metastasis, died of disease 18 months following diagnosis and the remaining two are with no evidence of disease (NED) at 9 and 13 months. *BRAF* mutations were not detected in any of the 15 pediatric and 5 young adult GISTs.

BRAF Mutation is a Potential Alternative Mechanism of Imatinib Resistance in GIST

We identified 28 GIST tumors from 26 imatinib resistant patients, lacking an identifiable mechanism of treatment failure. All except one patient had tumors harboring a primary mutation in either *KIT* or *PDGFRA* and developed secondary resistance to imatinib after an initial response. The only patient with a wild-type genotype showed primary resistance to imatinib. A *BRAF* V600E mutation was identified in 1 of the 28 samples tested. This patient is a 66-years-old man with a primary high

risk gastric GIST measuring 20 cm and a high mitotic count of 68 MF/HPFs. The tumor displayed a pure epithelioid morphology and showed diffuse staining for both *KIT* and *PDGFRA* by immunohistochemistry (Figs. 2A–2C). Mutation analysis of the primary tumor revealed a heterozygous *PDGFRA* exon 18 (4 amino acid deletion 842–845) mutation and absence of *BRAF* or *NRAS* mutations. Following resection of the primary tumor, he was treated with imatinib for 20 months for metastatic peritoneal disease. On resection of the peritoneal nodules, despite a good overall response with large areas of fibrosis, there were microscopic foci of viable and proliferating tumor cells, in keeping with a resistant phenotype. However, these foci did not resemble the pre-imatinib morphology and were composed of a spindle cell proliferation lacking both *KIT* and *PDGFRA* expression (Figs. 2D–2F). Moreover, these imatinib-resistant areas

closely resembled an embryonal rhabdomyosarcoma phenotype, with spindle cells containing abundant eosinophilic cytoplasmic and cross-striations, which were diffusely positive for desmin and myogenin (Figs. 2G and 2H), markers of skeletal muscle differentiation. Mutational analysis from DNA obtained from micro-dissection of this area showed the presence of a *BRAF* V600E mutation in addition to the primary *PDGFRA* exon 18 deletion (Fig. 2I). No mutations were identified in *BRAF* exon 11 in any of the imatinib-resistant patients tested.

This case was also subjected to FISH analysis for the presence of *KIT* or *PDGFRA* gene copy alterations, using paraffin sections from pre- and post-imatinib samples. This analysis is particularly relevant since loss of protein expression for both *KIT* and *PDGFRA* was seen in the post-imatinib sample, suggesting a possible large deletion encompassing both genes. However, there were no abnormalities seen by FISH, with two copies of *KIT* and *PDGFRA* probes in both the pre and post-treatment samples tested (data not shown).

No frozen tissue was available from any of the *BRAF* mutation positive GISTs to assess the phosphorylation status of downstream targets, MEK and ERK by Western blotting.

NRAS Mutations do not Appear to be Involved in GIST Pathogenesis

In 56 untreated wild-type GIST patients (15 children, 5 young adult, and 36 adults) and 26 imatinib-resistant patients without secondary *KIT* or *PDGFRA* mutations adequate material was available for detecting mutations in *NRAS* exons 2 and 3. However no *NRAS* mutations were identified in any of these GIST subsets.

CDKN2A (p16) Alterations may be Associated with BRAF Mutations in GISTs

We analyzed the protein expression status of both p16 and PTEN as examined by immunohistochemistry in 3 of the 4 *BRAF* mutated GISTs with available material. In two of the three cases tested cases, there was lack of P16 staining, while PTEN expression was retained (Figs. 1E–1H).

DISCUSSION

Melanoma is the prototypical example in which *BRAF* mutations have been detected in ~50% of the patients and *NRAS* in about 15%, with the two mutations typically being mutually exclusive (Brose et al., 2002; Gorden et al., 2003). More recently, activating *KIT* mutations were identified

in a small subset of melanomas lacking *BRAF* mutations (Curtin et al., 2006). As the progenitor cells for both melanoma and GIST, i.e., the melanocyte and the ICC, are *KIT*-expressing and *KIT*-dependent during embryologic development, it is possible that GIST pathogenesis may follow similar oncogenic signaling pathways observed in melanomas. Thus our working hypothesis was that alterations in the RAS-RAF-MEK signaling cascade may occur in GIST lacking identifiable mutations in *KIT* and *PDGFRA*. Furthermore, half of the imatinib-resistant GIST patients lack an identifiable mechanism of resistance, such as a secondary *KIT* mutation or *KIT* gene amplification (Antonescu et al., 2005; Debicc-Rychter et al., 2005; Heinrich et al., 2006). In this subset the acquisition of mutations in other signaling pathways, which are not targeted by imatinib, may occur. Thus our second hypothesis was that *BRAF* or *NRAS* mutations, commonly involved in other cancer types, may trigger an alternate mechanism of imatinib resistance in GIST patients.

To our knowledge this is the first report identifying *BRAF* mutations in either imatinib-naïve or imatinib-resistant GISTs. Although detected only in a minority of *KIT/PDGFRA* wild-type GISTs, *BRAF* mutations appear to be associated with a distinct clinicopathologic phenotype, occurring in middle-aged females and being preferentially located in the small bowel. Furthermore, the three tumors showed pathologic features consistent with a high risk of malignancy, as defined either by large tumor size or high mitotic count or both. *KIT* protein was consistently overexpressed in these cases and there were no other distinctive features microscopically that would differentiate them from *KIT* mutated GISTs. The mechanism through which *BRAF* activating mutations in GISTs may affect *KIT* signaling remains unclear. Similar to other tumor types where *BRAF* mutations are more commonly observed, the mutation in GIST was present in the exon 15 V600E hot spot. No other mutations were identified in the *BRAF* exon 11 or *NRAS* exons 2 and 3.

BRAF is a member of the RAF family of serine/threonine protein kinases that are important effectors of RAS activation and is involved in the RAS-RAF-ERK signaling pathway, which connects extracellular signals to transcriptional regulation. Activated RAF proteins phosphorylate MEK1 and 2 (MAPKKs), which in turn phosphorylate ERK1 and 2 (MAPKs), leading to the activation of several cytoplasmic and nuclear targets, including transcription factors such as ETS11, c-JUN, and c-

MYC. Thus the MEK-ERK effector pathway is commonly dysregulated in cancer, often through gain-of-function mutations of either RAS or RAF family members (Davies et al., 2002). The RAS/MAP kinase pathway is activated by many receptor tyrosine kinases, including KIT. The role of oncogenic KIT signaling on ERK1/2 activation has been recently evaluated by our group in a *KIT*^{V558Δ/+} GIST mouse model, produced by a knock-in strategy introducing a *KIT* exon 11-activating mutation into the mouse genome (Rossi et al., 2006). Although untreated tumors show consistent activation of ERK1/2 phosphorylation, there is no effect of imatinib treatment on ERK activation (Rossi et al., 2006). Thus *BRAF* mutated GIST patients may be similarly resistant to imatinib inhibition.

BRAF mutations are known to occur in melanocytic nevi and other benign or precursor lesions like colonic serrated polyps. This observation raises the possibility that *BRAF* mutations are an early event in the tumor development and subsequent gene alterations may be required for tumor progression to a malignant phenotype. Additional alterations in tumor suppressor genes, such as *CDKN2A* or *PTEN* have been shown to play a synergistic role along with the existing *BRAF* mutations, necessary for tumor development and/or progression (Daniotti et al., 2004; Curtin et al., 2005; Michaloglou et al., 2007). Melanocytic nevi typically show increased levels of *CDKN2A*, whereas most melanomas lose P16 expression (Curtin et al., 2005). *CDKN2A* encodes a 16 kDa nuclear protein (P16INK4A or P16) that specifically binds to the cyclin-dependent kinases 4 (CDK4) and 6 (CDK6), which are involved in the G1 checkpoint of the mammalian cell cycle. The preserved P16 function in melanocytic nevi maintains a stable cell cycle arrest even in the presence of a *BRAF* mutation, while loss of P16 leads to development of melanoma. *PTEN* mutations have also been implicated along with *BRAF* mutations in a subset of melanomas. *PTEN* encodes for a lipid and protein phosphatase and regulates intracellular levels of the lipid phosphatidylinositol phosphate (PIP3), involved in the control of apoptosis via AKT (Bonneau and Longy, 2000). In our study, loss of P16 expression by immunohistochemistry was noted in two of the *BRAF* mutated GISTs. *PTEN* expression was maintained in two of the three cases tested.

The most frequent mechanism of imatinib-resistance in GIST is the acquisition of secondary mutations either in *KIT* or *PDGFRA* which are

detected in half of the patients. These 2nd site mutations occur after a longer period of imatinib therapy (median 27 months), compared with resistant tumors without secondary mutations (median 14.5 months) and are typically seen in patients with *KIT* exon 11 mutated GISTs (Antonescu et al., 2005). Our patient with a high risk gastric GIST showed expression of both *KIT* and *PDGFRA* proteins in the primary tumor and direct sequencing demonstrated a primary *PDGFRA* exon 18 deletion in the absence of *BRAF* or *NRAS* mutations. The imatinib resistant tumor was resected after 20 months of imatinib therapy and showed not only loss of *KIT* and *PDGFRA* protein expression, but trans-differentiation into a rhabdomyosarcoma phenotype. These foci showed a high proliferation rate and diffuse expression for skeletal muscle markers, such as desmin and myogenin. A similar phenotypic switch or dedifferentiation phenomenon, with loss of *KIT* expression and acquisition of aberrant lines of differentiation has been previously reported in rare cases of imatinib-resistant patients, but thus far was not attributed to a particular genotype (Pauwels et al., 2005).

The detection of *BRAF* mutations in GIST patients is highly relevant clinically. First, it identifies a subset of *KIT/PDGFRA* wild type GIST patients who are less sensitive to imatinib and could benefit from the inhibition of alternative pathways. Second, it has been recently shown that mutation in *BRAF* is associated with enhanced and selective sensitivity to MEK inhibition when compared to "wild-type" cell or cells harboring RAS mutations. This MEK dependency was noted in *BRAF* mutant cells regardless of tissue lineage (Solit et al., 2006). Thus newer compounds targeting *BRAF* mutations and the RAS-RAF-MEK pathway now under study in clinical trials in melanoma or other tumor types, may show efficacy in *BRAF*-mutated GIST patients. Sorafenib, an inhibitor of RAF, VEGFR and PDGFR has been studied in melanoma and thyroid cell lines and has shown an inhibitory effect (Haluska et al., 2007). In in vitro studies modeling imatinib resistant GIST patients carrying the gatekeeper *KIT* T670I secondary mutation, sorafenib showed the highest potency in inhibiting cell proliferation and inducing apoptosis compared to other kinase inhibitors (Guo et al., 2007). Similar results have been obtained using the MEK inhibitor CI-1040 (Solit et al., 2006). Heat shock protein 90 (HSP90) inhibitors (geldanamycin or 17AAG) have also shown to be effective in *BRAF* mutants based on in vitro studies (Sharp and Workman, 2006).

In summary, this is the first report detecting *BRAF* mutations in imatinib-naïve or imatinib-resistant GISTs. They occur in a small subset of intestinal high risk GISTs lacking *KIT/PDGFRA* mutations. This finding delineates a new molecular group of patients who may benefit from selective *BRAF* inhibitors, as an alternative therapeutic option to imatinib. Furthermore, the acquisition of *BRAF* mutations may play a role in triggering imatinib-resistance in GIST patients and may induce a *KIT*-negative trans-differentiation phenotype in these tumors. Since our observations were made on a limited number of cases, additional cases are thus required to confirm that *BRAF* mutated GIST indeed show a distinct clinicopathologic phenotype.

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