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Novel Oncogene and Tumor Suppressor Mutations in *KIT* and *PDGFRA* Wild Type Gastrointestinal Stromal Tumors Revealed by Next Generation Sequencing

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Abstract

Among gastrointestinal stromal tumors (GISTs) of 10–15% are negative for *KIT* and *PDGFRA*, and most of these cases are SDH deficient. Recent studies have provided data on additional molecular alterations such as *KRAS* in *KIT* mutant GISTs. We aimed to assess the frequency and spectrum of somatic mutations in common oncogenes as well as copy number variations in GISTs negative for *KIT* and *PDGFRA* mutations. GISTs with wild type *KIT/PDGFRA* were tested via next generation sequencing for somatic mutations in 341 genes. SDHB immunohistochemistry to evaluate for SDH deficiency was also performed. Of 267 GISTs tested for *KIT* and *PDGFRA* mutations, 15 were wild type, of which eight cases had material available for further testing. All eight cases had loss of SDHB expression and had various molecular alterations involving *ARID1A*, *TP53*, and other genes. One case had a *KRAS* G12V (c.35G>T) mutation in both the primary gastric tumor and a post-imatinib recurrence. This tumor had anaplastic features and was resistant to multiple tyrosine kinase inhibitors, ultimately resulting in cancer-related mortality within 2 years of diagnosis. In conclusion, *KRAS* mutations occur in rare GISTs with wild type *KIT*and *PDGFRA*. These tumors may display immunohistochemical positivity for KITand primary resistance to tyrosine kinase inhibitors.

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal neoplasms of the gastrointestinal tract. These tumors are characteristically positivity for KIT immunohistochemistry as well as mutations within the *KIT* or *PDGFRA* receptor tyrosine kinase genes (Miettinen and Lasota, 2006). The majority of these tumors are sensitive to imatinib or second generation tyrosine kinase inhibitors such as sunitinib or sorafenib.

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However, 10–15% are *KIT* and *PDGFRA* wild type, some of which harbor mutations in *NF1*, *BRAF* (V600E), or succinate dehydrogenase *SDH* complex genes (Wang et al., 2011; Falchook et al., 2013).

KRAS mutations have recently been described in GISTs, and unlike mutually exclusive *BRAF* V600E mutations, have only been reported to date in the presence of either *KIT* or *PDGFRA* mutations (Miranda et al., 2012; Antonescu et al., 2013; Serrano et al., Epub ahead of print). Further, their presence and prevalence of *KRAS* mutation in GIST has been disputed (Lasota et al., 2013). While numerous recent studies have yielded a high number of *KIT* mutant GISTs tested for *KRAS* mutation, wild type GISTs have not been held to such rigorous study, likely due to relative rarity of these tumors. Accordingly, *KRAS* mutations have never been reported in *KIT*/*PDGFRA* wild type GISTs. In this study, we have surveyed GISTs wild type for *KIT* and *PDGFRA* for the presence of *KRAS* mutations. We report the clinicopathologic and molecular features of a case of a *KRAS* G12V mutant, malignant gastric GIST with immunohistochemical positivity for *KIT* and we review the literature on *KRAS* mutations in GIST.

MATERIALS AND METHODS

Patient Selection

After approval by our local institutional review board, molecular results for *KIT* and *PDGFRA* mutation testing performed in the Laboratory of Diagnostic Molecular Pathology were reviewed for all 267 GISTs clinically analyzed between January 2009 and December 2013. The diagnosis of GIST was based on morphology and positive immunohistochemical staining for either KIT or DOG1. Cases with wild type *KIT* and *PDGFRA* results ($n = 15$) underwent both SDHB immunohistochemistry and *KRAS* sequencing.

Mutation Analysis

Genomic DNA was extracted from formalinfixed paraffin-embedded tissue after macrodissection using the DNeasy Tissue KIT (Qiagen, Valencia, CA), following the manufacturer's standard protocol. *KIT* and *PDGFRA* mutations were routinely detected by a sizing assay, Sanger sequencing, and next generation sequencing (NGS). *KRAS* mutation was detected via NGS and mass spectrometry.

Briefly, insertions and deletion of exons 9 and 11 of *KIT* were detected by length analysis of fluorescently labeled polymerase chain reaction products on a capillary electrophoresis instrument (ABI 3730) (Pan et al., 2005). Negative cases underwent Sanger sequencing of *KIT* exons 9, 11, 13, and 17 and *PDGFRA* exons 12, 14, and 18. The coding regions of these exons were amplified using Hot-Start *Taq* DNA polymerase and appropriate primers. The PCR products were purified using Spin Columns (Qiagen) and sequenced using BigDye Terminator v3.1 Cycle Sequencing *KIT* (Applied Biosystems) according to the manufacturer's protocol on an ABI 3730 running ABI Prism DNA Sequence Analysis Software. All PCR products were sequenced with forward and reverse primers. Concordant results in duplicate in forward and reverse directions were required for mutation calling.

NGS of *KIT*/*PDGFRA* wild type GISTs ($n = 8$) was performed with the clinically validated NGS assay, MSK-IMPACT (Memorial Sloan Kettering- Integrated Mutation Profiling of Actionable Cancer Targets). MSK-IMPACT is a custom hybrid capture-based deep sequencing assay that interrogates 341 cancer-associated genes (including all exons of *KIT*, *PDGFRA*, *BRAF*, and *KRAS*, see Supporting Information Table 1 for a full list of genes) for single nucleotide variants (SNVs), small indels, and somatic copy number alterations (Cheng et al., in press). Briefly, DNA isolated from FFPE samples were subjected to shearing, followed by Library preparation. Matched normal tissue was processed in the same manner where available and samples were pooled together for sequencing on an Illumina HiSeq 2500. 100bp paired end sequence reads were aligned to reference human genome (hg19) using Burrows-Wheeler Aligner BWA (Li and Durbin, 2010). SNVs were detected using MuTect (Cibulskis et al., 2013); small indels were identified using SomaticIndelDetector. Matched normal DNA was used to filter out germ line variants. In cases where matched normal tissue was not available ($n = 3$), a mixture of pooled FFPE normal DNA was used as unmatched normal for mutation calling. SNVs and indels required at least 5% alternate allele frequency for novel mutations and 2% for hotspot mutations. Minimum read coverage, at the position where the mutation is called, required was 20 for both classes of mutations. To detect copy number changes, an in-house developed algorithm was used. Briefly, Loess normalized coverage values for each exon sequenced were used to derive tumor/normal ratios, which were subsequently log-transformed. The log-ratio values were used in segmentation by circular binary segmentation (Olshen et al., 2004). Segmented values were used to derive a null distribution to estimate significant changes. Fold change >2.0 (gain) and <2.0 (loss) along with $P < 0.05$ (FDR corrected) were used as criteria for calling copy number changes. All candidate mutations were manually reviewed using IntegratedG-enomicsViewer (Robinson et al., 2011). Technical details of this NGS assay are described elsewhere (Jelinic et al., 2014).

KRAS mutation testing on a separate sample of any positive GIST was performed with separate methodology for confirmation and assessment of heterogeneity. The separate methodology used was the MassARRAY system (Sequenom) with primers as previously described (Arcila et al., 2011; Chaft et al., 2012) at the following hotspots in duplicate with forward and reverse primers: c.34, 35 and forward only primers: 37, 38, 181, 182, 183, 351, and 437. Allele frequency was estimated by dividing mutant peak height by the sum of mutant and nonmutant peak height.

RESULTS

Prevalence of SDH Deficiency and KRAS Mutation

Of 267 GISTs analyzed for *KIT* and *PDGFRA* mutation, 15 were negative for mutations in these genes. Eight *KIT*/*PDGFRA* wild type GISTs had material available for *KRAS* testing, of which one case was positive for a *KRAS* G12V (c.35G>T) mutation. All eight cases wild type for *KIT* and *PDGFRA* by previously performed sizing assays and Sanger sequencing were also negative for *KIT* and *PDGFRA* mutations via NGS. The details of this case are presented below. All eight cases, including the *KRAS* mutant, were positive for *KIT* expression by immunohistochemistry and showed absence of immunohistochemical

expression of SDHB. Two cases harbored frame-shift mutations in *TP53*, making *TP53* the most commonly mutated tumor suppressor in the cohort. One case had an *ARID1A* mutation with loss of ARID1A immunohistochemical expression. Somatic alterations from all cases are summarized in Table 1.

KRAS Mutant GIST Clinical History

The patient displaying a *KRAS* mutation was a 67-year-old male with a 25-pack year history of smoking, a negative family history, and a remote history of prostate cancer, presented with abdominal pain. A computed tomography scan revealed massive lymphadenopathy involving the chest, abdomen, and pelvis as well as a 4.7 cm gastric mass. Biopsies revealed stage IV follicular lymphoma and synchronous GIST. The patient received chemotherapy for his lymphoma with positron emission tomography negative response.

KRAS Mutant GIST Pathology Findings

Initial wedge gastrectomy revealed a 6.5 × 6.5 × 4.5 cm encapsulated, lobulated, and partially cystic fleshy tan yellow mass within the wall of the stomach. The patient's second, post-imatinib, gastric resection specimen on the recurrent GIST revealed multiple nodules of similar appearance (Fig. 1A) Surgical pathology revealed mostly solid architecture with lymphovascular invasion (Fig. 1B), epithelioid to rhabdoid morphology with areas of anaplasia, multinucleation, intracytoplasmic vacuolization, and an increased mitotic rate of 99 mitoses per 50 high powered fields (Fig. 1C). Multinodular or plexiform architecture was not present. The tumor demonstrated immunohisto-chemical reactivity for KIT (Fig. 1D), CD34 (Fig. 1E), desmin, smooth, muscle actin, and MDM2 (Fig.1F) and was negative for S100 and lost expression of SDHB. Histologic examination and immunohistochemistry for CD20, CD10, and BCL2 revealed the resected GIST to be negative for microscopic involvement by the patient's known lymphoma.

KRAS Mutant GIST Molecular Findings

The original resection was analyzed first. Sizing assay and Sanger sequencing were negative for mutations in both *KIT* and *PDGFRA*. NGS (MSK-IMPACT assay) supported these results and also revealed the missense mutation *KRAS* G12V(c.35G>T) at an allele frequency of 25% (Fig. 2A). Mass spectrometry analysis of the post-imatinib gastric recurrence revealed the same *KRAS* mutation that was found in the original resection at similar estimated allele frequency of 29% (Fig. 2B). Amplification of *IGF1* and *MDM2* was also identified within the primary tumor by MSK-IMPACT (Fig. 2C). The patient progressed on imatinib at 400 mg and sunitinib at 37.5 mg, with widespread disease in the stomach, colon, and liver at 12 months after diagnosis. After secondary resection, the patient failed multiple other therapies including sorafenib plus temozolomide, pazopanib, and gemcitabine. The patient died of complications related to metastatic GIST 24 months after initial diagnosis.

DISCUSSION

In summary, we describe a lethal gastric GIST with *KRAS* mutation that did not respond to multiple lines of therapy. The identification of *KRAS* mutations in GIST is still relatively

new, and recent previously reported cases of *KIT*-mutated, *KRAS*-mutated GISTs are summarized in Table 2.

The molecular profile of the tumor is summarized in Table 1. Several other mutations in tumor suppressors including *ATRX, NF1, MSH2, PMS1*, and *DICER1* were also detected in this case. The contribution of several of these mutations to GIST development, progression, and imatinib resistance has not been well established. Interestingly, amplification of the proto-oncogenes *MDM2* and *IGF1* was identified in the GIST. The *MDM2* gene, perhaps most well known in association with well-differentiated liposarcoma and rarely reported in GISTs, encodes an E3 ubiquitin protein ligase, a negative regulator of TP53. In vitro studies have suggested that *MDM2* cooperates with RAS in tumor formation (Alarcon-Vargas and Ronai, 2002). The IGF1 gene encodes the ligand, insulin-like growth factor. The IGF system has been indicated to be activated in GISTs, and recent data have shown that increased IGF1 correlates with poor prognosis in GISTs (Braconi et al., 2008). Interaction between this ligand and its receptor has been shown to activate the PI3K and MAPK pathways, leading to cellular proliferation (Yu and Rohan, 2000). Like *MDM2*, recent data have shown that the IGF system cooperates with *KRAS* mutation in lung cancer (Molina-Arcas et al., 2013).

The other seven KIT/PDGFRA wild type GISTs harbored various other mutations in tumor suppressor genes were detected, including *TP53* frameshift mutations (two cases), and an *ARID1A* frameshift mutation with resulting loss of ARID1A immunohistochemical expression. *TP53* mutations in GIST occur in approximately 8% of GISTs and are associated with shortened progression free survival as well as rare cases of dedifferentiated GIST (Romeo et al., 2009, Antonescu et al., 2013). To our knowledge, this is the first report of *ARID1A* mutations in GISTs. Loss of ARID1A has recently been linked to increased phosphorylation of AKT and activation of the PI3K/AKT pathway, making it a potential target for targeted therapy (Samartzis et al., 2014).

The prevalence of *KRAS* mutation in GISTs ranges from <0.2% of all GISTs (Lasota et al., 2013) to, in our series of eight wild type GISTs, 11%. In 2012, Miranda et al. first described the occurrence of *KRAS* mutations in GISTs in three patients, two of which harbored *KIT* mutations and one of which harbored a *PDGFRA* mutation. The estimated incidence of *KRAS* mutation in GIST was 5% in their series (Miranda et al., 2012). A year later, Lasota et al. described a paucity of *KRAS* mutations in a cohort of 514 GISTs (Lasota et al., 2013), including 117 *KIT*/ *PDGFRA* wild type cases while Antonescu et al described another *KIT*-mutant, *KRAS*-mutant GIST that developed in a patient receiving imatinib for chronic myelogenous leukemia (Antonescu et al., 2013). In 2014, Serrano et al. described a *KIT* exon 11 mutant (554–559del) gastric GIST with a *KRAS* G12R mutation limited to 1 of 3 nodules of tumor tested. While the *KRAS* wild type, *KIT* mutant areas responded to imatinib, the *KRAS* mutated area did not (Serrano et al., Epub ahead of print). This case thus represents the sixth report of *KRAS* mutation in GIST and the first report of *KRAS* mutant GIST in the absence of *KIT* or *PDGFRA* mutation.

Factors contributing to the development of a *KRAS*-mutant GIST are currently unknown. This patient had a history of multiple other cancers, as do many patients with GIST. However, he did not have either a family history of GIST or of any known cancer syndrome.

Of interest, the patient had a smoking history of 25-pack years. Smoking has been linked to multiple types of cancer other than pulmonary and oropharyngeal, including pancreatic and colorectal carcinomas (Liang et al., 2009; Edderkaoui and Thrower, 2013). Transversion mutations are frequently seen in smoking-related malignancies. The particular missense transversion mutation present within this GIST, *KRAS* G12V (c. 35G>T) is frequently seen in smoking- associated pulmonary adenocarcinoma (Dogan et al., 2012).

Aside from this case, the case by Serrano et al. was the only other GIST with multiple intrapatient samples tested for *KRAS* mutation. Interestingly, the *KIT*/*KRAS* co-mutant case presented by Serrano et al. demonstrated *KRAS* heterogeneity, with only 1 of 3 GIST nodules positive for *KRAS* mutation (Serrano et al., Epub ahead of print). Not unsurprisingly, the *KRAS* mutant nodule demonstrated imatinib resistance while the nodule with only *KIT* mutation was sensitive. In contrast, both the pre- and post-imatinib samples from this case, which was *KRAS* mutation positive without activating mutation in *KIT* or *PDGFRA*, showed *KRAS* mutation homogeneity, with the same mutation of similar allele frequency in both samples tested. The differences and similarities between these two cases illustrate the following points: (1) *KRAS* mutation in *KIT*/*PDGFRA* wild type GISTs may be present as a part of the driving intracellular signaling alterations shared between multiple intrapatient samples, or as a resistance clone in a polyclonal background of *KIT* mutant GIST with imatinib resistance; and (2) *KRAS* mutant GISTs do not respond to imatinib.

Shortfalls of our study include small sample size as well as potential selection bias. *KIT*/*PDGFRA* sequencing is ordered by request of clinician or surgical pathologist as opposed to reflex testing. Therefore, cases with clinicopathologic features of SDH deficiency may undergo immunohistochemical staining without molecular analysis of *KIT*/ *PDGFRA*, resulting in a lower number of *KIT*/ *PDGFRA* wild type GISTS being tested in our clinical molecular lab. Additionally, low risk GISTs may not be tested if adjuvant therapy is not being considered. These factors may account for the fact that only 5.6% of out cohort was *KIT*/ *PDGFRA* wild type.

Regarding the use of next-generation sequencing in GISTs, recent studies have demonstrated this technique's potential applications. Whole exome sequencing of a *BRAF* mutant GIST treated with dabrafenib revealed possible mechanisms of resistance including a *PIK3CA* p. H1047R mutation and a *CDKN2A* aberration (Falchook et al., 2013), and whole exome sequencing of a select set of GISTs was performed to elucidate recurrently altered candidate genes discovered as regions of interest of microarray, leading to the discovery of potentially prognostically significant genes *SYNE2, DIAPH1*, and *RAD54L2* (Schopp-mann et al., 2013).

In conclusion, SDH deficient GISTs often have mutations or loss of known oncogenes including *TP53* and *ARID1A*. *KRAS* mutation can occur in *KIT*/*PDGFRA* wild type GISTs that immunohisto-chemically express the KIT protein and, not unexpectedly, such cases may not respond to receptor tyrosine kinase inhibition. Molecular screening for mutations downstream of *KIT* and *PDGFRA* including *KRAS* and *BRAF* may be useful to help elucidate factors contributing to imatinib primary resistance to imatinib in *KIT*/*PDGFRA*

wild type GIST patients as well as provide better-suited targeted therapy options in the future, such as small molecular *KRAS* inhibitors (Zimmermann et al., 2013).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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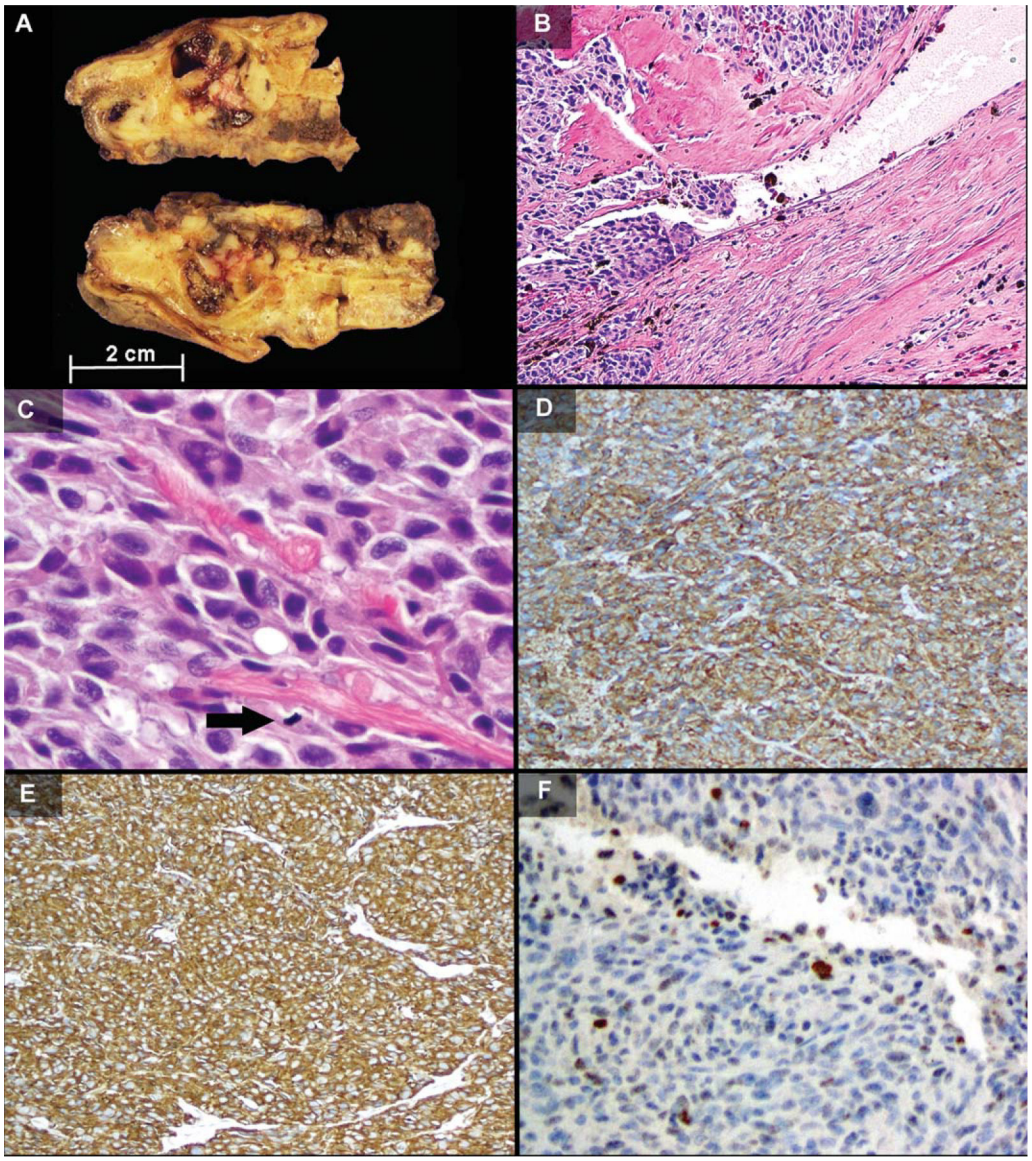

Figure 1.

Surgical pathology findings. A) Gross pathology of the recurrent post-imatinib gastric tumor showed multiple tan fleshy nodules with cystic hemorrhage within the gastric wall. B–F) The original resection demonstrated areas of lymphovascular invasion (B, H&E, 103), anaplasia with large tumor cells with multinucleation, intracytoplasmic vacuoles, and mitotic figure (arrow) (C, H&E, 40×), diffuse immunohistochemical positivity for KIT and CD34 (D and E, 10×), and patchy nuclear expression of MDM2 (F, 20×). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

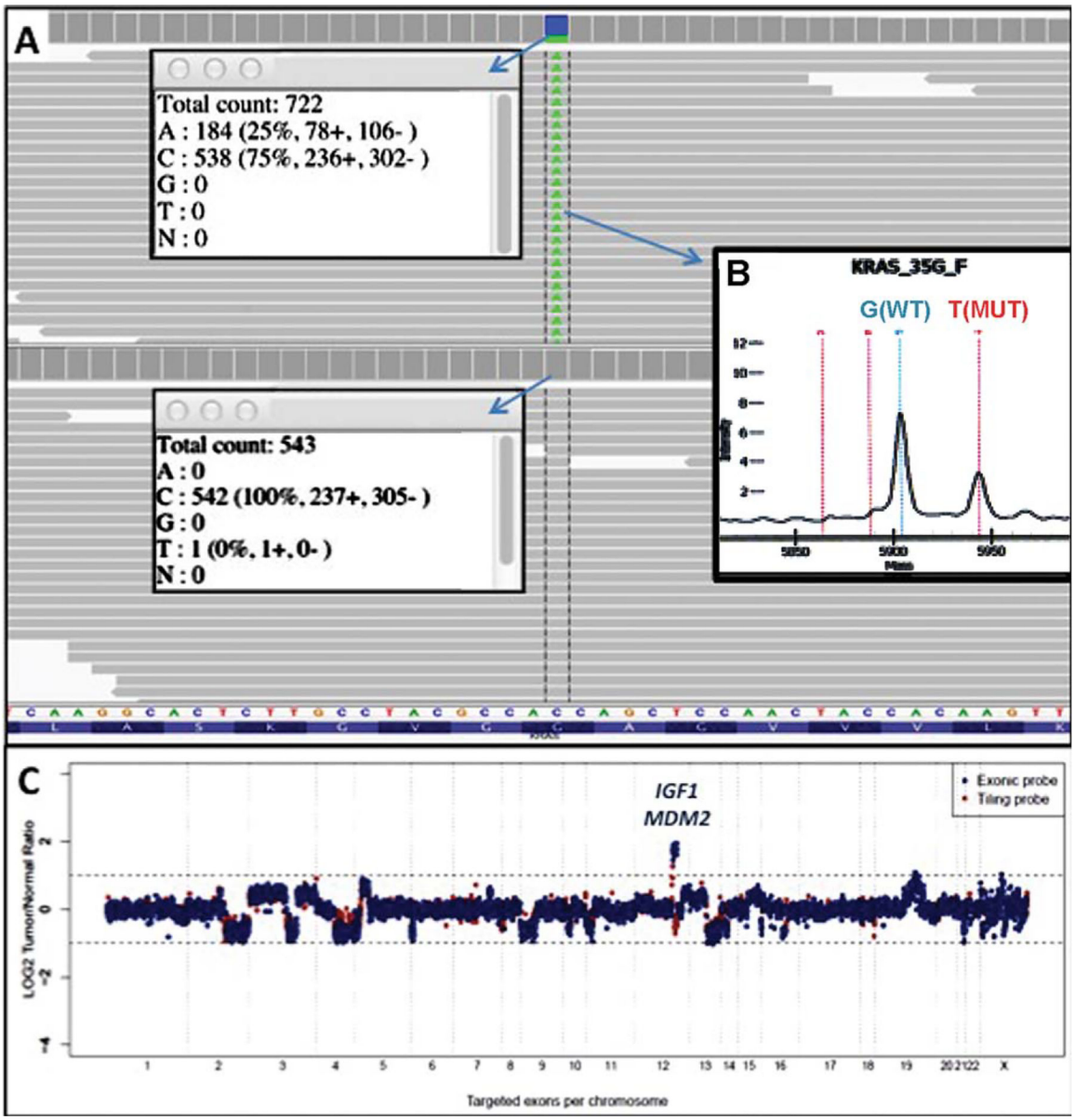

Figure 2.

Molecular pathology findings. A) IntegrativeGenomics-Viewer of KRAS sequencing by MSK-IMPACT assay from original tumor shows 25% of bidirectional reads show a KRAS c.35G>T (p.G12V) mutation in reverse direction in tumor (top, arrow pointing to total and nucleotide-specific reads) but not matched normal (below, arrow pointing to total and mutant specific reads). B) Confirmatory Sequenom Mass Spectrometry (arrow) results show the wild type (WT) *KRAS* peak, as well as the same mutant (MUT) peak (c.35G>T) in the GIST recurrence. C) Log ratio of copy number alterations graph show *MDM2* and *IGF1* amplification relative to normal tissue, with fold changes of 3.3 and 3.2, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE 1.

Clinicopathologic Characteristics and Somatic Mutations in SDH-Deficient Wild Type Gastric GISTs

Age	Sex	Morphology	Size (cm)	Mitotic rate (50 HPF)	AJCC stage, 7th ed.	Mean sample coverage	Somatic alterations (Allele frequency)
67	M	Epithelioid	4.7	99	II	440	*KRAS* p. G12V (26%) *ATRX* p. G75X (74%) *ATRX* p. EI509_1510delinsX (65%) *DICER1* p. L805Q (70%) *MSH2* p. T568fs (28%) *NF1* p.Q4234fs (8%) *PMS1* p. WI98L (27%) *IGF1* amplification (3.3 fold change) *MDM2* amplification (3.2 fold change)
35	M	Epithelioid	8	71	IV, liver metastasis	343	*ARID1A* p. S499fs (43%) with loss of ARID1A immunohistochemical expression
32	F	Spindle	2	12	II	379	none
43	F	Epithelioid	3.5	1	IV, nodal metastases	501	*SDHA* p. R352X (26%) *DAXX* p. T5681 (5%) *BMPR1A* splice site (c. 6751+1G>T) (27%)
59	F	Mixed	4	4	IA	999	[a]*BRD4* p.1113del (43%) [a]*CREBBP* p.686Q (52%) [a]*FLT1* p. L927S (49%) [a]*HNF1A* p. N62S (46%) [a]*SDHB* p. D204fs (63%) [a]*TP53* p. A138V (6%) [a]*TP53* p. R306fs (6%) [a]*TP53* p. 191_192del (29%)
28	F	Epithelioid	4	>5	IV, nodal metastases	395	*TP53* p. R342fs (29%) *TP53* p. 191-192del (28%)
61	F	Epithelioid	7.5	1	IV, nodal metastases	1034	[a]*SDHA* p. R210X (31%) [a]*RAD52* p. G118D (46%) [a]*BRCA2* p. T598A (46%) [a]*BRCA2* p. T2097M (49%) [a]*AXL* p. T328M (44%)
32	F	Epithelioid	10	2	IV, liver metastasis	776	[a]*NOTCH2* p. R2105W (46%)

Age	Sex	Morphology	Size (cm)	Mitotic rate (50 HPF)	AJCC stage, 7th ed.	Mean sample coverage	Somatic alterations (Allele frequency)
							[a]*MSH2* p. M813W (48%)
							[a]*KDR* p. N205S (49%)
							[a]*SDHA* p. C311F (41%)

[a]Sequenced against pooled normal due to unavailability of matched normal DNA.

HPF= High-powered field, AJCC= American Joint Committee on Cancer, M= Male, F= Female

TABLE 2.

Previously Reported Cases of *KRAS*-Mutant GISTs

Case	*KRAS* mutation	*KIT/PDGFRA* mutation	Organ	Risk	*KIT* IHC	Response to Imatinib
Miranda et al. (2012)	G12D	*KIT* Δ570–576	Stomach	High	+	Unknown
Miranda et al. (2012)	G12A/ G13D	*KIT* Δ 579	Small bowel	Intermediate	+	Unknown
Miranda et al. (2012)	G13D	*PDGFRA* D842V	Stomach	Low	+	Unknown
Antonescu et al. (2013)	G12V	*KIT* Δ 557–558	Small bowel	High	−	None
Serrano et al. (2014)	G12R	*KIT* Δ 554–559	Stomach	High	+	Limited to *KRAS* wild type nodule
Current	G12V	None	Stomach	High	+	None