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ORIGINAL REPORT

Molecular Correlates of Imatinib Resistance in Gastrointestinal Stromal Tumors

Michael C. Heinrich, Christopher L. Corless, Charles D. Blanke, George D. Demetri, Heikki Joensuu, Peter J. Roberts, Burton L. Eisenberg, Margaret von Mehren, Christopher D.M. Fletcher, Katrin Sandau, Karen McDougall, Wen-bin Ou, Chang-Jie Chen, and Jonathan A. Fletcher

From the Division of Hematology/Oncology, Department of Pathology, Oregon Health & Science University Cancer Institute, Oregon Health & Science University; Portland Veterans Affairs Medical Center, Portland, OR; Dana-Farber Cancer Institute; Harvard Cancer Center; Department of Pathology, Brigham & Women's Hospital, Boston, MA; Division of Oncologic Surgery, Hitchcock-Dartmouth Medical Center, Lebanon, NH; Division of Medical Oncology, Fox-Chase Cancer Center, Philadelphia, PA; Novartis Pharmaceuticals Corporation, Hanover, NJ; University of Helsinki, Helsinki; and the Department of Surgery, University of Turku, Turku, Finland.

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Address reprint requests to Michael C. Heinrich, MD, R&D-19 3710 U.S. Veterans Hospital Rd, Portland, OR 97239; e-mail: Heinrich@ohsu.edu.

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ABSTRACT

Purpose
Gastrointestinal stromal tumors (GISTs) commonly harbor oncogenic mutations of the KIT or platelet-derived growth factor alpha (PDGFRA) kinases, which are targets for imatinib. In clinical studies, 75% to 90% of patients with advanced GISTs experience clinical benefit from imatinib. However, imatinib resistance is an increasing clinical problem.

Patients and Methods
One hundred forty-seven patients with advanced, unresectable GISTs were enrolled onto a randomized, phase II clinical study of imatinib. Specimens from pretreatment and/or imatinib-resistant tumors were analyzed to identify molecular correlates of imatinib resistance. Secondary kinase mutations of KIT or PDGFRA that were identified in imatinib-resistant GISTs were biochemically profiled for imatinib sensitivity.

Results
Molecular studies were performed using specimens from 10 patients with primary and 33 patients with secondary resistance. Imatinib-resistant tumors had levels of activated KIT that were similar to or greater than those typically found in untreated GISTs. Secondary kinase mutations were rare in GISTs with primary resistance but frequently found in GISTs with secondary resistance (10% *v* 67%; $P = .002$). Evidence for clonal evolution and/or polyclonal secondary kinase mutations was seen in three (18.8%) of 16 patients. Secondary kinase mutations were nonrandomly distributed and were associated with decreased imatinib sensitivity compared with typical KIT exon 11 mutations. Using RNAi technology, we demonstrated that imatinib-resistant GIST cells remain dependent on KIT kinase activity for activation of critical downstream signaling pathways.

Conclusion
Different molecular mechanisms are responsible for primary and secondary imatinib resistance in GISTs. These findings have implications for future approaches to the growing problem of imatinib resistance in patients with advanced GISTs.

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common type of sarcoma arising in the digestive tract and are generally distinguished from other abdominal sarcomas by the expression of KIT receptor tyrosine kinase . This kinase is important not only as a diagnostic marker for GISTs, but serves as a primary oncogene in approximately 80% of these tumors, as evidenced by activating mutations of the *KIT* gene.

Imatinib (Gleevec; Novartis Pharmaceuticals, Basel, Switzerland) inhibits the enzymatic activity of several oncogenic tyrosine kinases, including KIT and platelet-derived growth factor alpha (PDGFRA). In clinical studies, 75% to 90% of patients with advanced GISTs treated with imatinib experienced a clinical benefit (stable disease + complete/partial objective response).[1-3] These imatinib-induced responses correlated with tumor kinase mutational status.[4,5] Patients with *KIT* exon 11-mutant GIST have a higher response rate and a significantly longer median survival compared with patients with exon 9-mutant GISTs, and those whose GISTs lack *KIT* or *PDGFRA* mutations.[4]

Although imatinib has revolutionized the treatment of advanced GISTs, clinical resistance to this drug has proved to be a significant problem with more prolonged follow-up. Secondary kinase mutations acquired during imatinib treatment have been reported in several small series.[6-15] In this study we evaluated molecular

markers of imatinib resistance in a series of well-documented GIST samples from a phase II study of imatinib.

PATIENTS AND METHODS

Patient Materials

Tumor samples were obtained from patients enrolled in the CSTI571 B2222 phase II trial of imatinib (sponsored by Novartis, Basel, Switzerland) for the treatment of advanced GIST.[2,4] Of 147 original patients, 92 had documented disease-related treatment failure as of May, 2005. Samples were obtained from 43 progressive-disease patients who consented to analysis of their tumors. Per the clinical protocol, tumor progression was defined according to traditional Southwest Oncology Group response criteria.[16] Specifically, progressive disease was defined as either a 50% or greater increase in the of sum of the products of all measurable lesions over the smallest sum observed (or baseline if no decrease was observed); clear worsening from previous examinations of any assessable disease; reappearance of any lesion that had disappeared; appearance of a new lesion; or the failure to return for evaluation due to death or deteriorating clinical condition. The appearance of a nodule within a mass on follow-up imaging studies was not considered to meet the protocol-specified definition of progression. The study was approved by the local institutional review board of each participating institution, and written informed consent was obtained from each patient. In addition, informed consent for the analysis of tumor-associated genetic alterations was obtained independently of patient consent for participation in the clinical study.

Imatinib response correlates were obtained using paired GIST biopsies taken during the week before initiation of imatinib and biopsies taken during the first week of therapy. Imatinib resistance correlates were evaluated in biopsies taken at the time of GIST progression while patients were still receiving imatinib. Routine pathology review, including KIT immunostaining, was performed on all biopsies.

Reagents and Cell Lines

The GIST430 and GIST48 cell lines were established from imatinib-resistant GISTs. Site-directed mutagenesis was used to generate relevant single, double, or triple mutations of *KIT* or *PDGFRA* cDNA.[4,17,18] Experiments involving recombinant DNA were performed using biosafety level 2 conditions in accordance with National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules.[19] Imatinib mesylate was purchased from the Oregon Health & Science University Hospital pharmacy (Portland, OR).

KIT *Short Hairpin RNA Studies*

A *KIT* lentiviral short hairpin RNA (shRNA) was obtained from William Hahn, MD, PhD (Dana-Farber Cancer Institute, Boston, MA, and Broad Institute RNAi consortium, Cambridge, MA). This shRNA was assembled by ligating KIT forward 5′-CCGGCCATAAGGTTTCGTTTCTGTACTCGAGT ACAGAAACGAAACCTTATGGTTTTTG-3′ and reverse 5′-AATTCAAAAA CCATAAGGTTTCGTTTCTGTACTCGAGTACAGAAACGAAACCTTAT GG-3′ oligomers into the *AgeI* and *EcoRI* sites of a pLKO.1puro lentiviral vector. Lentiviral preps were produced by cotransfecting pLKO.1puro empty vector or pLKO.1puro-KIT (shRNA), pCMVΔR8.91, and pMD.G helper virus packaging plasmids (at a 10:10:1 ratio) into 293T cells. These transfections were performed using lipofectamine and PLUS reagent (Invitrogen, Carlsbad, CA), and lentivirus supernatants were harvested at 24, 36, 48, and 60 hours. Viral titers were determined in GIST882 cells, according to a protocol from Invitrogen. GIST882 or GIST430 cells were infected, in the presence of 8 μg/mL of polybrene, and were then lysed for western blot analysis at 72 hours postinfection.

Immunoblotting Studies

Whole cell lysates were prepared from GIST biopsies or GIST cell lines.[20,21] The quality of each lysate was determined by immunoblotting for the GIST markers KIT and PKCθ, as described previously.[20,21] Immunoblotting was performed as described previously. In addition, hamster monoclonal anti–bcl-2 (the kind gift of Stan Korsmeyer, MD, Dana-Farber Cancer Institute) was utilized in the current report. Protein expression studies of mutant KIT or PDGFRA isoforms were performed as previously described by transient transfection of Chinese Hamster ovary cells. The transfected cells were treated with various concentrations of imatinib before preparation of protein lysates. KIT protein was assayed for activation status (phosphorylation) by

Table 1. PCR Primers Used for Detection of Mutations of *KIT* or *PDGFRA*

Exon	Forward Primer	Reverse Primer	D-HPLC Temperatures
K8-18	TTGGTATTTTTGTCCAGGAACTGAGC	AGAGAACAGCTCCCAAAGAAAAATCC	NA
K9	ATGCTCTGCTTCTGTACTGCC	CAGAGCCTAAACATCCCCTTA	50.0 °C
K11	CCAGAGTGCTCTAATGACTG	ACCCAAAAAGGTGACATGGA	50.0 °C/56.2 °C
K11-13	CCCTTTCTCCCCACAGAAACCCATG	ACACGGCTTTACCTCCAATG	NA
K11-13	ACACGGCTTTACCTCCAATG	AGACAATAAAAGGCAGCTTGGACGAC	NA
K12	CTGCACAAATGGTCCTTCAA	CAAAAAGCACAACTGGCAAAC	60.5 °C
K13	CATCAGTTTGCCAGTTGTGC	ACACGGCTTTACCTCCAATG	59.5 °C
K14	CTCACCTTCTTTCTAACCTTTTCTT	CCCATGAACTGCCTGTCAAC	57.2 °C
K15	GACCCATGAGTGCCCTTCT	TCTCTGTAAAGTTACTCTTGGTTG	59.1 °C
K16	AGTGATCTGCCTGCAAGTTCACATTAG	GCTCTAAAATGCTCTGTTCTC	60.8 °C
K17	TGTATTCACAGAGACTTGGC	GGATTTACATTATGAAAGTCACAGG	58.0 °C
K18	TTCTGTTCAATTTTGTTGAGCTTC	GCAGGACACCAATGAAACTT	58.7 °C
K19	GCAAACTGTGTCTCAGGAAGC	CCCTCAACATCTGGGTTTCT	56.9 °C
K20	AAAACAAGCTGAGGGCATTG	TGGGAGAAGGGGGATTCTAT	59.3 °C
K21	TGTTCGTTGTAGGGACTGCT	AACCATCATGGAAGCCAAAG	63.0 °C
K11-13	CCCTTTCTCCCCACAGAAACCCATG	ACACGGCTTTACCTCCAATG	NA
K11-13	CCAGAGTGCTCTAATGACTG	AGACAATAAAAGGCAGCTTGGACGAC	NA
P10	GGCCCTATACTTAGGCCCTTT	TCAGCTGATGAGTTGTCCTGA	50.0 °C/62.0 °C
P12	TCCAGTCACTGTGCTGCTTC	GCAAGGGAAAAGGGAGTCTT	50.0 °C/59.7 °C
P14	TGGTAGCTCAGCTGGACTGAT	GGGATGGAGAGTGGAGGATT	59.1 °C
P15	CCATCTCCTAACGGCTTTTG	CAGGACATGGGTCTTTCCAT	57.9 °C
P17	ACCTGATGATTTCCTGCTGC	CGTCCACACTCCACTCACTG	58.2 °C
P18	ACCATGGATCAGCCAGTCTT	TGAAGGAGGATGAGCCTGAC	50 °C/61.6 °C
P19	TGCTGTGGATCATCAGTGAGT	TTCCTTCCAGTGGTGTTTCA	60.1 °C
P20	CAATGCACTGAGCGTTTGTT	CTCCCCCTAGACCCACAGAC	59.8 °C

Abbreviations: PDGFRA, platelet-derived growth factor alpha; D-HPLC, denaturing high-performance liquid chromotography; K, KIT exon; NA, not available; P, *PDGFRA* exon.

immunoprecipitation using an anti-KIT antibody, followed by sequential immunoblotting for phosphoKIT (using antiphosphotyrosine antibody) or total KIT (anti-KIT monoclonal antibody). In the case of PDGFRA mutations, an identical methodology was used except for substitution of an anti-PDGFRA antibody for the immunoprecipitation and total PDGFRA immunodetection steps. IC_{50} (50% inhibitory concentration) was measured by densitometry of the phosphoKIT bands and normalization using total KIT expression (to correct for variations in loading of KIT protein in the various lanes).[4,17,18]

Mutation Detection Methods

Mutational analyses were performed on genomic DNA extracted from paraffin embedded or fresh frozen tumor tissue using a combination of polymerase chain reaction (PCR) amplification, denaturing high-performance liquid chromatography (D-HPLC) screening, and automated sequencing, as described previously.[4,17,22] PCR primer pairs and D-HPLC conditions are listed in Table 1.

PCR Evaluations of the Allelic Relationships of Primary and Secondary Mutations

Allelic relationships of primary and secondary *KIT* mutations were evaluated by reverse transcriptase-PCR (exons 8-18) or genomic PCR (exons 11 to 13) using primer pairs listed in Table 1. The resultant PCR products were cloned and individual bacterial colonies were bidirectionally sequenced.[23]

RESULTS

Description of Patient Population

The study population consisted of 147 patients treated in a randomized, phase II study of imatinib for patients with advanced GISTs.[2,24] As of May 2005, the median time to treatment failure for the entire phase II study population was 19.3 months and the median survival was estimated to be 57 months. Late treatment failure was primarily due to disease progression rather than discontinuation of therapy related to medication intolerance.

Treatment failures were divided into two groups. Patients who never achieved a partial remission and showed continued tumor growth during the first 180 days of treatment were defined as having primary imatinib resistance. Disease progression after a minimum of 6 months of partial remission or stable disease was defined as secondary imatinib resistance. This study is based on comparative analyses of tumor samples obtained from both these groups before and after treatment failure.

KIT Kinase Is Inhibited by Imatinib in Responding GISTs, but Not in Tumors With Primary Resistance

Activation of KIT was evaluated in fresh frozen metastatic GIST lesions from two patients (11 and 34) who underwent biopsy in the week before starting imatinib therapy and again after 5 or 7 days of treatment (Fig 1 and Table 2). Both patients had GISTs with *KIT* exon 11 mutations. KIT phosphorylation was evident in the preimatinib biopsies, but became nearly undetectable during the first few days of imatinib therapy. KIT inhibition was accompanied by dramatic decreases in the phosphorylated forms of mTOR, AKT, and mitogen-activated protein kinase (MAPK). Imatinib therapy also decreased expression of a proliferation marker (PCNA) and a critical antiapoptotic protein (bcl-2).

We analyzed the activation status of KIT-dependent signaling pathways in biopsies obtained from two patients whose GISTs had a *KIT* exon 9 mutation and showed primary imatinib resistance (Fig 2A and Table 3). Frozen tumor samples were not available from either patient pretreatment; nevertheless, on-treatment biopsies contained phosphorylated KIT protein in quantities equivalent to that typically present in untreated GIST specimens.[21] The pattern of intracellular signaling was similar to that seen in untreated GISTs (Fig 1 and Duensing et al[21]), indicating that primary resistance is associated with persistent KIT phosphorylation and activation of downstream AKT and MAPK pathways.

Reactivation of KIT and KIT-Dependent Signaling in Secondary Resistance

Two patients who had objective responses to imatinib underwent tumor biopsy after documented progression at 18 and 32 months. Matched pretreatment biopsies were available for both patients (Fig 2B and Table 2). Patient 1 had a primary *KIT* exon 11 deletion. Patient 14 had two activating *KIT* mutations in multiple pretreatment specimens (K642E and N822K). This is the only such example of double mutations in our series of more than 1,000 imatinib-naïve patients with GISTs.[17,25] We speculate that one mutation was the initiating mutation and the other developed during malignant progression. The imatinib-resistant tumors contained abundant amounts of activated KIT. Notably, the ratio of phosphorylated-AKT (P-AKT) to AKT was markedly increased in the progression samples

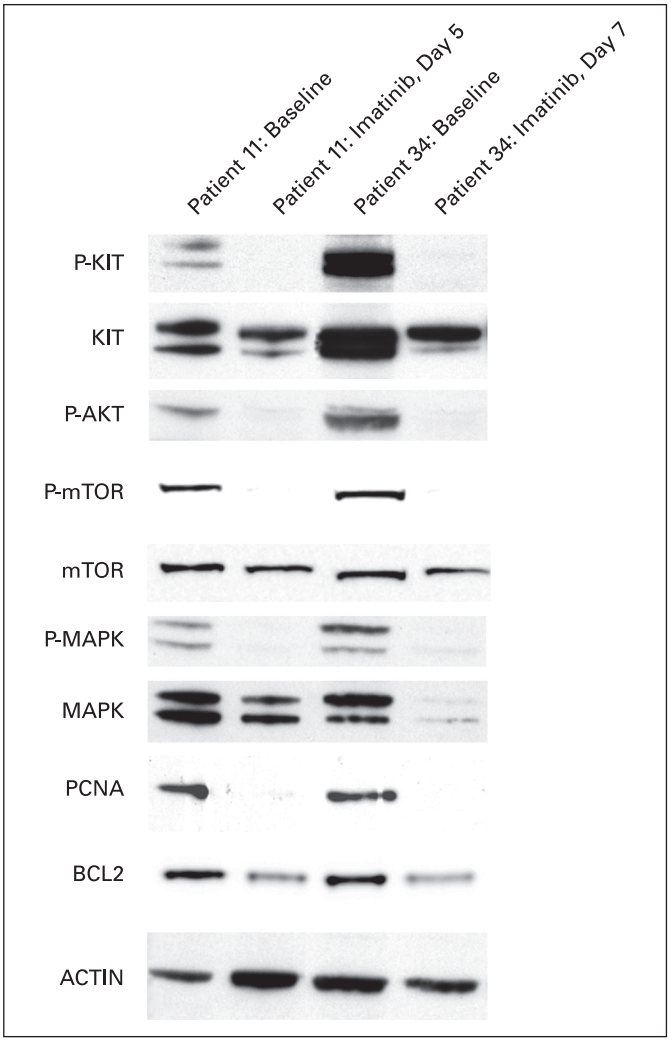

Fig 1. Imatinib inhibits KIT phosphorylation and activation of KIT-dependent signaling in responding gastrointestinal stromal tumors. Paired biopsy specimens were obtained before initiation of imatinib (baseline) and within the first week of therapy. The on-treatment samples have marked decreases in the levels of phosphorylated (P-) forms of KIT, AKT, mitogen-activated protein kinase (MAPK), and mTOR.

Table 2. Patients With Secondary Imatinib Resistance

Patient	Age/Sex	Initial Imatinib Dose (mg)	Primary Site	Best Response	Time From Diagnosis to Imatinib (days)	TTF (days)	Primary Mutation	Secondary Mutation (mutations/samples)	Status
1	64/F	400	SI	PR	664	930	*KIT* exon 11 Del WKVVE557-561	*KIT* exon 17 N822K (4/4)	AWD
2	53/F	400	Stomach	PR	2,921	883	*KIT* exon 11 Del WK557-558	*KIT* exon 17 Y823D (1/1)	DOD
3	50/M	400	SI	PR	348	717	*KIT* exon 11 Del PMYE551-554	*KIT* exon 13 V654A (3/3)	AWD
4	25/M	600	Stomach	SD	403	508	None	None (0/1)	AWD
5	39/M	400	SI	PR	591	504	*KIT* exon 11 V560D + KIT exon 13 insertion	*KIT* exon 13 insertion & V654A (3/4) & *KIT* exon 17 D816H (1/4)	DOD
6	54/M	600	SI	PR	1,037	512	*KIT* exon 11 W557G	KIT exon 17 Y823D (1/3)	DOD
7	43/M	400	IAB	PR	1,252	497	*PDGFRA* V561D	*PDGFRA* D842V (1/1)	DOD
8	49/M	600	Stomach	PR	2,120	1,580	*KIT* exon 11 Del PMYE551-554	None (0/2)	DOD
9	58/F	600	Stomach	PR	2,329	379	*KIT* exon 11 Del 554-558EVQWK	None (0/1)	AWD
10	49/M	600	SI	PR	643	1,564	*KIT* exon 11 Del KV558-559N	*KIT* exon 17 N822K (1/1)	AWD
11	50/F	400	SI	PR	3,615	349	*KIT* exon 11 Del VV559-560	None (0/1)	DOD
12	62/F	600	SI	PR	1,609	584	*KIT* exon 11 Del WKVV557-560C	*KIT* exon 17 Y823D (1/4)	DOD
13	46/M	600	SI	PR	58	1,297	*KIT* exon 11 Del MYEVQW552-557	*KIT* exon 13 V654A (1/1)	AWD
14	63/M	600	Stomach	PR	380	500	*KIT* exon 13 K642E + exon 17 N822H	*KIT* exon 17 C809G (2/2)	DOD
15	67/M	400	IAB	SD	155	215	None	None (0/1)	AWD
16	52/M	400	Stomach	PR	27	246	*KIT* exon 11 Del KPMYEVQWK550-558	None (0/2)	DOD
17	54/F	400	IAB	PR	778	766	*KIT* exon 11 V560G	*KIT* exon 13 V654A (3/3)	AWD
18	67/M	400	IAB	PR	104	498	*KIT* exon 11 V559D	None (0/2)	DOD
19	59/M	400	IAB	PR	125	587	*KIT* exon 11 V560D	*KIT* exon 17 D820A (2/2)	DOD
20	59/F	600	SI	PR	613	417	*KIT* exon 9	*KIT* exon 17 D820G (1/2)	DOD
21	54/M	400	Stomach	PR	952	366	*KIT* exon 11 Del KPMYEVQWK550-558	*KIT* exon 13 V654A (1/1)	DOD
22	65/M	400	IAB	PR	164	701	*KIT* exon 11 DelYEVQWK553-558	*KIT* exon 13 V654A (2/2)	DOD
23	70/F	400	Stomach	PR	2,285	846	*KIT* exon 11 Del EVQWK554-558	*KIT* exon 17 Y823D (1/1)	AWD
24	45/F	600	Stomach	PR	60	384	*KIT* exon 11 DelKPMYEVQWK550-558	*KIT* exon 14 T6701 (2/3)	DOD
25	44/F	400	Stomach	PR	411	1,028	*KIT* exon 11 Del WKVV557-560F	*KIT* exon 17 Y823D (1/1)	AWD
26	37/M	600	SI	PR	141	499	*KIT* exon 11 Del EVQWKVVEEINGNNYVYI554-571	None (0/1)	DOD
27	53/F	600	SI	PR	1,267	766	*KIT* exon 9	None (0/1)	AWD
28	63/M	600	SI	PR	167	462	*KIT* exon 9	*KIT* exon 13 V654A (2/2)	DOD
29	60/M	400	Stomach	PR	122	237	*KIT* exon 11 insertion K558NP	None (0/1)	DOD
30	63/M	400	Stomach	PR	138	505	*KIT* exon 11 Del KV558-559	None (0/1)	DOD
31	71/M	600	IAB	PR	507	747	*KIT* exon 11 L576P	*KIT* exon 13 V654A (1/1)	DOD
32	38/M	400	SI	SD	33	416	*KIT* exon 11 Del KPMYEVQW550-557FL	*KIT* exon 17 D820G (1/1)	DOD
33	60/M	400	Stomach	PR	275	252	*KIT* exon 13 K642E	*KIT* exon 17 D816H (2/4)	DOD
34	77/M	400	Stomach	PR	448	881	*KIT* exon 11 V560D	NP	AWD

Abbreviations: TTF, time to treatment failure; F, female; SI, small intestine; PR, partial response; Del, deletion; AWD, alive with disease; DOD, dead of disease; M, male; SD, stable disease; IAB, intra-abdominal; NP, not performed.

compared with matched pretreatment samples. In contrast, MAPK was only significantly activated in one of the progression samples (patient 14).

Secondary Kinase Mutations Are Common in Secondary but Not Primary Imatinib Resistance

To determine whether imatinib resistance is associated with acquired (secondary) kinase mutations, we screened the cytoplasmic domain exons of *KIT* and *PDGFRA* in DNA prepared from 79 tumor samples obtained from 43 patients with primary or secondary drug resistance (Tables 2 and 3). In 90.7% of patients (39 of 43), the pretreatment kinase genotype had been previously determined.

In 10 patients with primary resistance the median time to treatment failure was 3.6 months (range, 0.8 to 5.8; Table 3). No secondary mutations of *KIT* or *PDGFRA* kinase domain were found in specimens obtained from these patients at the time of progression. Patient 39 had primary progression on a dose of 400 mg of imatinib. No secondary kinase mutations were found at the time of progression on 400 mg, and cross-over to 600 mg led to disease stablilization. Twenty-three months later, the patient had tumor progression and ultimately underwent surgical debulking. Specimens from this procedure had multiple secondary kinase mutations (Table 3 and Fig 3).

In thirty-three patients with secondary resistance, the median time to treatment failure was 20.2 months (range, 7.2 to 52.7). Sixty-one progression specimens obtained from these 33 patients were judged to contain GISTs. The original mutation, which was known for 29 of 33 patients based on analysis of preimatinib specimens, was confirmed in all progression biopsies (54 of 54 samples).

Overall, 22 (67%) of 33 patients with secondary resistance had one or more secondary kinase mutations (21 *KIT* and 1 *PDGFRA*; Table 2 and Fig 3). This frequency is distinctly higher than that in patients with primary resistance (10% *v* 67%; $P = .002$). Notably, all secondary KIT kinase mutations were found in GISTs with an

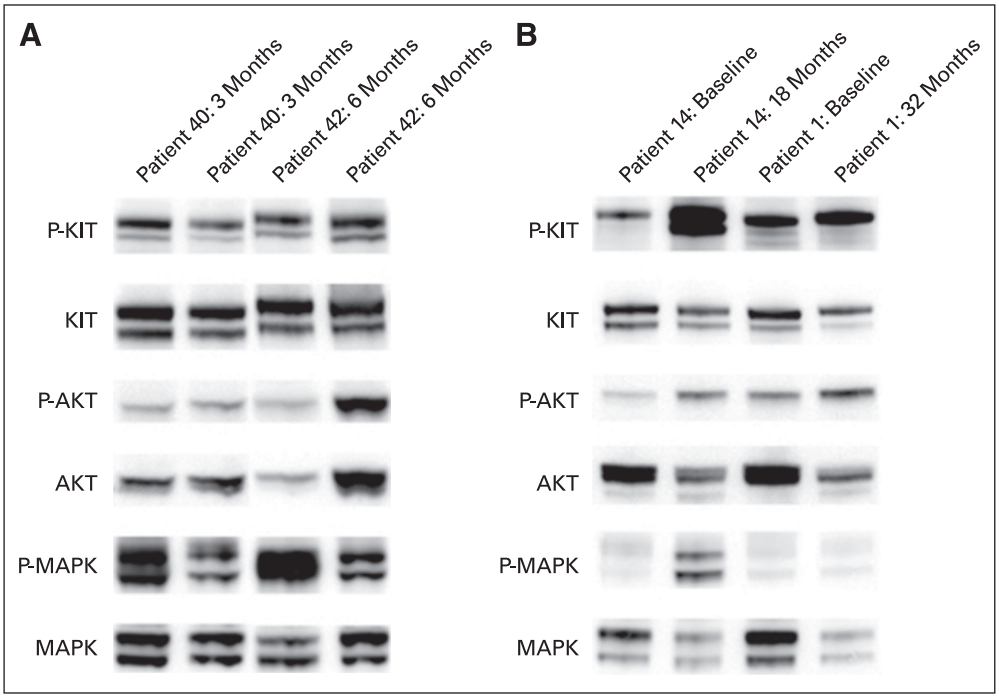

Fig 2. KIT phosphorylation and activation of KIT-dependent signaling pathways in gastrointestinal stromal tumors with (A) primary and (B) secondary imatinib resistance. Two parts of the same minimally progressing metastasis were evaluated at month 3 in patient 40 (lanes 1 and 2). Two different progressing metastases were evaluated at month 6 in patient 42 (lanes 3 and 4). Paired tumor specimens were obtained before treatment (baseline) and at the time of secondary imatinib resistance. P-, phosphorylated; MAPK, mitogen-activated protein kinase.

underlying primary *KIT* mutation and these secondary mutations were not present in pretreatment specimens. No secondary mutations were identified in one GIST lacking a primary *KIT* or *PDGFRA* mutation. In addition, the only secondary *PDGFRA* mutation identified was in a GIST with a primary *PDGFRA* V561D mutation. The secondary KIT kinase mutations were nonrandom and involved either the adenosine triphosphate (ATP) binding pocket of the kinase domain (V654A, T670I) or the kinase activation loop (C809G, D816H, D820A/E/G, N822K/Y, Y823D). Figure 3 summarizes the spectrum and frequency of secondary KIT mutations in this series and other published reports.

In contrast to primary resistance, two of three *KIT* exon 9 mutant GISTs were found to have additional *KIT* kinase mutations in biopsies obtained after the development of secondary imatinib resistance. However, the frequency of secondary *KIT* mutations in *KIT* exon 9 mutant GISTs was not obviously different between cases with primary resistance versus secondary resistance (1 of 7 *v* 2 of 3; $P = .18$).

Evidence for Clonal Evolution and/or Polyclonal Secondary Resistance Mutations in Individual Patients

Multiple biopsy and/or resection specimens were available from 16 patients with progressive disease on imatinib. In three of these

Table 3. Patients With Primary Imatinib Resistance

Patient	Age/Sex	Initial Imatinib Dose (mg)	Primary Site	Best Response	Time From Diagnosis to Imatinib (days)	TTF (days)	Primary Mutation	Secondary Mutation	Status
35	52/M	600	LI	NE	829	NE*	*PDGFRA* D842V	None (0/2)	DOD
36	48/F	600	IAB	SD	294	156	*KIT* exon 9	None (0/1)	DOD
37	45/M	400	SI	PD	620	23	*KIT* exon 9	None (0/1)	DOD
38	33/M	600	SI	SD	2,415	166	*KIT* exon 9	None (0/1)	DOD
39	36/F	400	SI	PD	638	74	*KIT* exon 9	N822K† (1/6) N822Y† (1/6) D820E† (2/6)	AWD
40	41/F	600	SI	SD	849	107	*KIT* exon 9	None (0/3)	AWD
41	65/M	400	IAB	PD	1,609	84	*KIT* N822K	None (0/1)	DOD
42	52/M	600	SI	SD	1,378	173	*KIT* exon 9	None (0/1)	DOD
43	51/M	400	SI	SD	1,145	173	*KIT* exon 9	None (0/1)	AWD
44	54/M	600	Stomach	PD	1,590	89	*PDGFRA* D842V	None (0/1)	DOD

Abbreviations: TTF, time to treatment failure; M, male; LI, large intestine; NE, not assessable; DOD, dead of disease; F, female; IAB, intra-abdominal; SD, stable disease; SI, small intestine; PD, progressive disease; AWD, alive with disease.

*TTF could not be defined due to use of different imaging modalities in staging exams.

†This patient had no detectable secondary mutation at the time of initial progression. However, secondary kinase mutations were found in specimens obtained more than 1 year after initial progression.

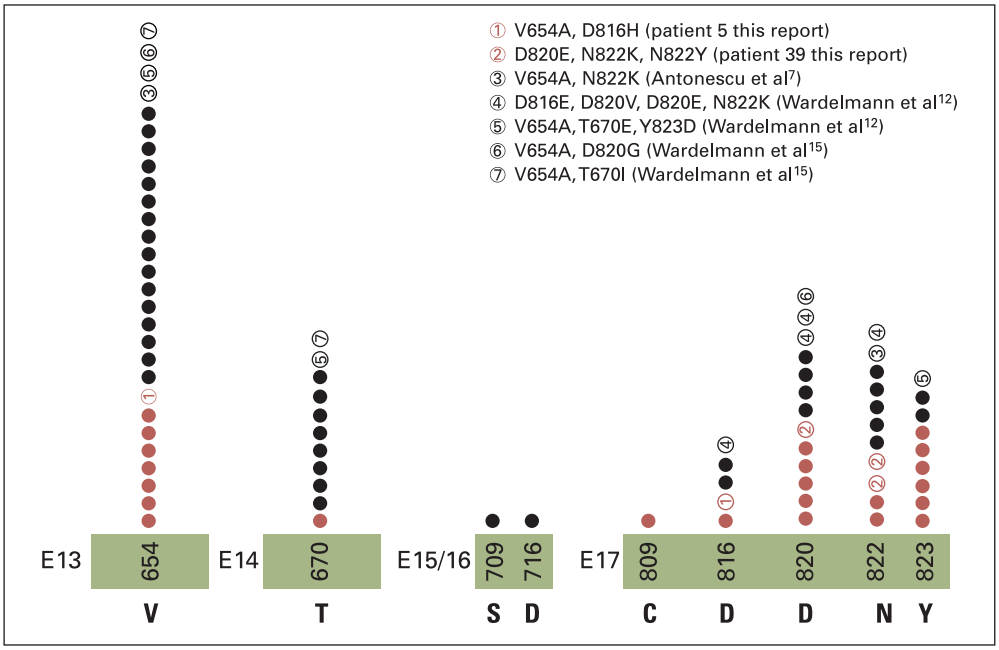

Fig 3. Secondary *KIT* mutations in imatinib-resistant gastrointestinal stromal tumors. Data from the current report (21 patients) and other published reports (43 patients) are indicated with red and black symbols, respectively.[6-14] Patients with different secondary mutations in geographically separate metastases are indicated by numbered symbols. *KIT* codons are listed in the shaded boxes.

patients (3 of 16; 18.8%), we found evidence of clonal evolution and/or polyclonal secondary kinase mutations (Tables 2 and 4).

Patient 5 had a baseline *KIT* V560D substitution on one allele and a single adenine base insertion in *KIT* exon 13 of the other allele, resulting in a frameshift and protein truncation. Thus, this patient's tumor was functionally homozygous due to loss of the wild-type allele. In specimens obtained at the time of imatinib resistance, a V654A mutation was present in two biopsy specimens and D816H in a third specimen. All three specimens had the original preimatinib *KIT* exon 11 and 13 mutations.

Allelic Distribution of Secondary Kinase Mutations

Most *KIT* mutant GISTs (≈ 90%) are heterozygous for the original activating mutation and therefore have one wild-type and one mutant *KIT* allele. Without exception, all secondary mutations were on the same allele as the primary mutation (Table 4). As noted above, patient 14 (Tables 2 and 4) had a GIST with two monoallelic preimatinib *KIT* mutations (K642E and N822H). An acquired C809G substitution found at the time of imatinib resistance was on the same allele as the K642E and N822H mutations.

Table 4. Alleleic Distribution Studies

Patient	Primary Mutation	Secondary Mutation	Allelic Distribution of Mutations
5	*KIT* V560D	V654A	Cis
5	*KIT* V560D	D816H	Cis
5	*KIT* V560D	Exon 13 insertion leading to premature stop codon	Trans
14	*KIT* K642E + N822H	C809	All Cis
19	*KIT* V560D	D820A	Cis
23	*KIT* Del554-558EVQWK	Y823D	Cis
28	*KIT* exon 9	V654A	Cis
33	*KIT* K642E	D816H	Cis

Abbreviations: Cis, same allele as primary mutation; Trans, opposite allele from primary mutation.

Secondary Kinase Mutations Lead to Imatinib Resistance In Vitro

To determine if secondary kinase mutations were the cause of clinically observed imatinib resistance, we tested the in vitro sensitivity of selected single, double, or triple mutant kinases to imatinib. Because GISTs manifest a wide range of mutations in exon 11 (Table 2), we chose a representative KIT exon 11 point mutation, V560D, as the standard for comparing imatinib sensitivity of single and double mutant KIT kinases.[25] Secondary mutations of interest (for example, V654A) were then engineered into the V560D isoform as well as into a wild-type cDNA, allowing comparative analyses of the effects of secondary mutations on imatinib sensitivity.

Several of the secondary KIT mutations identified in our series have been previously identified in human cancers: D816H, D820A, N822H/K, and Y823D.[4,17,18,26-30] In contrast, there are no reports of V654A, T670I, or C809G mutations occurring as a primary mutation.

The isolated KIT V560D mutation was very sensitive to imatinib (IC_{50} ≈100 nmol/L; Fig 4). Both V654A and T670I resulted in strong imatinib resistance, with IC_{50s} of 5 and 10 μmol/L, respectively—either in isolation or when coexpressed with KIT V560D. Similar results were obtained using *KIT* exon 9 AY as the primary mutation (Fig 4).

A number of amino acids located in or near the KIT activation loop were mutated in imatinib-resistant tumors (Fig 3 and Table 2). In vitro profiling of isolated activation loop mutations revealed a spectrum of imatinib sensitivity ranging from relatively sensitive (IC_{50} of 100-200 nmol/L, N882K, Y823D) to highly resistant (IC_{50} > 5,000 nmol/L, D816H). When coexpressed with V560D, all of the double mutant kinases were extremely resistant to imatinib, with the exception of V560D + N822K which was only moderately imatinib resistant (Fig 5A). Similar results were obtained using *KIT* exon 9 AY-insertion or K642E as the primary mutation (Fig 5B). Notably, both primary mutations were intrinsically imatinib sensitive, but

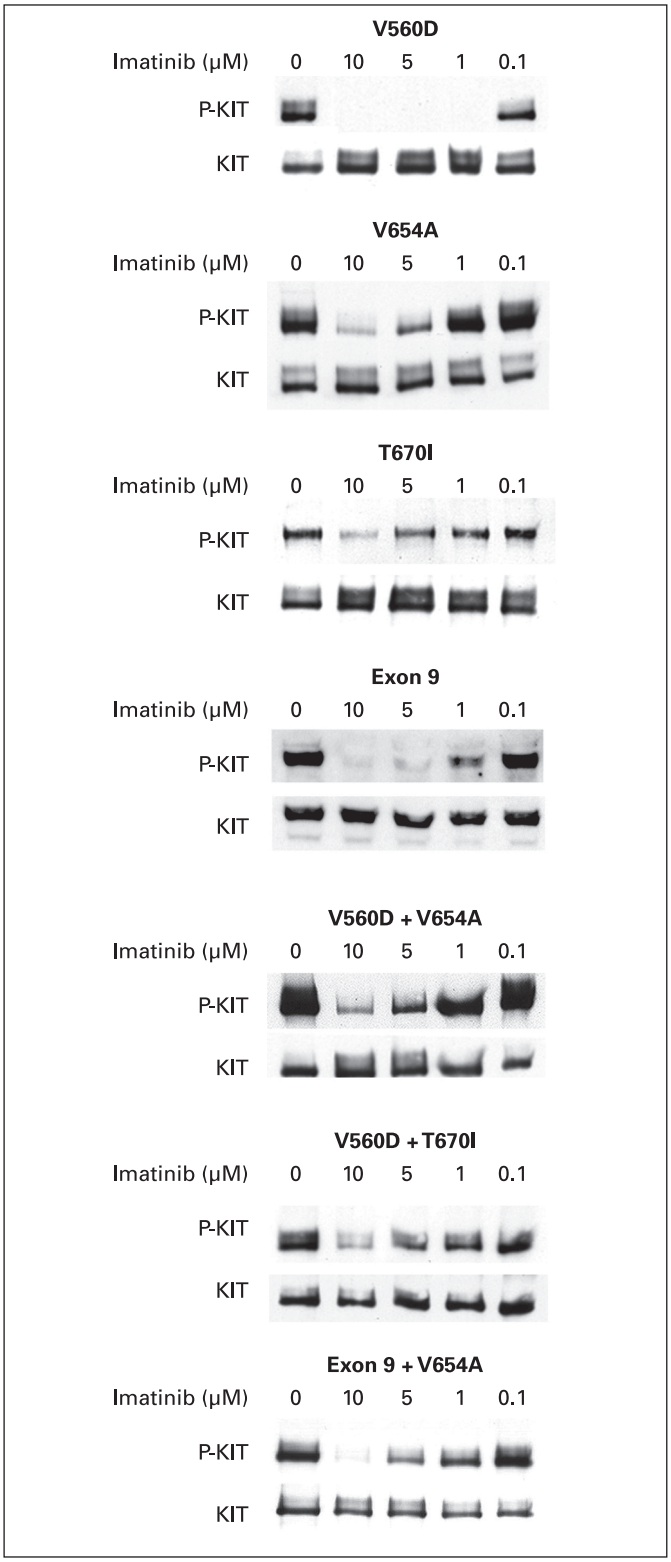

Fig 4. Biochemical characterization of secondary mutations involving the adenosine triphosphate (ATP) binding pocket of KIT. Secondary mutations involving the ATP binding pocket were tested for in vitro sensitivity to imatinib when expressed in isolation or when coexpressed with KIT exon 11 V560D or KIT exon 9 mutations. P-, phosphorylated.

coexpression of D820G with *KIT* exon 9 resulted in moderate imatinib resistance. Coexpression of the imatinib-sensitive K642E mutation with D816H resulted in extreme imatinib resistance.

A doubly mutant (K642E + N822H) kinase modeled on patient 14 was moderately resistant to imatinib (IC_{50} of ≈2 μmol/L). Notably, patient 14 received a dose of 600 mg daily of imatinib and experienced an objective partial response that lasted 500 days. The C809G mutation found at the time of progression proved resistant in vitro ($IC_{50} > 5$ μmol/L), both in isolation and when coexpressed with KIT K642E + N822H.

In patient 7, a primary PDGFRA V561D mutation (known to be sensitive to imatinib) was accompanied by a PDGFRA D842V mutation at the onset of progression. The doubly mutant V561D + D842V kinase proved imatinib-resistant with an IC_{50} similar to the isolated D842V isoform (Fig 6).[17]

GIST Cell Lines With Secondary Kinase Mutations Have In Vitro Imatinib Resistance

To further validate our biochemical studies, we generated cell lines from two patients with secondary imatinib resistance (neither patient was part of the phase II study). The GIST48 cell line has a combination of mutations (homozygous V560D + heterozygous D820A) similar to that documented in samples from patient 19. Concentrations of imatinib higher than 1 μmol/L were required for complete inhibition of KIT activation in this cell line (Fig 7A). This concentration is 10-fold greater than that necessary to block exon 11 mutant isoforms of KIT in GIST cells.[8,21,31] Activation of AKT was partially but not completely inhibited by imatinib doses of 0.1 to 5 μmol/L.

The GIST430 cell line is heterozygous for a *KIT* exon 11 deletion mutation and the V654A substitution (both on the same allele). Eight samples from imatinib-resistant tumors in our study had a similar combination of *KIT* exon 11 and V654A mutations. The doubly mutant KIT isoform expressed by GIST430 cells was imatinib resistant (IC_{50} of ≈ 2.5 μmol/L; IC_{90} of > 5 μmol/L). AKT activation in this cell line was only partially inhibited by imatinib doses higher than 2.5 μmol/L (Fig 7A).

We used RNAi to determine whether KIT expression was still required for activation of signaling pathways in imatinib-resistant GIST cells. shRNA knockdown of total KIT expression in the previously described imatinib-sensitive GIST882 cell line (homozygous KIT K642E mutation)[31] resulted in parallel decreases in phospho-KIT, phospho-AKT, phospho-p70S6K, and the proliferation marker cyclin A expression (Fig 7B). In contrast, there was no change in the expression of p70S6K, AKT, or PI3K. Comparable findings were obtained after KIT shRNA knockdown in GIST430 cells, demonstrating that activation of proliferation/survival signaling pathways remains KIT dependent in this imatinib-resistant cell line. *KIT* knockdown in the cell lines also induced flow-cytometric evidence for G1 block, decreased S phase, and markedly increased apoptosis (data not shown).

DISCUSSION

We analyzed genomic mechanisms of imatinib resistance in a cohort of patients that were part of a randomized phase II study of imatinib for treatment of advanced, unresectable GIST. Forty-three (46.7%) of 92 patients with disease-related treatment failures consented to studies of their tumor samples. The resulting collection of 83

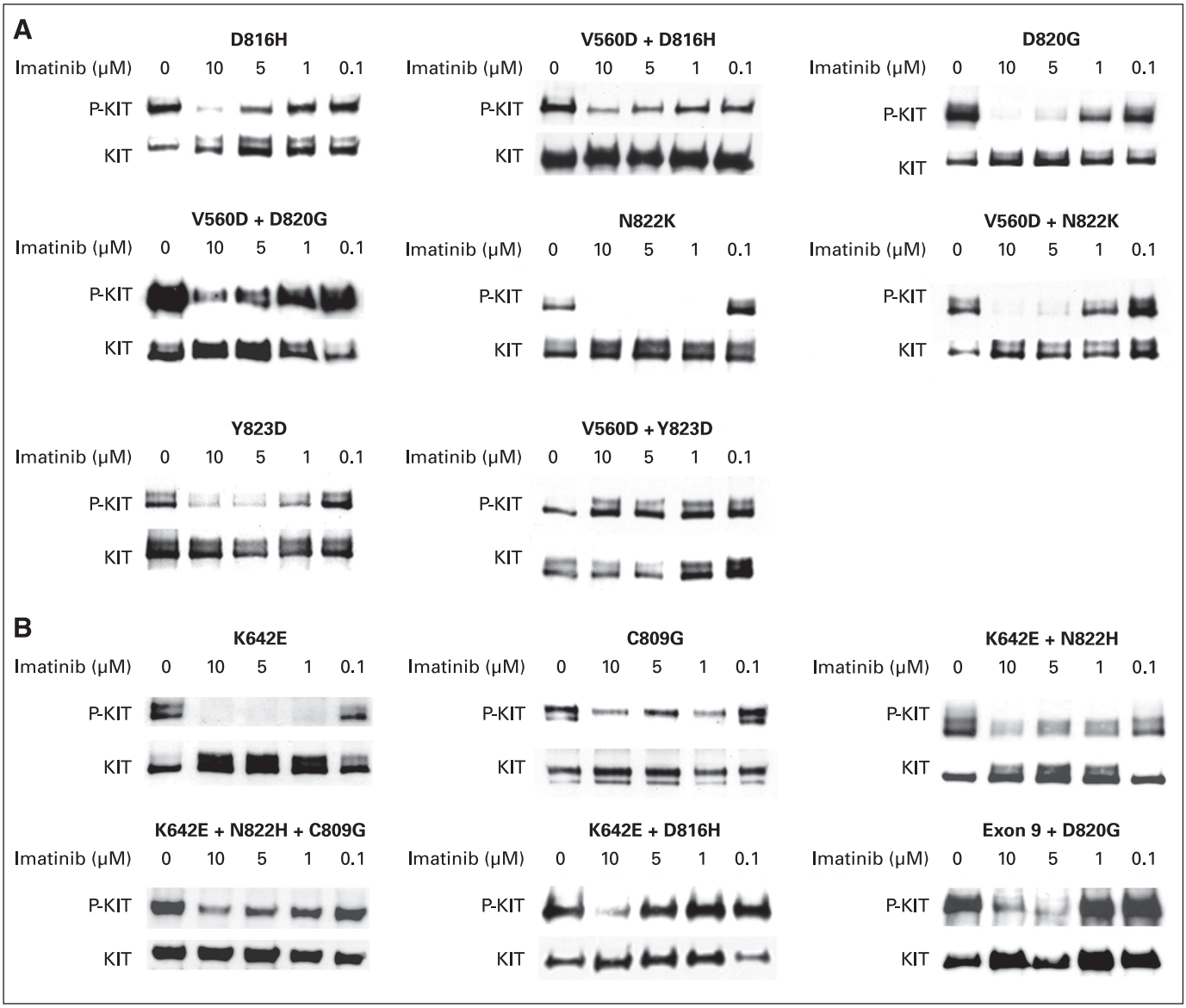

Fig 5. Biochemical characterization of secondary mutations involving the KIT activation loop. Secondary mutations involving the KIT activation loop were tested for in vitro sensitivity to imatinib when expressed in (A) isolation or when coexpressed with the KIT exon 11 V560D or (B) in isolation or coexpressed with KIT exon 13 K642E or KIT exon 9 mutations. P-, phosphorylated.

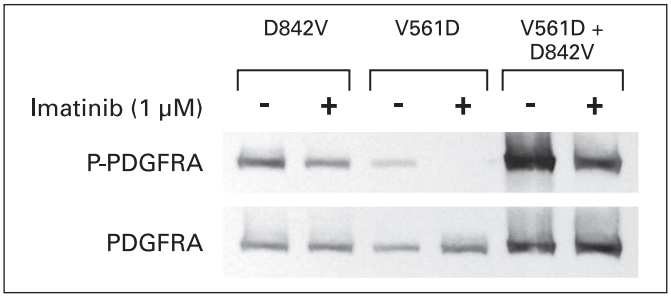

Fig 6. Platelet-derived growth factor alpha *(PDGFRA)* D842V mutation is associated with primary and secondary imatinib resistance. Imatinib potently inhibits the phosphorylation of the primary *PDGFRA* exon 12 V561D mutation (patient 17, pretreatment), but not *PDGFRA* D842V—either expressed in isolation (patients 1 and 10) or when coexpressed with V561D (patient 17, progression specimen).

specimens from well-monitored patients receiving standardized imatinib treatment is the largest examined to date for molecular mechanisms related to drug resistance.

Imatinib-resistant GISTs show activation of the same signaling pathways that are operative in untreated GISTs. Interestingly, primary imatinib resistance is infrequently associated with secondary kinase mutations (1 of 10; 10%). *PDGFRA* D842V and *KIT* exon 9 mutations were significantly over-represented in this group compared with secondary imatinib resistance. GISTs with these genotypes are less responsive to imatinib therapy than GISTs with an associated *KIT* exon 11 mutation.[4,5] The *PDGFRA* D842V mutation has moderate to high level in vitro resistance to imatinib explaining why this mutation was responsible for primary imatinib resistance in the two patients with a primary *PDGFRA* D842V mutation.[4,8,32] This conclusion is strengthened by our

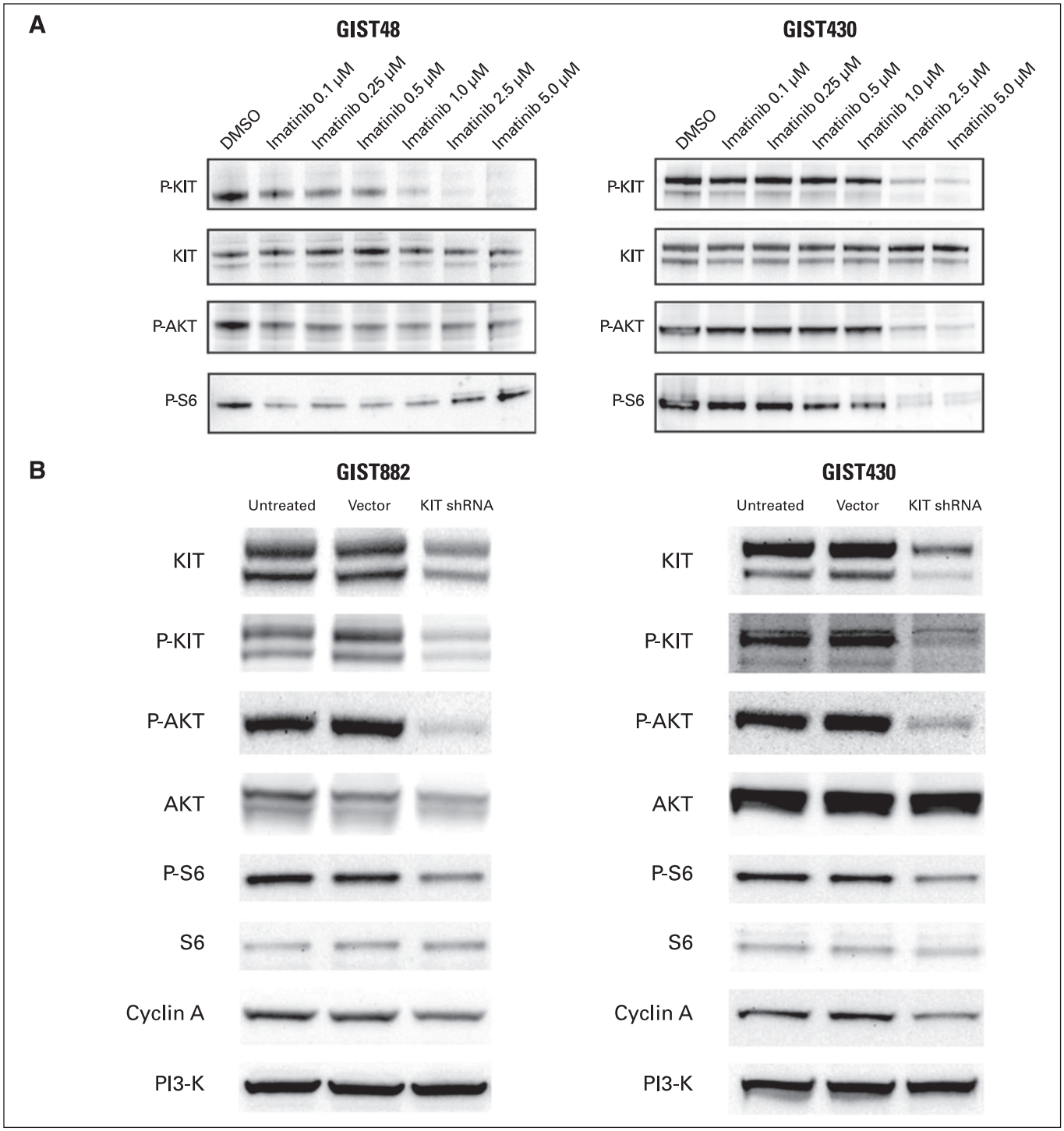

Fig 7. Biochemical characterization of secondary KIT mutations expressed in imatinib-resistant gastrointestinal stromal tumor (GIST) cell lines. GIST48 is an imatinib-resistant cell line with a homozygous *KIT* V560D and a heterozygous D820A mutation. GIST430 is an imatinib-resistant cell line with a heterozygous *KIT* exon 11 deletion mutation and V654A mutation (same allele). GIST882 is an imatinib-sensitive GIST cell line expressing a homozygous K642E mutation. (A) Effects of imatinib treatment. (B) Effects of RNAi inhibition of *KIT*. P-KIT, phosphorylated KIT; P-AKT, phosphorylated AKT; shRNA, short hairpin RNA.

observation that the *PDGFRA* D842V mutation was associated with secondary imatinib resistance in a GIST harboring a primary *PDGFRA* V561D mutation. Debiec-Rychter et al[8] also reported a case of imatinib-resistant GIST associated with acquisition of a *PDGFRA* D842V mutation in a GIST with a primary KIT exon 11 mutation.

The molecular mechanisms underlying primary resistance in GISTs with *KIT* exon 9 mutations are as yet unidentified, but may be related to differences in the underlying biology of this subset of GISTs. We speculate that some *KIT* exon 9 mutant GISTs have an alternative mechanism of KIT activation that does not require the enzymatic activity of KIT. Notably, imatinib only binds to the inactive form of KIT.[33,34] Therefore,

KIT-independent mechanisms of KIT phosphorylation could stabilize the protein in an active, imatinib-resistant conformation.

GISTs with *KIT* exon 11 mutations commonly become imatinib resistant due to acquisition of secondary kinase mutations located on the same allele as the original gain of function KIT mutation. These secondary kinase mutations appear to be the predominant mechanism for late imatinib resistance in *KIT* exon 11 mutant GISTs. Fluorescence in situ hybridization assays (data not shown) revealed low-level *KIT* amplification (3 to 4 copies per cell), accompanied by KIT protein overexpression, in only two patients, suggesting that genomic *KIT* amplification has only a minor role in GIST imatinib resistance. We believe that the difference in the frequency of secondary mutations between GISTs with underlying *KIT* exon 11 versus *KIT* exon 9 mutations is reflective of the underlying biology of these tumors—namely, that *KIT* exon 11 mutant GISTs are more addicted to KIT signaling than *KIT* exon 9 mutant GISTs. In this model, *KIT* exon 9 mutant GISTs are able to use alternative signaling mechanisms to drive cellular proliferation. In contrast, *KIT* exon 11 mutant GISTs are more restricted in their signaling requirements and the most common mechanism of resistance is expansion of a clone with an imatinib-resistant kinase mutation.

Using RNAi *KIT* knockdown, we demonstrated that imatinib-resistant GIST cell lines with secondary kinase mutations remain dependent on KIT signaling for growth and survival. These findings have implications for future approaches to the growing problem of imatinib resistance in patients with advanced GISTs.

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Authors	Employment	Leadership	Consultant	Stock	Honoraria	Research Funds	Testimony	Other
Michael C. Heinrich			Novartis (A); Pfizer (A)		Novartis (A); Pfizer (A)			
Christopher L. Corless			Novartis (A)		Novartis (A)			
George D. Demetri			Novartis (A); Pfizer (A)		Novartis (A); Pfizer (A)		Novartis Pfizer (N/R)	
Heikki Joensuu			Novartis (A)		Novartis (A)			
Burton L. Eisenberg					Novartis (A)			
Margaret von Mehren			Novartis (A)					
Katrin Sandau	Novartis (N/R)							
Karen McDougall	Novartis (N/R)							
Jonathan A. Fletcher						Novartis (B)		

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Author Contributions

Conception and design: Michael C. Heinrich, Charles D. Blanke, George D. Demetri, Heikki Joensuu, Jonathan A. Fletcher
Financial support: Michael C. Heinrich, Katrin Sandau, Karen McDougall, Jonathan A. Fletcher
Administrative support: Michael C. Heinrich
Provision of study materials or patients: Michael C. Heinrich, Charles D. Blanke, George D. Demetri, Heikki Joensuu, Peter J. Roberts, Margaret von Mehren, Christopher D.M. Fletcher
Collection and assembly of data: Michael C. Heinrich, Christopher L. Corless, George D. Demetri, Peter J. Roberts, Christopher D.M. Fletcher, Wen-bin Ou, Chang-Jie Chen, Jonathan A. Fletcher
Data analysis and interpretation: Michael C. Heinrich, Christopher L. Corless, Charles D. Blanke, George D. Demetri, Katrin Sandau, Wen-bin Ou, Chang-Jie Chen, Jonathan A. Fletcher
Manuscript writing: Michael C. Heinrich, Christopher L. Corless, George D. Demetri, Heikki Joensuu, Wen-bin Ou, Chang-Jie Chen, Jonathan A. Fletcher
Final approval of manuscript: Michael C. Heinrich, Christopher L. Corless, Charles D. Blanke, George D. Demetri, Heikki Joensuu, Peter J. Roberts, Burton L. Eisenberg, Margaret von Mehren, Christopher D.M. Fletcher, Katrin Sandau, Karen McDougall, Wen-bin Ou, Chang-Jie Chen, Jonathan A. Fletcher

GLOSSARY

AKT: A transforming serine-threonine kinase involved in cell survival.

bcl2: First discovered as a translocated locus [t(14;22)] in B-cell leukemias, bcl-2 is an antiapoptotic protein that protects cells from programmed cell death by preventing the activation of pro-apoptotic caspase proteins.

KIT: A member of the PDGFR family, c-kit is a tyrosine kinase receptor that dimerizes following ligand binding and is autophosphorylated on intracellular tyrosine residues.

MAPK(mitogen-activated protein kinase) : MAPKs are a family of enzymes that form an integrated network influencing cellular functions such as differentiation, proliferation, and cell death. These cytoplasmic proteins modulate the activities of other intracellular proteins by adding phosphate groups to their serine/threonine amino acids.

PDGFRA (platelet-derived growth factor alpha) : The receptor for PDGF exists distinctly as the dimeric $\alpha\alpha$ or $\beta\beta$ form. All dimer combinations of PDGF A and B signal through PDGFR-$\alpha\alpha$; PDGF BB signals through PDGFR-$\beta\beta$; PDGF CC signals through the $\alpha\alpha$ and $\alpha\beta$ receptors; and PDGF DD signals through the $\beta\beta$ and $\alpha\beta$ receptors.

PKC theta (PRKCQ) : A member of the protein kinase C family. PKC theta is highly expressed by T cells, interstitial cells of cajal, and gastrointestinal stromal tumors.

RNAi: Post-transcriptional gene silencing that regulates gene expression.

shRNA (short hairpin RNA; short interfering hairpin) : shRNA contains sense and antisense sequences from a target gene connected by a loop, and is expressed in mammalian cells from a vector by a pol III–type promoter. The shRNA is transported from the nucleus into the cytoplasm, where Dicer processes it. Once in the cell, the shRNA can decrease the expression of a gene with complementary sequences by RNAi.

Tyrosine kinase: Generic name for enzymes that transfers a terminal phosphate group from ATP to specific protein tyrosine residues. Tyrosine kinases are a member of the family of protein kinases.