Acquired Resistance to Imatinib in Gastrointestinal Stromal Tumor Occurs Through Secondary Gene Mutation

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Abstract Most gastrointestinal stromal tumors (GIST) have an activating mutation in either *KIT* or *PDGFRA*. Imatinib is a selective tyrosine kinase inhibitor and achieves a partial response or stable disease in about 80% of patients with metastatic GIST. It is now clear that some patients with GIST develop resistance to imatinib during chronic therapy. To identify the mechanism of resistance, we studied 31 patients with GIST who were treated with imatinib and then underwent surgical resection. There were 13 patients who were nonresistant to imatinib, 3 with primary resistance, and 15 with acquired resistance after initial benefit from the drug. There were no secondary mutations in *KIT* or *PDGFRA* in the nonresistant or primary resistance groups. In contrast, secondary mutations were found in 7 of 15 (46%) patients with acquired resistance, each of whom had a primary mutation in *KIT* exon 11. Most secondary mutations were located in *KIT* exon 17. KIT phosphorylation was heterogeneous and did not correlate with clinical response to imatinib or mutation status. That acquired resistance to imatinib in GIST commonly occurs via secondary gene mutation in the *KIT* kinase domain has implications for strategies to delay or prevent imatinib resistance and to employ newer targeted therapies.

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the intestinal tract. After surgical removal of primary GIST, tumor recurrence is frequent, and previously, about 50% of patients died within 5 years of initial diagnosis (1). In 1998, Kitamura and colleagues reported that GIST is commonly associated with activating mutations in the *KIT* receptor tyrosine kinase (2). More recently, we showed that a knock-in *KIT* activation mutation, $KIT^{V558\Delta}/+$, is sufficient to induce GIST in mice (3). Imatinib mesylate (STI571, Gleevec, Novartis Pharmaceuticals, Basel, Switzerland) is an oral agent that specifically inhibits the BCR-ABL and ABL tyrosine kinases as well as the KIT and PDGFR receptor tyrosine kinases (4). Initially, imatinib was applied to the treatment of chronic myelogenous leukemia (CML), where it achieves a 90% complete response rate (5, 6). Subsequently, imatinib was tested in metastatic or unresectable GIST and found to induce a partial response or stable disease in >80% of patients (7, 8). Primary resistance to imatinib, in which GIST continues to progress despite the institution of therapy, occurs in about 15% of patients. Remarkably, the 2-year survival of patients with metastatic GIST is now ~70% (9).

In up to 90% of cases, GISTs have activating mutations in either the KIT or PDGFRA receptor tyrosine kinases (10–13). The most common site of *KIT* mutation is in the 5′ end of the exon (11), which encodes the juxtamembrane domain, and usually deletions or substitutions of codons 550 to 560 occur. *KIT* exon 9 mutation occurs in 10% to 15% of patients. It defines a distinct subset of GISTs that are often located in the small bowel and have an aggressive clinical behavior (11, 14). Infrequently, a mutation is identified in *KIT* exon 13 or 17 (15, 16). Another 5% of patients with GIST have a *PDGFRA* mutation that typically involves exon 12 or 18 (12, 13). About 10% of patients do not have a detectable mutation in either *KIT* or *PDGFRA*. In particular, GISTs that occur in pediatric patients are nearly always wild-type for both genes (11A). Recent data suggest a possible correlation between imatinib response and the type of mutation, as tumors with an exon 9 mutation or wild-type *KIT* are less likely to respond to imatinib (17, 18).

Although most patients with advanced GIST benefit from imatinib treatment, it is now clear that many patients subsequently develop resistance to the agent. The median time to progression is about 24 months (9). The mechanism of acquired resistance to imatinib in GIST has not been well defined. In CML, second site mutation in *BCR-ABL* is the predominant mechanism of imatinib resistance (19, 20). Therefore, we postulated that acquired resistance to imatinib in GIST is due to secondary site mutation in the *KIT* or *PDGFRA* genes. We did molecular analysis of 65 tumor nodules from 31 patients who were treated with imatinib.

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Three patients showed primary resistance to imatinib, whereas 15 patients acquired resistance to imatinib during therapy.

Materials and Methods

Clinicopathologic analysis. Patients with the diagnosis of GIST who were treated with imatinib and underwent surgical resection of their tumor at Memorial Sloan-Kettering Cancer Center were identified from our prospective sarcoma database. There were 31 patients who had adequate tumor tissue for molecular analyses following treatment. Patient, tumor, and treatment information was obtained from the database and by reviewing medical charts. Primary resistance to imatinib was defined as continued growth of any tumor despite the institution of imatinib therapy. Acquired resistance was defined as new tumor growth that occurred subsequent to an initial period during 2 to 3 months on imatinib in which the patient had stable or responding disease. Tumor size was determined by computed tomography scan or magnetic resonance imaging. This study was approved by the Institutional Review Board.

Pathologic material was examined and the diagnosis was confirmed using standard H&E staining and CD117 immunohistochemistry on formalin-fixed, paraffin-embedded tissue as previously described (11). Histologic response to imatinib was based on gross and microscopic findings of necrosis and fibrosis and was scored for each tumor nodule as: minimal (<10% response), low (10-50% response), moderate (50-90% response), or high (≥90% response) degree of response.

KIT/PDGFRA genotyping. Mutation analysis was done as described previously (11). Genomic DNA was isolated from snap-frozen tumor tissue samples stored at −70°C, using a standard phenol-chloroform organic extraction protocol. Adequate DNA for mutational analysis was obtained in 65 tumor nodules from 31 patients. All cases were tested for the known sites of *KIT* (exons 9, 11, 13, 14, and 17) and *PDGFRA* (exons 12 and 18) mutations. One microgram of genomic DNA was subjected to PCR using Platinum TaqDNA Polymerase High Fidelity (Life Technologies, Inc., Gaithersburg, MD). Primer sequences and annealing temperatures were as described (11), with the addition of primers for *KIT* exon 14 (GTCTGATCCACTGAAGCTG at 50°C and ACCCCATGAACTGCCTGTC at 51°C), *PDGFRA* exon 12 (TCCAGTCACTGTGCTGCTTC and GCAAGGGAAAAGGGAGTCTT at 54°C), and *PDGFRA* exon 18 (ACCATGGATCAGCCAGTCTT and TGAAGGAGGATGAGCCTGACC at 55°C). Direct sequencing of PCR products was done for all exons tested and each ABI sequence was compared with the National Center for Biotechnology Information human *KIT* and *PDGFRA* gene sequences. In four patients, including three with acquired resistance, there was adequate tumor tissue available for mutation analysis from a surgical resection that occurred prior to initiation of imatinib. In each case, including one patient who developed a second site mutation, we confirmed the same primary mutation as in the recurrence.

cDNA sequencing and cloning. For tumors with secondary *KIT* mutations by genomic DNA analysis, we then amplified and sequenced the *KIT* cDNA from exons 10 to 18. In this way, we confirmed our initial genotype results and also determined whether the secondary mutation occurred on the same or opposite allele as the primary mutation. Adequate RNA was obtained in 32 tumor nodules from 18 patients by using the RNA Wiz reagent (Ambion, Inc., Austin, TX) and the guanidinium isothiocynate-phenol chloroform method. Five micrograms of RNA were transcribed using reverse transcriptase superscript II (Invitrogen, Carlsbad, CA). The cDNA was subjected to PCR using primers in *KIT* exon 10 (GCCGGATCCTTTCGTAATCGTAGCT) containing a *Bam*HI linker and exon 18 (GGCGAATTCTGTATACACAGTTGAAAATG) with an *Eco*RI linker. Amplification of the *KIT* insert was done separately with Platinum Taq (Invitrogen) and Pfu ultra high-fidelity DNA polymerase (Stratagene, La Jolla, CA) in order to exclude false-positive mutations due to errors in polymerase proofreading. For cloning, the *KIT* insert and the chloramphenicol-resistant pBC KS+ vector (Stratagene) were digested with *Bam*HI and *Eco*RI and then ligated using the rapid DNA ligation kit (Roche Applied Science, Penzburg, Germany). Plasmid transformation of XL-1 blue supercompetent cells (Stratagene) and colony selection on chloramphenicol Luria-Bertani agar plates were done using standard methods. For each sample, 10 distinct clones were picked and expanded overnight in a shaking incubator. Colony PCR was done using Taq polymerase and the same *KIT* exon 10 and 18 linker primers. Clones containing the insert were selected and DNA was isolated with a DNA Miniprep kit (Qiagen, Inc., Valencia, CA) and then directly sequenced.

Western blotting. Adequate tissue for protein extraction was available in 24 patients (43 tumor nodules). We also included for comparison 8 untreated GISTs with *KIT* exon 11 mutations, 4 of which were in-frame deletions, and 4 were substitutions. A CD117-negative and desmin-positive high-grade gastric leiomyosarcoma was included as a negative control. For preparation of whole lysates, 1 g of snap-frozen tumor was ground to powder in liquid nitrogen using a PowerGen 700 Homogenizer (Omni International, Marietta, GA), and resuspended in radioimmunoprecipitation assay lysis buffer (Upstate, Lake Placid, NY) containing a cocktail of protease and phosphatase inhibitors (Sigma, St. Louis, MO). Protein concentrations were determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Protein extracts were subjected to electrophoresis and immunoblotting using a standard protocol. Antibodies included mouse anti-phospho-Tyr^{20} and Tyr^{99} (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-phospho-KIT Y721 (Zymed Lab, Inc., San Francisco, CA), rabbit anti-KIT (Oncogene Science, Boston, MA), mouse anti-actin (Santa Cruz), donkey anti-mouse secondary antibody (Santa Cruz), and anti-rabbit secondary antibody (Calbiochem, La Jolla, CA). Blots were incubated with Immun-Star horseradish peroxidase luminol/enhancer (Bio-Rad) and exposed to Kodak BioMax MR Film (Eastman Kodak Company, Rochester, NY). Results with phospho-KIT were scored as: 1, strong; 2, weak; and 3, negative.

Fluorescence* in situ *hybridization analysis. Touch preps of frozen tumor tissue were done in nine nodules from eight patients. The slides were fixed in 3:1 methanol/acetic acid then stored at −20°C. Fluorescence *in situ* hybridization (FISH) was done according to standard procedures. Briefly, the slides were pretreated with pepsin-HCl (0.007 mol/L HCl, 8 μg/mL pepsin) at 37°C for 3 to 5 minutes, rinsed in PBS, fixated in 1% formaldehyde for 10 minutes, then rinsed, dehydrated, and air-dried. The slides were then denatured in 70% formamide at 68°C for 2 to 4 minutes, quenched, dehydrated, and air-dried. 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, IL). A chromosome 4 centromeric probe labeled with Spectrum Orange (CEP 4, Vysis) was used as reference. The probe mix, 50 to 80 ng of each KIT BAC and 2 μL Cot-1 DNA (Invitrogen), was ethanol-precipitated, and resuspended in hybridization buffer. The KIT probe mix was denatured at 70°C for 10 minutes, followed by pre-annealing at 37°C for 30 minutes. The KIT probe was then combined with the denatured CEP 4 probe on the slide, coverslipped and incubated overnight at 37°C. After standard posthybridization washes, the slides were stained with 4′,6-diamidino-2-phenylindole and mounted in antifade (Vectashield, Vector Laboratories). Analysis was done using a Nikon E800 epifluorescence microscope with MetaSystems Isis 3 imaging software. A minimum of 100 cells was scanned over separate regions for each slide.

Results

Imatinib resistance may develop during chronic imatinib therapy. We identified 31 patients with GIST who were treated with imatinib and subsequently underwent surgical resection. There were 8 females and 23 males, and the median

age at initial diagnosis was 56 (range 31-84) years. The extent of disease at the original diagnosis of GIST was localized in 19 patients and metastatic in 12. Disease at the start of imatinib therapy consisted of a primary tumor alone in 6 patients, a primary GIST with liver and/or peritoneal metastasis in 7 patients, and recurrent liver and/or peritoneal disease in the remaining 18 patients. Imatinib was administered at a starting dose of 400 to 600 mg per day.

Based on the responsiveness of their disease to imatinib at the time of surgery, we categorized patients into "nonresistant," "primary resistance," or "acquired resistance." Elective removal of residual GIST following either a partial response or stable disease on imatinib was done in the 14 nonresistant patients (Table 1). Their median duration of imatinib therapy was 8 (1-22) months. Surgery was done in 3 patients with primary resistance to imatinib. Each had progression of disease detected on computed tomography within 3 months of starting therapy (Table 2). There were 15 patients who developed acquired resistance. Each had either a partial response (n = 10) or stable disease (n = 5) in response to imatinib and then subsequently acquired resistance and radiologic progression of disease. The median length of imatinib treatment in patients with acquired resistance was 18 (8-32) months (Table 2). One of these patients (#14) first had an operation to remove stable disease and later underwent another resection to remove resistant disease. Two patients required surgery because of a complication during imatinib therapy, involving either bleeding or perforation.

Histologic response correlates with clinical response to imatinib. The primary tumor was located in the stomach in 8 patients, small bowel in 17, rectum in 5, and intra-abdominal in 1 patient. The median size of the primary GIST was 9.5 (2.6-24) cm. There were 13 tumors larger than 10 cm. The morphology of the primary tumor was spindle-shaped in 25 cases, epithelioid in 4, and mixed in 2 tumors. Histologic response was graded based on the combined extent of tumor necrosis and fibrosis, because both are usually absent in untreated GIST, although central necrosis may be present in large tumors. Patients with primary resistance uniformly lacked any histologic evidence of response to imatinib (patients 29-31, Table 2). Six of 14 patients in the nonresistant group had a histologic response of >90%. Moderate histologic responses (50-90%) occurred in four others. Only three nonresistant patients had a minimal histologic response despite a clinical partial response or stable disease. In contrast, in the acquired resistant group, all but two patients had had at least one nodule lacking any response (Table 2).

Secondary KIT mutation occurs in acquired imatinib resistance. In the nonresistant group, there were 11 patients with a *KIT* mutation (3 in exon 9, 7 in exon 11, and 1 in exon 13), 1 patient with a *PDGFRA* mutation (exon 18), and 2 were wild-type. There were no identifiable secondary mutations in the nonresistant group. However, there was one patient (#2) who had a homozygous exon 11 deletion in 6 of 15 nodules tested. The significance of this finding is unclear as the patient had stable disease and currently has no evaluable tumor. As expected, the 3 patients with primary resistance to imatinib also had only a single mutation, which involved *KIT* exon 9 or 11 or *PDGFRA* exon 18.

Of the 15 patients with acquired resistance to imatinib, 14 had a common primary *KIT* mutation (11 in exon 11 and 3 in exon 9) and 1 patient was wild-type. Secondary mutations

Table 1. Patients with nonresistant GIST

	Primary tumor location	Histology	Disease at imatinib start	Time on imatinib* (months)	Best clinical response	Histologic response	Primary mutation	Secondary mutation	Phospho-KIT Y721
1	SB	S	P, L	22	PR	2, 3	Ex 11 VYIDPTQL569-576del	absent (0 of 2)	2, 3
2A†	SB	S	P, PE, L	15	PR	2, 3	Ex 11 P551H MY552-554del	absent (0 of 6) LOH (3 of 6)	1
2B			L	20	PR	1, 2	Ex 11 P551H MY552-554del	absent (0 of 3) LOH (3 of 3)	3
3A	SB	S	PE	12	SD	1	Ex 9 INS502AY	absent (0 of 1)	NA
3B†			PE	18	SD	1	Ex 9 INS502AY	absent (0 of 3)	1, 3
4†	SB	S	P, L	3	SD	1	Ex 9 INS502AY	absent (0 of 3)	1
5	rectum	S	P	9	PR	4	WT	absent (0 of 1)	NA
6	SB	S	P	4	SD	2	Ex 9 INS502AY	absent (0 of 1)	1
7	rectum	S	P	6	PR	4	Ex 11 VQWKV555-559del	absent (0 of 1)	3
8	SB	S	P	2	PR	1	Ex 11 K558N, INS559Q	absent (0 of 1)	NA
9	SB	S	PE, L	1	PR	3, 4	WT	absent (0 of 1)	NA
10	stomach	S	P	8	PR	4	Ex 11 WK557-558del	absent (0 of 1)	NA
11	stomach	E	PE	5	SD	2, 3	PDGFRA D842V	absent (0 of 2)	1
12	SB	S	P, PE, L	9	PR	4	Ex 11 INGNNYVYIDPTQL563-576del	absent (0 of 2)	3
13	rectum	S	P	8	PR	3	Ex 13 K642E	absent (0 of 1)	2
14A	SB	S	PE	3	PR	4	Ex 11 VYIDPTQL569-576del	absent (0 of 1)	2

Abbreviations: SB, small bowel; S, spindle; E, epithelioid; P, primary tumor; PE, peritoneal; L, liver; PR, partial response; SD, stable disease; del, deletion; LOH, loss of heterozygosity; INS, insertion; NA, not available.

*Time from the start of imatinib to surgical resection.

†Tested for KIT amplification by FISH.

Table 2. Patients with either acquired or primary resistance to imatinib

	Primary tumor location	Histology	Disease at imatinib start	Time on imatinib* (months)	Best clinical response	Histologic response	Primary mutation	Secondary mutation	Phospho-KIT Y721
14B†	SB	S	PE	29	PR	1	Ex 11 VYIDPTQL569-576del	D820Y (2 of 2)	2
15	rectum	S + E	CW	28	SD	1	Ex 11 KVVEE558-562del-LOH	Y823D (1 of 1)	3
16†	SB	S	PE	13	PR	1	Ex 11 L576P	absent (0 of 1)	1
17†	rectum	S	PE, L	15	PR	1	Ex 9 INS502AY	absent (0 of 1)	1
18	SB	S	PE	26	SD	1, 2	Ex 9 INS502AY	absent (0 of 4)	1, 2
19	stomach	S	PE, L	27	PR	1, 4	Ex 11 WK557-8del	T670I (2 of 2)	1, 2
20	I-abd	S	PE, L	13	PR	1	Ex 11 V559D	absent (0 of 2)	2
21	stomach	E	PE	8	SD	1, 2	WT	absent (0 of 1)	2
22†	stomach	S + E	PE	25	PR	2, 4	Ex 11 K558N, INS559P	N822K (1 of 3)	2
23	SB	S	L	14	SD	1	Ex 9 INS502AY	absent (0 of 1)	NA
24A	SB	S	PE	9	PR	1, 2	Ex 11 V560del	V654A (1 of 2)	2
24B			PE	16	PR	1	Ex 11 V560del	N822K (1 of 2)	1
25	SB	S	L	32	SD	2, 4	Ex 11 V559D	D820Y (1 of 2)	3
26	stomach	E	L	20	PR	1	Ex 11 INS574TQLPYD	absent (0 of 2)	NA
27	SB	S	P, L	16	PR	1, 3	Ex 11 NGNNYVYIDPTQLPY564-578del	N822K (1 of 2)	1
28	stomach	S	P, PE, L	25	PR	1, 4	Ex 11 WK557-558del	absent (0 of 3) LOH (1 of 3)	2
29	stomach	E	L	12	PrimR	1	PDGFRA D842V	absent (0 of 1)	NA
30	SB	S	P, L	3	PrimR	1	Ex 11 PY577-558del	absent (0 of 1)	1
31†	SB	S	PE, L	8	PrimR	1	Ex 9 INS502AY	absent (0 of 2)	1

Abbreviations: SB, small bowel; S, spindle; E, epithelioid; P, primary tumor; PE, peritoneal; L, liver; PR, partial response; SD, stable disease; del, deletion; LOH, loss of heterozygosity; INS, insertion; NA, not available; I-abd, intra-abdominal; PrimR, primary resistance; CW, chest wall.

*Time from the start of imatinib to surgical resection.

†Tested for KIT amplification by FISH.

were detected in 10 tumor nodules samples from seven (46%) patients and involved five different residues. The majority were *KIT* exon 17 mutations and occurred in six of seven patients with secondary mutations. All involved substitutions and three were N822K, two were D820Y, and one was Y823D (Table 2; Fig. 1). The other two secondary mutations included substitutions in exon 13 (V654A) and exon 14 (T670I). We again identified loss of the wild-type allele (loss of heterozygosity), WK557-8del in one patient (#28, one of three nodules tested) as we had in one nonresistant patient (#2).

In 10 of 15 patients with acquired resistance, more than one nodule was available for molecular analysis. In two of them an identical secondary mutation was identified in both nodules tested. In three patients, the secondary mutation was present on one but not all nodules. Interestingly, in one other patient (#24) a nodule showed an exon 13 V654A mutation, whereas a subsequent nodule removed 7 months later showed an exon 17

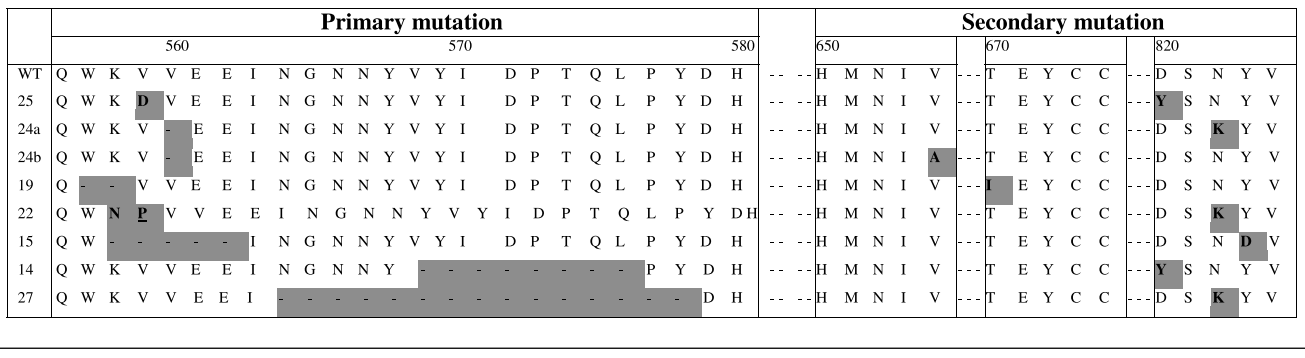

Fig. 1. Primary and secondary *KIT* mutations in seven patients who acquired resistance to imatinib. All the primary mutations occurred in exon 11 and they tended to be deletions. Secondary mutations were detected predominantly in exon 17 but also in exons 13 and 14. The wild-type sequence is shown for comparison. Numbers in the first column refer to the individual patients.

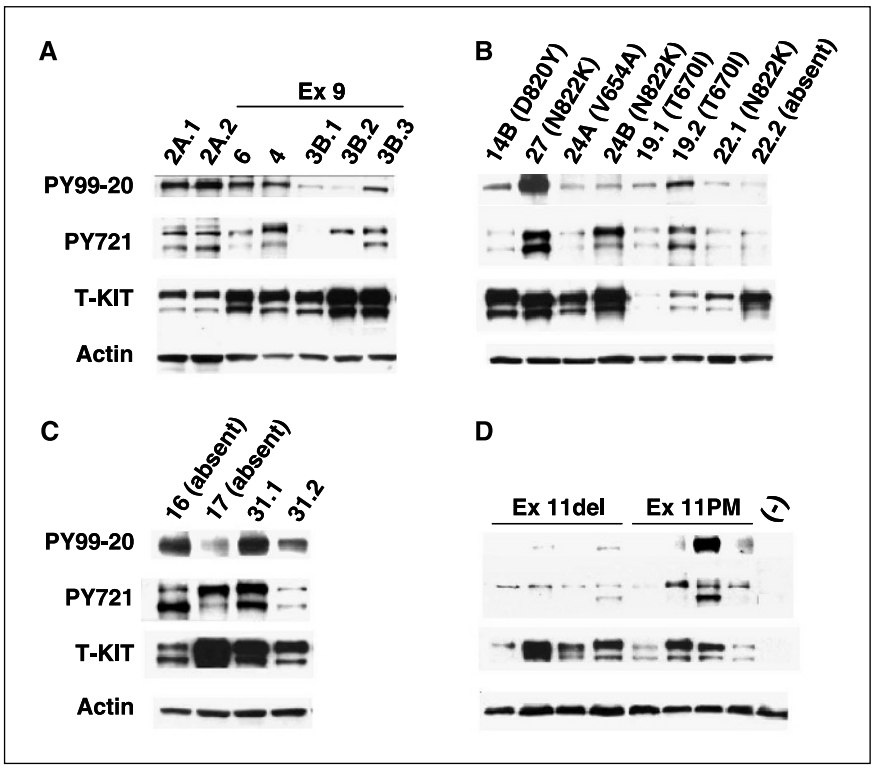

Fig. 2. Western blot analysis of activated and total KIT. *A,* nonresistant group; *B,* acquired resistance patients with secondary mutations; *C,* acquired resistance patients without secondary mutations (16 and 17) and primary resistance; and *D,* untreated GISTs with *KIT* exon 11 mutations compared with the negative control of a leiomyosarcoma.

N822K substitution. The remaining four patients had only a primary mutation identified.

To confirm the secondary mutations found at the genomic level and to exclude the possibility of polyclonal resistance and/or the presence of additional *KIT* mutations that were missed, amplification of the *KIT* cDNA from exons 10 to 18 was done followed by subcloning and direct sequencing. A total number of 32 samples from 18 patients were analyzed, including 2 pre-imatinib samples, 13 nonresistant nodules from 6 patients, 14 samples from 9 patients with acquired resistance and 3 nodules from 2 primary resistance patients. This analysis included 10 nodules from 5 patients with acquired resistance and second site mutations. Secondary mutations were confirmed in 7 of 7 nodules and the absence of mutation in the 3 other nodules. No additional *KIT* mutations were detected. Notably, the primary and secondary mutations were all located on the same allele.

KIT activation is variable regardless of response to imatinib. KIT activation as measured by phosphorylation was heterogeneous and did not consistently correlate with histologic response or response to imatinib (Table 1; Fig. 2A). Surprisingly, from the 11 operations in 10 nonresistant patients tested, only three specimens lacked evidence of KIT activation. Meanwhile, five nonresistant patients had strong KIT activation (Fig. 2A) and three had moderate expression. From 14 operations in 13 patients in the acquired resistance group, 6 patients had strong KIT activation, 5 had moderate expression, and 2 had none. Notably, KIT activation was also variable in the subset of patients with a second mutation, as there was strong activation in three, moderate expression in three, and no detectable phospho-KIT in two instances. In fact, the three patients (#22, 24, 27) with an identical N822K secondary mutation had either weak (n = 1) or strong (n = 2) phospho-KIT expression. There was also intraindividual variability, because in patient #19, carrying an identical T670I secondary mutation in the two nodules tested, the phospho-KIT was strong and weak (Fig. 2B). Furthermore, the patient (#24) with distinct secondary *KIT* mutations in two metachronous nodules had strong KIT activation in one and weak activation in the other (Fig. 2B). KIT activation variability was also noted within the resistant nodules without secondary mutations (Fig. 2C). Both patients with primary resistance did have strong phospho-KIT staining (Fig. 2C; Table 2). Inconsistent KIT activation was also observed in the untreated control group. Although, phospho-KIT was detected in all eight tumors tested, it had a weak pattern in five, including all four GISTs with *KIT* exon 11 in-frame deletions, and was strong in three (Fig. 2D). All three untreated GISTs with identical 557-8 WK deletion showed a weak expression only of the mature isoform.

KIT amplification is not a frequent event in imatinib resistance. Tissue for FISH analysis was available in nine nodules from eight patients: three nonresistant (#2, 3, 4), four with acquired resistance (three with no secondary mutations #16, 17, 22, and one carrying a D820Y, #14B) and one patient with primary resistance (two nodules, #31). Copy number ratio for KIT: CEP 4 in the nine nodules tested did not differ from the normal control liver sample and no evidence of KIT amplification was identified in any of the tumors tested (Fig. 3).

Discussion

We attempted to determine the mechanism of acquired resistance to imatinib in patients with GIST in order to identify strategies to prevent or delay its development. We did a molecular analysis of *KIT* and *PDGFRA* mutations in a cohort of patients who were treated with imatinib and then underwent surgical resection. We found second site *KIT* mutations to be

common in acquired resistance to imatinib. Of the 15 patients with responsive or stable disease who then experienced disease progression, 7 had an identifiable second mutation. In each case, both the first and the second mutation were located in the *KIT* gene. In fact, they were located on the same allele. In contrast, we have not identified two simultaneous *KIT* or *PDGFRA* mutations in >300 untreated GIST tumors that we have analyzed.

All resistant tumors that had a second mutation had a primary *KIT* exon 11 mutation. These primary mutations were mainly in-frame deletions but there was one insertion and one substitution mutation (Fig. 1). The eight second site mutations were all substitutions that involved five different residues. By comparison, the incidence of point mutations in nontreated GIST is at most 15%. The five residues derived from either the first (exons 13 or 14) or second (exon 17) KIT kinase domain. In contrast, the incidence of exons 13, 14, or 17 mutations in untreated GIST is about 1% (11, 15). Six of the secondary mutations were located in exon 17 between amino acids 820 and 823, the most common being N822K, which was seen in three cases. Although N822K and D820Y have been previously reported as a primary sporadic mutation (10) and germ line mutation in a familial GIST syndrome (21), respectively, we believe that in these resistant nodules, N822K and D820Y substitutions represent a secondary mutation because they were associated with an exon 11 mutation and only one of the multiple nodules analyzed from the same patient carried this mutation. Similar *KIT* kinase mutations, N822K and Y823D, have been described recently in a subset of seminomas (22). To date, all primary mutations reported in *KIT* exon 13 have been a K642E substitution (10, 15–17). However, we identified a V654A mutation in exon 13. Interestingly, a recent study by Chen et al. (23) reported a V654A mutation in all six resistant nodules taken from five GIST patients. Similarly, this second mutation in their study was also found on the same allele as the primary mutation. However, there are several differences between our findings and those of Chen et al. (23) including: (*a*) the incidence of second mutations in acquired resistance (100% versus 46%), (*b*) the location of second mutations (exon 13 versus different areas of the KIT kinase domain), and (*c*) the type of primary mutation of tumors developing second mutation (exon 9 and 11 versus only exon 11 mutations).

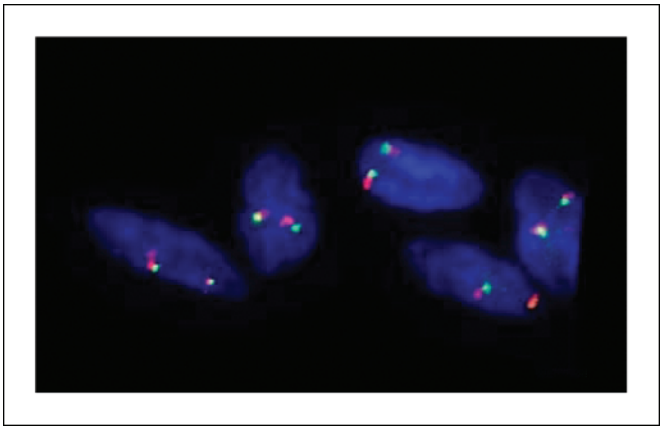

Fig. 3. FISH analysis on a patient with acquired resistance. Patient #18 who had no secondary mutation showed a normal chromosome copy number for both KIT (*green*) and reference centromeric chromosome 4 (*red*).

Some of these discrepancies might be due to a limited number of cases in both series. Although no primary mutations have been previously described in *KIT* exon 14, a recent report has shown a T670I substitution in a patient with acquired resistance (24). One of our seven patients with a second mutation had a similar T670I mutation. New activating kinase mutations were also reported in seven of nine (78%) GIST patients with a unique pattern of resistance, defined radiologically as a "nodule within a mass", and thought to represent the progression of a clone resistant to imatinib (25).

Recently, the crystal structures of the autoinhibited inactive and active conformations of the KIT kinase have been determined, as well as the structure of the KIT kinase in complex with imatinib (26). These structures provide insight into the mechanism of the normal regulation of KIT kinase function but moreover they provide explanations for the basis of constitutive activation of mutations found in neoplasms such as GIST. A critical feature in KIT kinase function is the role of the juxtamembrane domain of the KIT receptor in regulating kinase activity. In the inactive autoinhibited state, the juxtamembrane domain of KIT inserts into the kinase active site and thus disrupts the formation of the active conformation. Critical residues in these interactions are WK557-558 and VV559-560. Mutation of these residues disrupts these inhibitory interactions and destabilizes the inactive autoinhibited conformation of the KIT kinase. Thus, a diversity of juxtamembrane domain mutations represents the majority of primary oncogenic mutations found in GIST. The activation loop (A-loop) of the KIT kinase, which includes Y823, a pseudosubstrate of the KIT kinase, is another major site of oncogenic mutation. During KIT kinase activation, Y823 becomes phosphorylated and this seems to stabilize the open active conformation of the A-loop presumably by strong negative electrostatic interactions of the phosphate residue. Oncogenic activation loop mutations such as Y823D, which mimic Y823 phosphorylation, thus stabilizing the active conformation of the A-loop. The structure of the KIT-imatinib complex revealed that, similar to BCR-ABL, imatinib binds the inactive conformation of the kinase although the KIT-imatinib complex deviates somewhat from the autoinhibited inactive KIT kinase conformation. It is therefore not surprising that A-loop mutations are generally not inhibited by imatinib, although there seem to be exceptions. Therefore, there are two possible mechanisms of how resistance to imatinib therapy may develop. First, second site mutations may stabilize the active conformation of the KIT kinase which prevents imatinib binding. Alternatively, second site mutations may specifically interfere with imatinib binding without affecting the overall KIT kinase conformation. Our findings are in agreement with these predictions. First, five of the second site mutations in this study are located in the A-loop (26). Tyr^{823} was found to be substituted by aspartic acid in one of the resistant tumors and other mutations included D820Y and N822K. Although the Y823D mutation introduces a tyrosine-phosphate mimic (Fig. 4), the others may destabilize the inactive conformation by introduction of a positively charged side chain into a positively charged pocket formed on the COOH-terminal lobe of the kinase. Second, one mutation seems to block imatinib binding to KIT. In T670I, the gatekeeper residue Thr^{670} is replaced by an isoleucine residue. This mutation disrupts an important H-bond between imatinib and the kinase and the

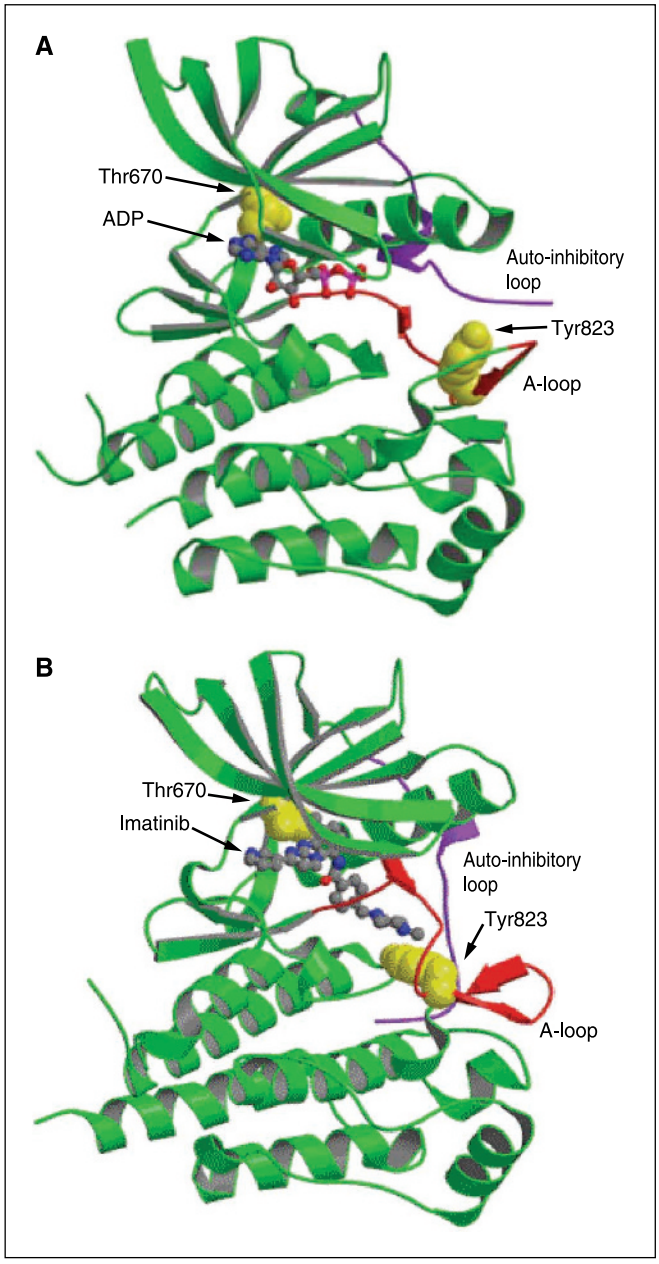

Fig. 4. Interaction of imatinib and the KIT kinase. The activation loop (A-loop; 809-831) is highlighted in red and the autoinhibitory domain (547-579) is highlighted in purple. The sites of mutation at Thr^{670} and Tyr^{823} are shown in space-filling representation in yellow. *A,* active form with ADP bound (ADP is shown as ball-and-stick representation). *B,* inactive form with imatinib bound (imatinib shown as ball-and-stick representation). Figures generated with the programs MOLSCRIPT (35) and RASTER3D (36). Coordinates correspond to those reported by Mol et al. (26).

isoleucine methyl group protrudes into the imatinib binding site precluding proper imatinib binding to the KIT kinase (Fig. 4).

Investigation of CML revealed that the predominant mechanism of acquired resistance to imatinib is second site mutation in *BCR-ABL* (19, 20). The *BCR-ABL* mutations described to date in resistant CML either alter the imatinib binding site or the residues responsible for the unique conformational change that permits imatinib binding and prevents the inactive conformation necessary for imatinib binding (20). *BCR-ABL* kinase domain substitutions occurred in 29 of 32 (90%) resistant tumors, whereas genomic amplification of *BCR-ABL* transcript was infrequent. Similarly, we found secondary *KIT* mutation in patients with acquired resistance. However, there are several distinct features between acquired resistance in CML and GIST (Table 3). First, preexisting *BCR-ABL* kinase mutations have been found in 2 of 4 patients (20) and in 5 of 24 patients (27) who did not respond to imatinib. Furthermore, *BCR-ABL* kinase mutations were also found in 4 of 13 patients with chronic phase CML who had stable disease on imatinib and this predicted subsequent clinical relapse (20). We did not find secondary mutations in any of the 14 GIST patients with nonresistant disease. Shah et al. (20) found polyclonal resistance, in which two to four clones harbored distinct mutant *BCR-ABL* kinase domains mutations in 12 of 32 CML patients. We did not detect polyclonal resistance in 10 samples from five patients with acquired resistance, because all cDNA subclones from one tumor nodule revealed only a single type of secondary mutation. Although we did not find coexisting, multiple second mutations from patients with acquired resistance, one patient had two different secondary mutations in resistant nodules at two different time points. Thus, tumor nodules within a patient may develop independent means of imatinib resistance.

The mechanism for the development of a second mutation in GIST is unclear. The long duration of imatinib therapy (median of 27 months) in patients with acquired resistance who developed a second mutation makes it less likely that a preexisting clone is responsible for acquired resistance (Fig. 5). In CML, the presence of *BCR-ABL* kinase domain mutations in pre-imatinib samples of patients with primary resistance, the relapse of patients in the blast phase is within 1 to 3 months, and the finding of multiple mutations in resistant patients suggest clonal selection of preexisting mutations that confer imatinib resistance. In GIST, the possibility of preexisting mutations remains to be evaluated carefully.

Our inability to find secondary mutations in eight resistant GIST patients suggests that other gene mutations, or altogether

Table 3. Comparison of imatinib resistance in CML and GIST

	CML*	GIST†
Acquired resistance		
Kinase domain mutations	29 of 32	7 of 15
Median duration of imatinib therapy	20 months‡	27 months
Gene amplification in acquired resistance	3 of 11	0 of 4
Activation of transcribed protein	11 of 11	heterogeneous
Primary resistance		
Kinase domain mutations pre-imatinib	7 of 28	0 of 4
Nonresistant disease		
Kinase domain mutations	4 of 13	0 of 14

NOTE: Results are listed as number of patients with feature over number tested.
*Based on refs. (19, 20, 27).
†Current report.
‡Chronic phase CML.

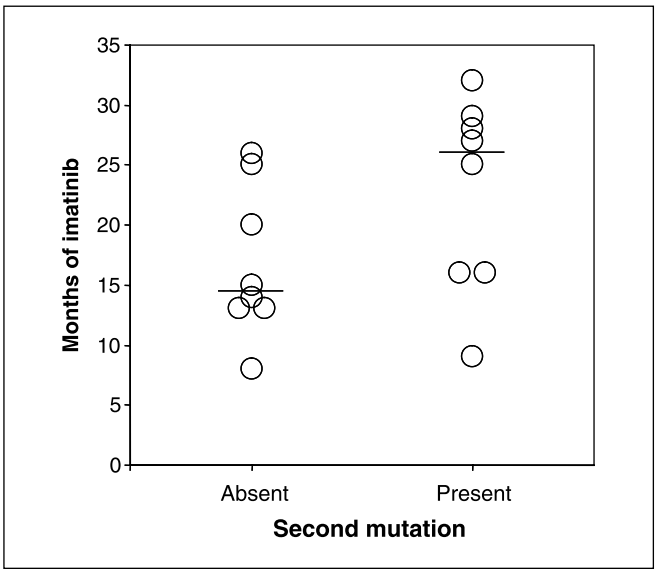

Fig. 5. Relationship of duration of therapy to the presence of a second *KIT* mutation in patients with acquired resistance to imatinib. Resistant patients who developed a second *KIT* mutation had a median duration of imatinib treatment of 27 months, whereas those who did not had a median treatment duration of 14.5 months. Patient 24 developed acquired resistance at 9 and 16 months of imatinib therapy and had a secondary mutation in each instance, which are both plotted.

different mechanisms, may be involved. Fletcher et al. (28) suggested additional mechanisms of resistance besides secondary *KIT* mutations in GIST, including *KIT* genomic amplification and activation of an alternative receptor tyrosine kinase protein in the absence of KIT expression. We have found no evidence for *KIT* genomic amplification in our five resistant patients. Based on experimental data, other possibilities are increased serum acid glycoprotein levels and increased multidrug resistance gene expression (29, 30). More recently, Van Oosterom and colleagues (17, 31) found preliminary evidence that bioavailability of imatinib may decrease during chronic therapy, possibly due to up-regulation of hepatic enzymes responsible for drug clearance. It is now well documented that cessation of imatinib during stable measurable disease results in tumor proliferation (32) and therefore subtherapeutic imatinib levels may also result in tumor progression.

Our analysis of phosphorylated KIT protein yielded heterogeneous results, signifying the complexity of KIT activation in treated GIST. Strikingly, the majority of nonresistant patients still had activated KIT. Most of the patients in the acquired resistance group also had phospho-KIT and almost half had strong expression. This finding suggests that GIST progression may still depend on KIT and alternative inhibitors of KIT, or its downstream pathway, may be of therapeutic benefit. Nevertheless, there were three patients with acquired resistance whose tumors lacked detectable KIT activation. There was no discernible pattern of KIT activation based on histologic response, the type of primary mutation, or the presence of a second KIT mutation. There was not even a correlation between phosphorylated KIT and total KIT. Our findings are consistent with those of Duensing et al. (33) who found that both phosphorylated and total KIT varied substantially among tumors, even within identical KIT genotype. Our data with KIT phosphorylation may be confounded by the fact that we stopped imatinib generally 2 to 5 days prior to surgery. Currently, there are no established guidelines for the perioperative use of imatinib but our approach seems to be safe.

Secondary mutations during chronic imatinib use has several implications for the clinical management of patients with advanced GIST. Our recommendations are based on the supposition that the risk of developing resistance is proportional to the amount of residual viable tumor in patients on imatinib. Therefore, we consider extirpation of all gross disease whenever possible. Indeed, that was the rationale for operating on most of the patients in this report who were nonresistant. The fact that several nonresistant patients had only minor histologic responses along with activated KIT underscores the potential for viable tumor to progress. An argument may even be made for debulking (removing as much gross disease as possible) in patients with extensive residual disease during imatinib therapy. Alternatively, optimal management may include the combined use of imatinib with other newer molecular agents. Although we did not detect any second mutations in nonresistant patients, Shah et al. (20) found second mutations in 4 of 13 patients with stable CML and that predicted subsequent relapse. Patients with stable GIST who may be found to have a second mutation (such as by biopsy) should certainly be considered for additional surgery or other molecular agents due to the likelihood of impending progression. Of course, serial tumor assessment is not practical in GIST as it is in CML. A variety of other molecular inhibitors are currently under investigation for advanced GIST that is refractory to imatinib. The furthest along is SU11248 (Pfizer Inc., New York, NY), an inhibitor of multiple tyrosine kinases. It seems to be effective in imatinib-resistant GIST, especially in patients with exon 9 mutations (34).

In summary, we report a clinical and molecular study of acquired resistance to imatinib in GIST. We found that secondary mutations are common in imatinib resistance. The mutations tend to be single amino acid substitutions in the KIT kinase domains and occur particularly in exon 17. Secondary mutations were not seen in the pre-imatinib, nonresistant, or primary resistant tumors. There was considerable heterogeneity between KIT activation and responsiveness of GIST to imatinib. Our findings have implications for strategies to treat or avert imatinib resistance and might be useful in the design of second-generation kinase inhibitors.

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