Allele-Specific Polymerase Chain Reaction for the Imatinib-Resistant *KIT* D816V and D816F Mutations in Mastocytosis and Acute Myelogenous Leukemia

Christopher L. Corless,^{*,†} Patina Harrell,[†]
Mario Lacouture,[‡] Troy Bainbridge,[†] Claudia Le,[†]
Ken Gatter,^{*} Clifton White, Jr.,[§] Scott Granter,[¶]
and Michael C. Heinrich^{†||}

From the Department of Pathology,^{*} Oregon Health and Science University Cancer Institute,[†] and the Divisions of Dermatology[§] and Hematology and Oncology,^{||} Oregon Health and Science University, Portland, Oregon; the Section of Dermatology,[‡] University of Chicago, Chicago, Illinois; and the Department of Pathology,[¶] Brigham and Women's Hospital, Boston, Massachusetts

Oncogenic mutations of the receptor tyrosine kinase *KIT* contribute to the pathogenesis of gastrointestinal stromal tumors, systemic mastocytosis (SM), and some cases of acute myelogenous leukemia (AML). The D816V substitution in the activation loop of *KIT* results in relative resistance to the kinase inhibitor imatinib (Gleevec). Because this mutation occurs in 80 to 95% of adult SM, its detection has diagnostic and predictive significance. Unfortunately, the fraction of mutation-positive cells in clinical SM samples is often below the 20 to 30% threshold needed for detection by direct DNA sequencing. We have developed an allele-specific polymerase chain reaction assay using a mutation-specific primer combined with a wild-type blocking oligonucleotide that amplifies D816V at the level of 1% mutant allele in DNA extracted from formalin-fixed, paraffin-embedded tissue. There were no amplifications among 64 *KIT* wild-type tumors and cell lines, whereas all D816V-mutant samples (eight AML and 11 mast cell disease) were positive. Other D816 substitutions associated with resistance to imatinib *in vitro* are rare in SM. Among these D816F was detectable with the assay whereas D816H, D816Y, and D816G did not amplify. Nine biopsies (bone marrow, skin, or colon) with suspected SM were negative by denaturing high performance liquid chromatography and/or DNA sequencing but positive by allele-specific polymerase chain reaction. Thus, the assay may be useful in confirming the diagnosis of SM. (*J Mol Diagn* 2006; 8:604–612; DOI: 10.2353/jmoldx.2006.060089)

The receptor tyrosine kinase *KIT* plays an important role in the development of bone marrow stem cells, mast cells, primary germ cells, and the interstitial (Cajal) cells of the gastrointestinal tract.¹ Correspondingly, gain of function mutations in the *KIT* gene are found in myeloproliferative disorders, including subtypes of mastocytosis and in seminomas and gastrointestinal stromal tumors (GISTs).^{1,2} With the introduction of *KIT* kinase inhibitors such as imatinib (Gleevec) into clinical practice, there is increasing recognition of the critical relationship between *KIT* gene mutations and the response to treatment. For example, the 60 to 70% of GISTs that harbor a mutation in the juxtamembrane domain of *KIT* (exon 11) are more responsive to imatinib therapy than are GISTs with no *KIT* mutation.^{3,4} Interestingly, these exon 11-mutant tumors may become resistant to imatinib by acquiring a secondary *KIT* mutation in the ATP-binding pocket (exon 13) or activation loop domain (exon 17).^{5–8} Exon 17 is of particular significance in this regard because primary mutations in this domain are found in systemic mastocytosis (SM) and acute myelogenous leukemia (AML), and there is clinical interest in the use of kinase inhibitors like imatinib to treat these diseases.

The first *KIT* gene mutation identified in human cells was the substitution of valine for aspartic acid at codon 816 (D816V; A81402T based on GenBank U63834; *KIT* cDNA A2468T) in a mast cell leukemia cell line.⁹ The resulting mutant form of *KIT* has a constitutively activated kinase, signaling from which appears to be essential for the survival and proliferation of mast cell precursors in the marrow. Studies from a number of groups have established that substitutions at codon 816 are common in systemic mast cell disease, and such mutations are defined by the World Health Organization as one of the minor criteria for the diagnosis of SM.^{10–15} The classification for SM includes indolent SM, aggressive SM, mast cell leukemia, and SM with associated hematological nonmast cell lineage disease (SM-AHNMD), which includes variants with associated myelodysplastic syndrome (SM-MDS), myeloproliferative disease (SM-MPD), and acute myelogenous leukemia (SM-AML).^{14,16} *KIT* gene mutations have been identified in all of these SM

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Address reprint requests to Christopher L. Corless, M.D., Ph.D., OHSU Dept. of Pathology (L471), 3181 SW Sam Jackson Park Rd., Portland, OR 97239. E-mail: corlessc@ohsu.edu.

subtypes, being detectable in samples of skin, bone marrow, gut, spleen, and blood, albeit to varying degrees.¹⁴

Among the KIT codon 816 substitutions reported, D816V is by far the most common, representing more than 90% of mutations in adult SM patients in a number of series.^{17–22} In the largest series to date, Garcia-Montero and colleagues¹⁵ observed *KIT* mutations in 93% of 113 cases, and nearly all of these (97%) were D816V. Other, more rare *KIT* mutations that have been reported in SM include alterations in exon 17 (D815K, D816F, D816H, D816Y, D820G, E839K), exon 11 (V560G), and exon 10 (K509I, F522C, V530I, A533D). Among these, D816F may be relatively more common in pediatric patients.^{22,23} Interestingly, in SM with excess eosinophils (SM-EOS, hypereosinophilic syndrome), the fusion oncogene *FIP1L1-PDGFRα* is the most commonly detected molecular alteration.^{24–26}

Detection of *KIT* D816V is significant not only because it can help confirm a suspected diagnosis of SM, but because this mutant form of KIT is fully resistant to inhibition by imatinib *in vitro*,^{4,27–29} and clinical reports suggest that this drug is not very effective in the treatment of D816V-positive disease.^{30–34} D816V is also observed in cases of AML, particularly in tumors with core binding factor translocations,^{35,36} and there is evidence that D816V confers resistance to imatinib treatment in this setting as well.³⁷ For these reasons, the *KIT* D816V mutation is of particular clinical interest.

Determining the *KIT* gene mutation status in cases of AML is generally straightforward because tumor cells typically dominate blood, bone marrow aspirate, and bone marrow biopsy samples. In contrast, mutation analysis in mastocytosis presents a greater challenge. The degree of mast cell infiltration in affected organs varies widely and may represent as little as 1% of the cell population in a given biopsy. In recent studies of GISTs, we have used denaturing high-performance liquid chromatography (HPLC) to screen *KIT* gene amplicons for mutations.^{4,38} With a sensitivity of ~10 to 20% mutant allele, HPLC works well in the analysis of DNA from the highly cellular samples of these sarcomas. However, analysis of biopsies from patients with possible SM is problematic using this methodology because the number of mast cells is often quite low (<10% of cells) and the probability of a false negative result is unacceptably high. In response to growing clinical demand for *KIT* mutation screening in patients with suspected SM, we looked at published methods that have been used to detect *KIT* mutations in this patient population. These include cDNA amplification and sequencing, analyses of genomic DNA by direct sequencing, restriction fragment length polymorphism or single strand conformation polymorphism, and real-time polymerase chain reaction (PCR) using peptide-nucleic acid probes.^{18–20,22,23,33} Because most clinical samples are formalin-fixed and paraffin-embedded, a cDNA-based approach would be technically challenging. Restriction fragment length polymorphism and single strand conformation polymorphism are somewhat labor intensive and have limited sensitivities, and our attempts to adopt the peptide-nucleic acid probe method

described by Sotlar and colleagues²³ were unsuccessful. Therefore, we developed a new allele-specific PCR (AS-PCR) method that is modeled on a *RAS* gene assay reported by McKinzie and Parsons.³⁹ This AS-PCR assay is based on a combination of a mutation-directed primer and a wild-type blocking oligonucleotide and can reproducibly detect either *KIT* D816V or D816F that is present at low levels in DNA extracted from paraffin-embedded tissue.

Materials and Methods

Tumor Samples and Cell Lines

Tumor samples were obtained from the Tissue Bank of the Oregon Health and Science University Cancer Institute, from the Oregon Health and Science University Departments of Pathology and Dermatology, the University of Chicago Section of Dermatology, and the Department of Pathology of Brigham and Women's Hospital. The use of these tissues in the study was performed in accordance with the regulations of the institutional review boards of these institutions. The human HMC-1.2 mast cell line, which harbors both V560D and D816V mutations of the *KIT* gene, was kindly provided by Dr. C. Akin (Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD).²⁸ LoVo cells (a colon cancer cell line that is wild type for *KIT*) were obtained from the American Type Culture Collection, Rockville, MD (<http://www.atcc.org>). In a previous study we generated stably transfected clones of Ba/F3 cells (murine interleukin 3-dependent hematopoietic pro-B cells) expressing wild-type, D816V, D816F, or D816Y mutant forms of human *KIT* cDNA.⁴⁰ DNA extracted from these Ba/F3 subclones was used in validation experiments.

DNA Preparation

DNA was extracted from HMC-1.2, LoVo, and Ba/F3 cells with the QIAmp DNA mini kit (no. 51306; Qiagen, Valencia, CA). The same kit was used to extract genomic DNA from blood and bone marrow aspirates, as well as from formalin-fixed tissue (unembedded), and formalin-fixed, paraffin-embedded tissue samples, in accordance with the manufacturer's recommendations.

Standard PCR

KIT exon 17 amplicons were generated from genomic DNA as previously described.⁴¹ For the standard (control) PCR reaction, the forward primer was 5'-TGTATTCA-CAGAGACTTGGC-3', and the reverse primer was 5'-TAATGTTCCAGCATACCATGCAA-3'. This reverse primer matches sequences in *KIT* intron 17 and was also used in the allele-specific PCR. To amplify *KIT* cDNA sequences from Ba/F3 subclones, the same forward primer was matched with the reverse primer 5'-GCTCCCAAAGAAA-AATCCCATAGG-3'.

Allele-Specific PCR

Allele-specific PCR reactions were performed using 200 ng of DNA and a master mix based on the Expand High Fidelity Polymerase kit (no. 11759078001; Roche, Indianapolis, IN) with 1.4 U of polymerase, 160 μ mol/L dNTP (Stratagene, Cedar Creek, TX), 400 nmol/L mutation-specific primer (Figure 1), 200 nmol/L blocking oligonucleotide (Figure 1), and 800 nmol/L reverse primer (5'-TAAT-GTTCAGCATACCATGCAA-3'). The cycling conditions were as follows: 95°C for 1 minute, followed by 45 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute, and a final 7-minute incubation at 73°C.

HPLC and DNA Sequencing

PCR products were routinely analyzed on a denaturing HPLC system (WAVE; Transgenomic, Inc., Omaha, NE), as previously described.^{4,38} This system allows the detection of DNA heteroduplexes that differ by as little as one nucleotide in 400 bp and is sensitive in the range of 15 to 20% mutant allele. DNA sequencing was performed using the BIGDye terminator kit (Applied Biosystems, Foster City, CA) and analyzed on an ABI 310 capillary sequencer.⁴

Topo Subcloning

To look for low-abundance *KIT* exon 17 mutations, amplicons were cloned into Topo plasmids and sequenced individually, as previously described.⁴²

Results

Assay Design

Our assay is modeled on the approach recently described by McKinzie and Parsons³⁹ to study human *KRAS* and mouse *Hras* mutations. The mutation-specific primer (MSP) and the blocking oligonucleotide are shown in Figure 1. The MSP is designed to prime from the mutant A of the T>A substitution of D816V (A81402T based on GenBank U63834; *KIT* cDNA A2468T) but includes an intentional mismatch in the penultimate 3' nucleotide. When aligned with wild-type (WT) sequence, the MSP is doubly mismatched at the 3' end, limiting its ability to prime. The MSP can also prime from the second nucleotide of the two-nucleotide substitution that results in D816F (double substitution G81401T and A81402T in GenBank U63834; *KIT* cDNA G2467T and A2468T) (Figure 1), which is another mutation associated with SM and is resistant to imatinib *in vitro*.²⁸ In theory, the MSP could also serve in amplifying D816I and D816M (both perfect matches), as well as D816L (penultimate mismatch), but to our knowledge these mutations have not been observed in SM. On the other hand, the known SM-associated mutations D816H (G81401C; *KIT* cDNA G2467C) and D816Y (G81401T; *KIT* cDNA G2467T) would not be expected to amplify, as both are doubly mismatched to

Mutant-specific primer

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5' GTGATTTTGGTCTAGCCAGAAT->
|||||*|
D816V Mutant sequence ---CACTAAAACCATCGGTCTCA---
5' GTGATTTTGGTCTAGCCAGAAT->
|||||*|
D816F Mutant sequence ---CACTAAAACCATCGGTCTCA---
5' GTGATTTTGGTCTAGCCAGAAT
|||||**
Wild-type sequence ---CACTAAAACCATCGGTCTCT---

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Blocking oligonucleotide

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5' GTGATTTTGGTCTAGCCAGAAAX
|||||**
D816V Mutant sequence ---CACTAAAACCATCGGTCTCA---
5' GTGATTTTGGTCTAGCCAGAAAX
|||||**
D816F Mutant sequence ---CACTAAAACCATCGGTCTCA---
5' GTGATTTTGGTCTAGCCAGAAAX
|||||*|
Wild-type sequence ---CACTAAAACCATCGGTCTCT---

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Figure 1. Design of mutant-specific and blocking primers. The mutant-specific primer has a single mismatch (indicated by an asterisk) immediately 5' to the 2447 T>A substitution that results in D816V but is matched to the mutant A and still allows extension. When bound to the wild-type allele, this primer is doubly mismatched at the 3' end and makes a poor substrate for extension. The blocking oligonucleotide is designed with a reverse strategy such that it has a double mismatch to the mutant allele. In addition, the 3' terminal nucleotide of this oligonucleotide is chemically reversed (3' to 5') so that it cannot serve as a primer (indicated by the ^X).

the last two MSP nucleotides. This was confirmed for both of these mutations, as detailed below.

The design of the blocking oligonucleotide (B-oligo) is the reverse of the MSP, such that it is doubly mismatched to the mutant D816V and D816F sequences, but only singly mismatched to WT. In addition, the 3' terminal nucleotide of the B-oligo was linked in a chemically reversed configuration (3' to 5') to eliminate any possibility of priming.

Pilot studies were performed using DNA purified from HMC-1.2 cells, which are heterozygous for the D816V mutation. The expected sequence of the amplified product was confirmed by bidirectional sequencing. Temperatures examined for primer annealing included 58°C, 57°C, and 55°C, with the latter providing the best results. We tested a reduced number of PCR cycles³⁵ but found that 45 cycles provided more consistent results. The addition of the B-oligo increased the sensitivity of the assay by 10- to 100-fold (data not shown). Molar ratios of MSP to B-oligo ranging from 2:1 to 1:2 were tested, and the most consistent results were observed at a 2:1 ratio, which was used in all subsequent assays. In addition, it was found that doubling the concentration of the reverse primer relative to the MSP yielded more consistent amplifications.

Parson and colleagues⁴³ reported that substitution of a modified DNA polymerase that lacks 5' to 3' exonuclease activity (Stoffel fragment) in their allele-specific mouse *Hras* assay increased allele discrimination. However, in our assay a Stoffel fragment polymerase (no. N8080038;

Figure 2. Sensitivity of AS-PCR for D816V in paraffin-derived DNA. DNA was extracted from formalin-fixed, paraffin-embedded seminoma heterozygous for *KIT* D816V (50% mutant allele by denaturing HPLC and direct sequencing) and was diluted with increasing amounts of *KIT* wild-type DNA from a formalin-fixed, paraffin-embedded GIST before amplification by AS-PCR and detection by HPLC.

Applied Biosystems) generated spurious products; therefore, we used the Expand High Fidelity polymerase (no. 11759078001; Roche).

Assay Sensitivity for KIT D816V

The sensitivity of the AS-PCR assay for *KIT* D816V was influenced by the quality of the template DNA. Positive signals were routinely detectable at a level of 0.1% mutant allele using DNA extracted from HMC-1.2 cells diluted into wild-type DNA from LoVo cells (not shown). The detection threshold was higher when paraffin-derived DNA from a seminoma heterozygous for D816V was diluted with the same wild-type LoVo DNA (not shown) or with genomic DNA from a formalin-fixed, paraffin-embedded GIST that was wild-type for *KIT* exon 17 by denaturing HPLC (Figure 2). The D816V mutation in paraffin-derived DNA was reproducibly detected at the level of 1% but not below this. The positive control used for all AS-PCR reactions was a parallel tube containing the same reverse primer matched to a generic exon 17 forward primer, which was 5' to the allele-specific primer and yielded a larger amplicon.

Validation Studies

The AS-PCR assay was validated through the analysis of five types of samples. First, 64 samples of genomic DNA wild type for *KIT* exon 17 were tested in the allele-specific reaction. No amplifications were observed with the MSP/B-oligo mixture, whereas control primers showed strong

Figure 3. AS-PCR of wild-type and mutant *KIT* cDNAs. cDNA prepared from stably transfected Ba/F3 subclones expressing various isoforms of *KIT* cDNA was tested by AS-PCR. Positive AS-PCR signals (minimum 2 mV) were obtained from cells expressing D816V or D816F cDNA but not from cells transfected with the wild-type or D816Y forms of *KIT*.

signals. The wild-type status of these 64 samples had been determined by denaturing HPLC screening, which in our experience is more sensitive than DNA sequencing. Thirty-one of the samples were also wild type by direct sequencing; the remaining cases were not sequenced. Because 20 of the samples were derived from cell lines or fresh-frozen tumor tissue (six AML, three GIST, seven myxoid chondrosarcoma, and one lymphoma), the quality of the DNA was probably not a factor in these AS-PCR reactions. The remaining 44 samples were from paraffin-embedded tumors (GIST, non-GIST sarcoma, hemangioma, thymic carcinoma, renal cell carcinoma, renal oncocytoma, and desmoid fibromatosis). Genomic DNA from a cell line wild type for *KIT* exon 17 (LoVo cells) was included as a negative control in all subsequent reactions.

Second, a group of Ba/F3 subclones expressing wild-type or mutant forms of human *KIT* cDNA were tested. As illustrated in Figure 3, positive AS-PCR signals were obtained from cells stably transfected with D816V or D816F cDNA, but not from cells containing WT or D816Y forms of *KIT*. Control PCR reactions for human *KIT* exon 17 sequences were positive in all cases. Because the amplification target in these cell lines was cDNA, a different reverse primer was used in the AS-PCR reactions. Nevertheless, the results support the specificity of the MSP/B-oligo combination for the D816V and D816F mutations. As we did not have any paraffin-derived samples with *KIT* D816F, which is a rare mutation in mast cell disease and has not been reported in seminoma or GIST, we could not confirm that *KIT* D816F can be amplified from genomic DNA. However, the positive result obtained from the D816F cDNA-expressing Ba/F3 clone suggests that any signal generated by MSP/B-oligo combination could represent either D816V or D816F.

Third, a series of tumors with sequence-confirmed *KIT* D816V was analyzed with the AS-PCR assay. These included eight samples of AML (DNA from fresh cells) and three samples of paraffin-embedded seminoma (Table 1,

Table 1. Cases with Known *KIT* D816 Mutation

Sample no.	Diagnosis	DNA source	Direct DNA sequence	AS-PCR
1	AML	Fresh cells	D816V	Positive
2	AML	Fresh cells	D816V	Positive
3	AML	Fresh cells	D816V	Positive
4	AML	Fresh cells	D816V	Positive
5	AML	Fresh cells	D816V	Positive
6	AML	Fresh cells	D816V	Positive
7	AML	Fresh cells	D816V	Positive
8	AML	Fresh cells	D816V	Positive
9	Seminoma	Paraffin	D816V	Positive
10	Seminoma	Paraffin	D816V	Positive
11	Seminoma	Paraffin	D816V	Positive
12	Seminoma	Paraffin	D816H	Negative
13	Seminoma	Paraffin	D816H	Negative
14	GIST*	Paraffin	D816H	Negative
15	GIST*	Paraffin	D816H	Negative
16	GIST*	Paraffin	D816G	Negative
17	GIST*	Paraffin	D816A	Negative
18	AML	Fresh cells	D816Y and D816V [†]	Positive
19	AML	Fresh cells	D816Y and D816V [†]	Positive
20	GIST*	Paraffin	D816H	Positive

*GIST samples from patients with acquired imatinib resistance.

[†]D816V detected at low levels (5 to 10%) among TOPO subclones from exon 17 ampimers of these cases.

cases 1 to 11). All of these samples yielded positive amplicons by AS-PCR. As noted above, we could not further validate the AS-PCR reaction for the D816F mutation because none of our tumor samples had this substitution.

We next examined a group of tumors with alternate mutations of codon 816 using the AS-PCR assay. Two seminomas with sequence-confirmed D816H did not amplify by AS-PCR, as expected (Table 1, samples 12 and 13). D816H is also a mutation found in GISTs from patients with acquired resistance to imatinib, and two such tumors were negative by AS-PCR despite robust amplifications with the control primers (samples 14 and 15). Imatinib-resistant GISTs with D816G or D816A were AS-PCR-negative as well (samples 16 and 17, respectively).

Interestingly, two AML samples that had *KIT* D816Y mutation by direct sequencing were positive by AS-PCR (Table 1, samples 18 and 19). Topo subclones were prepared and sequenced from these amplification products, and D816V-positive clones were detected at low levels (1 of 20 from case 18, 1 of 10 from case 19). These results are consistent with the estimated sensitivity of the AS-PCR assay and provide evidence of heterogeneity among *KIT* mutations that occur in AML. We suspect that mutation heterogeneity also accounts for the small positive AS-PCR signal observed in one of the imatinib-resistant GISTs that appeared to be D816H by direct sequencing (Table 1, sample 20). Although the AS-PCR signal in this case could represent a false positive, it is perhaps more likely that it reflects low-abundance D816V that accumulated under the selective pressure imatinib therapy. This view is supported by the fact that there were no positive signals among 25 untreated GISTs that were wild type for *KIT* exon 17 (see above) or among the four other D816H-mutant tumors examined (two seminoma, two GIST).

Finally, a group of biopsies from adult patients with suspected mast cell disease was screened for *KIT* exon

17 mutation using our standard PCR reaction and HPLC, with confirmatory sequencing as necessary, and by AS-PCR. Among 21 bone marrow samples (aspirates or cores), six were positive by both assays, seven were positive by AS-PCR only, and the remaining eight were negative by both assays (Table 2). The tested marrows were predominantly referral samples from patients with suspected SM; unfortunately, clinical details for most of these patients could not be obtained. Marrow samples from a patient with mast cell leukemia (Table 2, sample 29) and a patient with SM-AML (sample 30) were positive by both the standard and AS-PCR assays. In contrast, marrow samples from three patients with a clinical diagnosis of SM-MPD (samples 56 to 58) were all negative by both assays. Interestingly, one of these was found to be positive for a *JAK2* V617F mutation when analyzed in the Molecular Diagnostics Center of Oregon Health and Science University (data not shown).

Among 13 skin biopsies with mast cell infiltrates, three were positive by both the standard assay and AS-PCR, another was AS-PCR-positive only, and the remaining nine were completely negative (Table 2). Because no attempt was made to microdissect the areas involved by mast cells, it is possible that the negative cases had too few mast cells to allow detection, even by AS-PCR. Alternatively, the infiltrates may not have been related to a mutant *KIT*-related neoplasia. Other examples of tissues that yielded positive AS-PCR results included one colon biopsy, one lymph node, and one of two blood samples (Table 2).

The results summarized in Table 2 demonstrate an excellent correlation between the standard PCR/HPLC assay and AS-PCR results, but with a clear advantage for the latter. Sequential biopsies from one particular patient are of interest in this regard. A colon biopsy from this 61-year-old male (sample no. 32) was morphologically consistent with the clinical diagnosis of SM but was negative for an exon 17 mutation by the standard assay. The

Table 2. AS-PCR Results on Samples from Patients with Clinically Suspected Mast Cell Disease

Sample no.	Diagnosis	Age	Sex	Site	DNA source	HPLC	Direct sequence	AS-PCR
21	Mastocytosis	64	M	Skin	Paraffin	pm/snp	D816V	Positive
22	Mastocytosis	48	F	Skin	Paraffin	pm/snp	D816V	Positive
23	Mastocytosis	28	M	Skin	Paraffin	pm/snp	SNP I798I and D816V	Positive
24	Mastocytosis	22	F	LN	Paraffin	pm/snp	D816V	Positive
25	Mastocytosis	74	M	BM	Fresh aspirate	pm/snp	D816V	Positive
26	Mastocytosis	65	F	BM	Fresh aspirate	pm/snp	D816V	Positive
27	Mastocytosis	39	F	BM	Fresh aspirate	pm/snp	D816V	Positive
28	Mastocytosis	56	F	BM	Fresh aspirate	pm/snp	D816V	Positive
29	Mast cell leukemia	64	M	BM	Fresh aspirate	pm/snp	D816V	Positive
30	SM-AML	46	M	BM	Paraffin	pm/snp	D816V	Positive
31	SM-AML	61	M	Blood	Fresh	pm/snp	D816V	Positive
32	Mastocytosis	61	M	Colon	Paraffin	WT	WT	Positive
33	Mastocytosis	56	M	Skin	Paraffin	WT	ND	Positive
34	Mastocytosis	62	M	BM	Formalin-fixed	WT	ND	Positive
35	Mastocytosis	40	F	BM	Fresh aspirate	WT	ND	Positive
36	Mastocytosis	46	F	BM	Paraffin	WT	WT	Positive
37	Mastocytosis	26	F	BM	Paraffin	WT	WT	Positive
38	Mastocytosis	64	M	BM	Fresh aspirate	WT	WT	Positive
39	Mastocytosis	55	M	BM	Fresh aspirate	pm/snp	SNP I798I	Positive
40	Mastocytosis	61	M	BM	Fresh aspirate	?pm/snp?	WT	Positive
41	Mastocytosis	78	F	Skin	Paraffin	WT	WT	Negative
42	Mastocytosis	35	M	Skin	Paraffin	WT	WT	Negative
43	Mastocytosis	31	F	Skin	Paraffin	WT	WT	Negative
44	Mastocytosis	24	F	Skin	Paraffin	WT	ND	Negative
45	Mastocytosis	71	F	Skin	Paraffin	WT	ND	Negative
46	Mastocytosis	30	F	Skin	Paraffin	WT	WT	Negative
47	Mastocytosis	52	F	Skin	Paraffin	WT	ND	Negative
48	Mastocytosis	44	F	Skin	Paraffin	WT	ND	Negative
49	Mastocytosis	38	F	Skin	Paraffin	WT	ND	Negative
50	Mastocytosis	40	M	Blood	Blood	WT	ND	Negative
51	Mastocytosis	60	F	BM	Paraffin	WT	ND	Negative
52	Mastocytosis	35	F	BM	Fresh aspirate	WT	ND	Negative
53	Mastocytosis?	52	F	BM	Fresh aspirate	WT	ND	Negative
54	Mastocytosis	18	M	BM	Fresh aspirate	WT	ND	Negative
55	Mastocytosis?	65	M	BM	Formalin-fixed	WT	ND	Negative
56	SM-MPD	71	F	BM	Paraffin	WT	ND	Negative
57	SM-MPD	73	M	BM	Paraffin	WT	ND	Negative
58	SM-MPD	57	F	BM	Fresh aspirate	WT	ND	Negative

Abbreviations: pm/snp, point mutation or single nucleotide polymorphism; I798I, polymorphism at codon 798; BM, bone marrow; LN, lymph node; WT, wild-type; ND, not done; SM-AML, acute myelogenous leukemia arising in systemic mastocytosis; SM-MPD, systemic mastocytosis with myeloproliferative disorder.

AS-PCR assay on this sample was clearly positive. The patient subsequently developed AML, and a blood sample (no. 31) was positive by both the standard assay (D816V on sequencing) and the AS-PCR assay.

Discussion

Denaturing HPLC combined with DNA sequencing is a useful approach in screening for *KIT* gene mutations in solid tumors such as GIST and seminoma.^{4,38,41} Unfortunately, this method is not sufficiently sensitive to detect mutations in biopsies from patients with suspected mast cell disease because the target mast cells often represent only a small fraction of the total cellularity. Clinical demand for *KIT* gene mutation screening in such samples led us to develop the AS-PCR assay described in this study. This assay provides reproducible detection of D816V with an estimated sensitivity of 1% mutant allele for DNA extracted from paraffin-embedded tissue. Based on the analysis of a Ba/F3 clone expressing a *KIT* D816F cDNA, this substitution is also detectable by the assay, but the sensitivity for this mutation could not be estab-

lished because of its rarity and a lack of clinical case material. Regardless, any positive result with the assay may reflect either D816V or D816F. Because both these substitutions occur in SM and confer relative resistance to imatinib, we believe that the assay has clinical utility.

Allele-specific PCR with unmodified, mutation-specific primers is commonly used to detect low abundance mutations in research studies. However, such primers may yield false-positive amplifications even under optimized conditions, a circumstance that cannot be afforded in testing clinical samples. As demonstrated by McKinzie and Parsons,³⁹ partially mismatched allele-specific primers may offer greater specificity. In our assay, no false-positive amplifications were observed among 64 samples of *KIT* wild-type DNA from nonmast cell diseases. Positive signals were detected only among samples of AML, seminoma, or mast cell disease, in which *KIT* exon 17 mutations are known to occur.

The sensitivity of allele-specific assays may be increased by suppressing the amplification of the corresponding wild-type allele. For example, a restriction endonuclease can be used to cleave wild-type sequence

before amplification, if an appropriate site is available. Alternatively, a blocking oligonucleotide can be used to prevent either priming or elongation of a target sequence. Dominguez and Kolodney⁴⁴ showed that a blocking oligonucleotide that included locked nucleic acids substantially suppressed the amplification of wild-type *BRAF* exon 15 in an assay using Stoffel-fragment polymerase. This approach may prove useful when several mutations of interest occur in a given exon, although it remains to be seen how widely adaptable it is. The assay developed by McKinzie and Parsons⁴⁴ uses a blocking oligonucleotide to suppress priming of the wild-type allele. In our hands, addition of this type of blocker increased the sensitivity of the allele-specific PCR by more than 10-fold.

One significant drawback to any AS-PCR assay is that the mutation-specific primer introduces the mutation of interest into the amplified product. Thus, it is not possible to confirm a positive result by direct DNA sequencing of the amplicon. This illustrates the challenge of validating a new, more sensitive assay when the mutation of interest is diluted by a high background of WT DNA and falls below the detection threshold for conventional sequencing. Another challenge is the empirical nature of the assay design process. For example, we attempted to engineer a *KIT* exon 17 AS-PCR assay with a mutation-specific primer to the DNA strand opposite the one used in the final assay, but the sensitivity proved to be 10-fold lower. Another issue that must be kept in mind is selection of a positive control for the amplification of individual samples. In this study, we amplified *KIT* exon 17 sequences in parallel tubes using standard primers, but we are currently working toward multiplex amplification of another *KIT* exon together with the D816V/F AS-PCR reaction.

A number of other methodologies have been used to hunt for *KIT* gene mutations in patients with mast cell disease. The most common is analysis of cDNA from peripheral blood mononuclear cells or fresh bone marrow biopsy material, which allows selective amplification of *KIT* sequences from cells that are actively expressing this gene. Worobec and colleagues³³ used this approach in analyzing peripheral blood mononuclear cells from 65 cases of SM, among which 16 (25%) were found to have D816V. Interestingly, the mutations were limited to cases of SM and SM-AHNMD; no pediatric cases or adults with urticaria pigmentosa (UP) alone had mutations. Longley and colleagues²⁰ also used a cDNA-based approach in analyzing peripheral blood mononuclear cells and found D816V in 11 of 11 adults with SM or persistent UP and in one case of pediatric SM. Additional cases of pediatric SM or persistent UP were associated with D816Y or D816F. More recently, Fritsche-Polanz and colleagues¹⁹ prepared cDNA from bone marrow mononuclear cells and compared the frequency of D816V mutations in patients with SM versus myelodysplastic syndrome. They reported a strikingly high sensitivity of 0.5% for the mutant allele by direct sequencing and 0.05% by restriction fragment length polymorphism with fluorescence detection. Optimized protocols for cDNA analysis clearly have advantages over genomic approaches, but such protocols may be of more limited value in the analysis of paraffin-embedded material from clinical biopsies.

Buttner and colleagues¹⁸ used a restriction fragment length polymorphism-based approach to analyze genomic DNA from paraffin-embedded skin punch biopsies of mast cell disease patients. They estimated the sensitivity for D816V at 3%. The mutation was observed in six of six cases of adult SM but in none of 11 children with localized or diffuse UP, which matches the results of Longley and colleagues.²⁰ The authors emphasized that only skin samples that were heavily infiltrated with mast cells were analyzed. In contrast, Yanagihori and colleagues²² claim that there is a high incidence of D816 mutations in skin biopsies from pediatric patients with UP (10 of 12 cases). Because these authors used only direct DNA sequencing of amplified genomic DNA, their results are quite surprising given the relatively low abundance of mast cells illustrated in their study.

Because the goal of our study was simply to validate a new allele-specific approach to D816 mutations, we tested all available archival material from patients in whom there was a clinical suspicion of mast cell disease. In this population, which was not otherwise defined, skin biopsies yielded a somewhat lower frequency of *KIT* D816 mutations (4 of 13 cases), than did bone marrow samples (13 of 21 cases). We made no attempt to enrich for the target mast cells in any of the tested material, so it remains unclear whether the skin biopsies simply had too few mast cells or whether the mast cells that were present did not reflect a systemic form of disease.

In summary, we have developed an allele-specific assay for D816V/F for use on samples of paraffin-embedded tissue. These mutations confer resistance to imatinib, but there are other potentially more potent kinase inhibitors that look promising *in vitro* and in clinical trials.^{40,45–47} The sensitivity of our assay may provide an additional measure of confidence in testing specimens with low mast cell content, and may be useful in the diagnosis and management of patients with mast cell disease. Of course, a negative result by our assay, or any other assay, does not exclude the possibility of a *KIT* exon 17 mutation.

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