# *KIT* Gene Mutations and Copy Number in Melanoma Subtypes

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**Abstract**

**Purpose:** We recently identified a *KIT* exon 11 mutation in an anorectal melanoma of a patient who had an excellent response to treatment with imatinib. To determine the frequency of *KIT* mutations across melanoma subtypes, we surveyed a large series of tumors.

**Experimental Design:** One hundred eighty-nine melanomas were screened for mutations in *KIT* exons 11, 13, and 17. *KIT* copy number was assessed by quantitative PCR. A subset of cases was evaluated for *BRAF* and *NRAS* mutations. Immunohistochemistry was done to assess KIT (CD117) expression.

**Results:** *KIT* mutations were detected in 23% (3 of 13) of acral melanomas, 15.6% (7 of 45) of mucosal melanomas, 7.7% (1 of 13) of conjunctival melanomas, 1.7% (1 of 58) of cutaneous melanomas, and 0% (0 of 60) of choroidal melanomas. Almost all the *KIT* mutations were of the type predicted to be imatinib sensitive. There was no overlap with *NRAS* mutations (11.1% of acral and 24.3% of mucosal tumors) or with *BRAF* mutations (absent in mucosal tumors). Increased *KIT* copy number was detected in 27.3% (3 of 11) of acral and 26.3% (10 of 38) of mucosal melanomas, but was less common among cutaneous (6.7%; 3 of 45), conjunctival (7.1%; 1 of 14), and choroidal melanomas (0 of 28). CD117 expression, present in 39% of 105 tumors representing all melanoma types, did not correlate with either *KIT* mutation status or *KIT* copy number.

**Conclusions:** Our findings confirm that *KIT* mutations are most common in acral and mucosal melanomas but do not necessarily correlate with *KIT* copy number or CD117 expression. Screening for *KIT* mutations may open up new treatment options for melanoma patients.

The incidence of malignant melanoma has risen dramatically over the past 50 years, yet current therapies for advanced disease provide only modest benefit (1). There has been recent progress in unraveling the molecular genetics of this cancer, with the emergence of several subtypes that are distinguished by their site of origin and tumor genotype (2). For example, Curtin and colleagues (3) observed that 81% of melanomas arising in skin without chronic sun-induced damage have an oncogenic *BRAF* or *NRAS* mutation, whereas such mutations are less common in chronic sun-induced damage melanomas, acral melanomas, and mucosal melanomas (1). In contrast, melanomas arising in chronic sun-induced damage skin and acral sites are more likely to have increased *CCND1* copy number; loss of *CDKN2A* or gain of *CDK4* are frequent alterations in mucosal and acral melanomas (4, 5).

Melanomas with oncogenic mutations in *KIT* have been observed in several recent studies (4, 6–9). Although such mutations seem to be more rare than *BRAF* and *NRAS* mutations, they may reflect the important role of KIT tyrosine kinase in melanocyte development (10). *KIT* mutations seem to be more common in mucosal and acral melanomas compared with cutaneous melanomas and are sometimes accompanied by an increase in *KIT* copy number (4, 6–9). Importantly, most of the reported mutations are of the type predicted to be sensitive to KIT kinase inhibitors. Success in the treatment of gastrointestinal stromal tumors (GIST) with imatinib (Gleevec; Novartis Pharma) has led to speculation that *KIT*-mutant melanoma might also be managed by this approach. Increases in *KIT* copy number have also been observed in melanoma (4). In some cases, there is an accompanying mutation, but in others, none is detected. Whether a KIT inhibitor would be effective against tumors that have amplified *KIT* but no detectable mutation is another question of great interest.

A phase II trial of imatinib for patients with unresectable acral or mucosal melanoma is ongoing at Dana-Farber Cancer Institute. Prior trials have shown that kinase inhibitors such as imatinib have little or no activity in unselected melanoma patients (11–14). Therefore, the current trial requires that a *KIT* gene mutation be documented in the tumor before a patient is treated. The first patient treated on this trial, a woman with a recurrent rectal melanoma containing a *KIT* exon 11 insertion/duplication, showed a partial response to 400 mg/d of

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## Translational Relevance

Recent studies have revealed the presence of *KIT* gene alterations in some cases of malignant melanoma. These alterations (activating mutations and/or increased copy number) may be particularly common in melanomas arising in acral and mucosal sites. Treatment options for these types of melanomas are limited, but oncogenic mutations of *KIT* are good targets for clinically available tyrosine kinase inhibitors. We recently observed a dramatic response to imatinib treatment in a patient with a *KIT*-mutant rectal melanoma. This led us to survey the type and frequency of *KIT* alterations in 189 melanomas representing all the major subtypes. We have confirmed that *KIT* mutations are more common among acral (23%) and mucosal (15.6%) melanomas than among cutaneous (1.7%) and conjunctival (7.7%) tumors. Increased *KIT* copy number was also more common in acral (27%) and mucosal (26%) melanomas than in the other subtypes. Interestingly, not all tumors with *KIT* gene alterations were immunopositive for CD117. Our findings suggest that routine screening of acral and mucosal melanomas for *KIT* alterations may open up new treatment avenues for patients with these tumors.

imatinib after just 4 weeks of therapy (15). A positron emission tomography scan confirmed marked reduction in tumor metabolic activity, consistent with KIT inhibition. The excellent response in this patient with a *KIT*-mutant melanoma suggests that mutation screening may be critical in identifying patients who may benefit from kinase targeted therapy.

As there are only limited data on *KIT* alterations across the major subtypes of malignant melanoma, we surveyed a large collection of cases and, for comparison, examined *BRAF* and *NRAS*, as well. Our results confirm that a significant fraction of acral and mucosal melanomas harbor *KIT* gene mutations and/or increased *KIT* copy number. We also observed differences between these two melanoma subtypes with respect to the frequency of detectable *BRAF* and *NRAS* mutations. Consistent with previous reports (3, 4, 16–22), we observed *NRAS* mutations in both of these subtypes, whereas *BRAF* mutations were detected in acral but not mucosal melanomas. In our series, mutations in *KIT*, *BRAF* and *NRAS* were mutually exclusive, indicating that each genotype may represent a distinct molecular disease subtype. This observation has clear implications for the use of targeted therapeutics in the future.

## Materials and Methods

***Melanoma samples.*** Blocks of formalin-fixed, paraffin-embedded melanoma tissue were obtained from the Pathology department archives of Oregon Health & Science University, the University of Washington, and Emory University, in accordance with Institutional Review Board–approved protocols at each institution. *BRAF* and *NRAS* mutation status was previously reported for 44 of the cutaneous melanomas and 62 of the choroidal melanomas, but these cases had not been analyzed for *KIT* (23).

All of the melanomas were from adult patients, who ranged from ages 22 to 86 years. Most of the tumors analyzed were primary lesions, but recurrences or metastases (lymph node, small bowel, liver) were studied in cases where the primary was unavailable. Acral and mucosal melanomas from patients enrolled in the 06-056 phase II trial at Dana-Farber Cancer Institute were included in the study. A *KIT* mutation in one of these cases has been detailed in a prior case report (15).

***DNA preparation.*** Tumor-rich areas (>80%) were scraped from 5-μm unstained sections by comparison with a H&E-stained slide, and genomic DNA was extracted using a Qiagen mini-kit (Qiagen, Inc.) in accordance with the manufacturer's directions. As melanin pigment can copurify with genomic DNA and inhibit PCR reactions, we incubated samples that had obvious melanin contamination, or failed to amplify on initial testing, with Chelex-100 (Bio-Rad). Specifically, purified DNA was incubated for 10 min at room temperature with an equal volume of 5% Chelex-100 suspension equilibrated in Qiagen AE buffer. The mixture was heated to 95°C for 2 min, allowed to cool, and then the Chelex-100 resin was pelleted in a microfuge and the supernatant DNA used for PCR reactions. Approximately 70% of DNA samples that initially failed to amplify were recovered by this approach.

***Mutation screening.*** Screening for mutations in *KIT* exons 8, 9, 11, 13, and 17, and *BRAF* exons 11 and 15 was done using a combination of PCR amplification and denaturing high performance liquid chromatography, as described previously (23, 24). All suspected mutations were confirmed by bidirectional sequencing on an ABI 3130 automated sequencer.

Mutations in *NRAS* exons 1 and 2 were identified by melting curve analysis of PCR amplicons, measuring fluorescence energy transfer between an anchor probe and a wild-type reporter probe that covered the codons of interest. PCR primers and probes were prepared by Integrated DNA Technologies.[9] Primers and probes for the exon 2 assay were exactly as described by Nikiforova et al. (25). Primers and probes for the exon 1 assay were as follows:

*NRAS* 1 forward: CACTAGGGTTTTCATTTCCATTG
*NRAS* 1 reverse: TCCTTTAATACAGAATATGGGTAAAGA
*NRAS* 1 anchor: TGAAATGACTGAGTACAAACTGGTGGTGGTT-Fluorescein
*NRAS* 1 reporter: LC640-AGCAGGTGGTGTTGGGA-C3 blocker

Twenty-five microliter reactions were carried out in a Roche LC480 Lightcycler (Roche) using Roche Probe Master mix and including 80 nmol/L of the forward primer, 800 nmol/L of reverse primer, and 800 nmol/L each of the anchor and reporter probes. The PCR conditions for both assays were as follows: 95°C for 10 min, followed by 50 cycles of 95°C for 20 s/60°C for 20 s/72°C for 20 s, then 95°C for 1 min and 45°C for 2 min. Melting curve analyses were done in the same instrument. Based on dilution analyses, the sensitivity of the *NRAS* exon 1 and exon 2 assays was ~15% mutant allele each. Melanoma samples showing aberrant peaks by melting curve analysis were sequenced bidirectionally to confirm the presence of a mutation.

***Reverse transcription-PCR Assay for KIT copy number.*** *KIT* gene copy number was assessed in quantitative real-time PCR reactions by comparison with coamplified *GAPDH*. All primers and probes were purchased from Integrated DNA Technologies. The *KIT* exon 17 primers were as follows: forward AAAGATTTGTGATTTTGGTCTAGC, and reverse GAAACTAAAAATCCTTTGCA. The *KIT* exon 17 dual-labeled hydrolysis probe was FAM-TGTGGTTAAAGGAAACGTGAGTA-IABlkFQ. The glyceraldehyde-3-phosphate dehydrogenase (GAPD) primers were as follows: forward CACTAGGCGCTCACTGTTCT, and reverse GCGAACTCACCCGTTG. The GAPDH hydrolysis probe was TxRed-TGGGGAAGGTGAAGGTCGGA-IAbRQSp. The *KIT* exon 7 primers were as follows: forward AGGTAGAAACTGAAAAAGACATGC, and reverse GCCTTTTAATCTCGTTAGATGAAGT. The *KIT* exon 7 dual-labeled hydrolysis probe was FAM-AGGCATGCTATCCACAGGTGATTG-IABlkFQ.

PCR reactions were done in duplicate in a Roche LC480 Lightcycler, with a 20 μL total volume, 50 ng genomic DNA, 200 nmol/L primers

[9] http://www.idtdna.com

and probes, and 1× Probes Master Mix (Roche). The PCR conditions were 1 cycle of 95°C for 10 min, and 50 cycles of 95°C for 10 s, and 60°C for 20 s. The second derivative maximum algorithm included with the instrument software was used to determine cycle threshold (Ct) values for each target gene.

Relative copy numbers were calculated using the ΔΔCt method, where Ct is the threshold cycle for amplification. For each sample, ΔCt for *KIT* versus *GAPDH* was calculated as ΔCt = Ct(KIT) - Ct(GAPDH). The ΔCt value for each experimental test sample was calibrated to a reference pool of genomic DNA prepared from 9 samples of formalin-fixed, paraffin-embedded normal tissue that included colon, kidney, liver, prostate, and spleen (all archived 4 y or less), using the formula ΔΔCt = ΔCt(test sample) - ΔCt(reference pool). Relative DNA copy number was calculated using the formula $2^{-\Delta\Delta Ct}$. Relative copy numbers were converted to absolute copy numbers by assigning a value of 2 (diploid) to the reference pool and multiplying the relative copy number of test samples by a factor of 2.

Pilot reactions established that *KIT* Cts between 26 and 34 cycles were in the linear range of amplification. Samples that did not amplify by 34 cycles were deemed to be of insufficient quality. Increased *KIT* copy number was determined by comparison to nine individual formalin-fixed, paraffin-embedded normal tissue samples. The mean and SDs of the 9 samples, based on quadruplicate measurements, was 2.25 ± 0.68 *KIT* copies relative to *GAPDH*. The threshold for increased *KIT* copy number was set relative to these normal samples using the 95% confidence level according to Chebychev's inequality, with the formula mean ± (4.47 × SD). This threshold was 5.29 copies of *KIT* relative to *GAPDH*.

***Fluorescence* in situ *hybridization for KIT.*** To validate the results of the reverse transcription-PCR for *KIT* copy number, fluorescence *in situ* hybridization (FISH) was done on 5-μm sections of paraffin-embedded melanoma tissue using a cocktail of three probes to chromosome 4 (Vysis LSI 4q12 tricolor; Abbott Laboratories). These probes cover the genes *SCFD2* (Spectrum green), *LNX* (Spectrum orange), and *KIT* (Spectrum aqua; also includes a portion of the neighboring gene *PDGFRA*). The protocol was as previously described (26). Briefly, slides were baked overnight at 60°C, deparaffinized, and then dehydrated. Pretreatment washes included distilled water (10 mi), 2× SSC (3 min), and 10 mmol/L citric acid buffer (pH 6.0; 80°C, 45 min). Slides were protease treated at 37°C for 10 min, dehydrated, and air dried. After addition of the probe, the slides were denatured for 5 min at 73°C and hybridized for 14 to 18 h at 37°C. After washing, the slides were then counterstained with 4′,6-diamidino-2-phenylindole and visualized on a fluorescence microscope.

***CD117 immunohistochemistry.*** Deparaffinized 5-μm sections of tumor were heated in Dako Target Retrieval Solution (pH 6.0; Dako USA) for 10 min in a Russell Hobbs pressure cooker (model RHNPC800P) and allowed to cool. Immunostaining for CD117 (KIT) was done using the Dako polyclonal rabbit antibody (Dako A4502) at 1:400 dilution, followed by a standard avidin-biotin detection protocol using diaminobenzidine. Hematoxylin-counterstained slides were cover-slipped and examined for the intensity of staining (0, 1+, or 2+).

## Results

***KIT mutations.*** Among 189 melanoma cases that were fully screened for mutations in *KIT* exons 11, 13, and 17, the frequency of *KIT* mutations was 23% for acral tumors, 15.6% for mucosal tumors, 7.7% for conjunctival tumors, 1.7% for cutaneous tumors, and 0% for choroidal tumors (Table 1). Most of the 45 mucosal tumors were sinonasal ($n$ = 29), but 7 arose in the oral cavity, 7 in the anorectal area, and 2 in the vulva/vagina. Interestingly, the mutation frequency was higher among tumors of the anorectum/vulva/vagina (4 of 9; 44.4%) than among tumors arising in the head and neck (3 of 36; 8.3%; Table 1). However, more cases will need to be collected and analyzed to see if this trend is significant.

Point mutations were the most common, resulting in substitutions in exon 11 (W557R, K558N, V559A, V559D, L576P—4 cases) or exon 17 (Y823D; Table 2). However, one acral melanoma had an in-frame exon 11 deletion (EVQWKV 554-559), and we have previously observed an exon 11 insertion/duplication in a rectal melanoma (15). All of the *KIT* mutations identified in our cases also occur in GISTs. As *KIT* exon 9 is another site of mutation in GISTs (~10% of tumors), we screened 148 melanomas, including 6 acral and 27 mucosal tumors, for mutations in this exon, but none were found. We also examined *KIT* exon 8, in which mutations occur in acute myelogenous leukemias but did not observe any in 99 melanomas, including 3 acral and 25 mucosal melanomas.

***KIT copy number.*** *KIT* gene copy number (chromosome 4) was assessed by quantitative real-time PCR for exon 17 sequence and compared with coamplified *GAPDH* (chromosome 12) as an internal control. In pilot assays, comparable results were obtained using β-actin (*ACTB*, chromosome 7) as the internal control (data not shown); however, *GAPDH* was selected over *ACTB* because this chromosome is commonly overrepresented in melanoma (3, 27, 28). Differences in the amplification thresholds (Ct) of *KIT* and *GAPDH* were compared with those in a reference pool of normal genomic DNA prepared from 9 samples of formalin-fixed, paraffin-embedded benign tissue. The assay was validated by several approaches. (*a*) Substitution of a *KIT* exon 7 amplicon for exon 17 in the assay had no significant effect (data not shown). (*b*) A *KIT*-mutant melanoma cell line known to have increased copies of *KIT* by array CGH showed elevated copy number with the PCR assay.[10] (*c*) FISH for *KIT* was done on five of the melanomas in the study. FISH signals versus reverse transcription-PCR copy number for the five cases were as follows: 2 versus 2.6; 3 versus 5.5; 5 to 7 versus 5.0; 7 to 8 versus 7.8; and "too numerous to count" versus 28.8. Figure 1 illustrates the FISH results for two of the cases.

Increased *KIT* copy number was identified by reverse transcription-PCR in over one quarter of the acral and mucosal melanomas (Table 3). In 2 of 3 acral tumors and 7 of 10 mucosal tumors, no accompanying *KIT* mutation was detected. Extra copies of *KIT* were much less common among cutaneous and conjunctival tumors and were not observed in any of the choroidal melanomas tested.

***BRAF mutations.*** We previously reported *BRAF* mutations in 36.4% (16 of 44) of cutaneous melanomas and 0% (0 of 62) uveal melanomas (23). In this study, *BRAF* mutations were identified in 26.7% of conjunctival melanomas and 16.7% of acral melanomas but not in any of the mucosal melanomas (Table 1). All were the common V600E substitution in exon 15, with the exception of a single conjunctival tumor with V600R. In contrast, there were no *BRAF* exon 15 mutations among 47 mucosal melanomas, regardless of site. Twenty-six mucosal cases were additionally screened for mutations in *BRAF* exon 11 and none were found.

***NRAS mutations.*** In a previous study, we observed that mutations in *NRAS* exons 1 and 2 were uncommon in cutaneous melanomas (3.7%; 1 of 27) and absent in choroidal

[10] F.S. Hodi, manuscript submitted.

**Table 1.** Summary of mutations in melanoma subtypes

| Gene | Acral | | Mucosal | |
|---|---|---|---|---|
| | This study | Other studies | This study | Other studies |
| *KIT* | 23% (3/13) | 11% (3/28; ref. 4) | 15.6% (7/45) | 15% (3/15; ref. 6)<br>21% (8/38; ref. 4)<br>22.2% (4/18; ref. 30) |
| *BRAF* | 16.7% (2/12) | 9.5% (2/21; ref. 42)<br>15% (2/13; ref. 18)<br>15.4% (2/39; ref. 19)<br>21% (6/28; ref. 4)<br>23% (7/30; ref. 3)<br>33% (5/15; ref. 20) | 0% (0/45) | 0% (0/13; ref. 17)<br>3% (1/38; ref. 4)<br>5.6% (2/36; ref. 22)<br>5.9% (1/17; ref. 16)<br>9.5% (2/21; ref. 19)<br>11% (2/19; ref. 3) |
| *NRAS* | 11.1% (1/9) | 3.6% (1/28; ref. 21)<br>10% (3/30; ref. 3)<br>47.4% (9/19; ref. 43) | 24.3% (9/37) | 5% (1/19; ref. 3)<br>13.9% (5/36; ref. 22) |

*Melanomas arising in chronically sun-damaged skin.
† Preselected for CD117 positivity; sites not specified.

melanomas (0 of 47; ref. 23). Our expanded series of 60 cutaneous melanomas yielded a slightly higher frequency of 11.7% (Table 1). However, *NRAS* mutations were considerably more common (24.7%) among 37 mucosal melanomas analyzed. As expected from the literature, the affected codons were 12 (G12C, G12D, G12V), 13 (G13R), and 61 (Q61K, Q61L, Q61R). One interesting trend was that exon 1 mutations dominated among the mucosal melanomas (8 of 9 cases), whereas exon 2 mutations were more common in the cutaneous tumors (6 of 7 cases). There was one *NRAS* mutation among 9 acral tumors analyzed, and none in 11 conjunctival melanomas.

***Correlation of KIT alterations and CD117 expression.*** Immunohistochemistry for CD117 (KIT) was done on subsets of the melanoma subtypes, including examples with *KIT*, *BRAF*, or *NRAS* mutation, and cases with increased *KIT* copy number. Staining was graded as negative (0), weak/moderate (1+), or strong (2+). The results, tabulated in Table 4, showed no apparent correlation between CD117 and *KIT* mutation status or increased *KIT* copy number. Occasional tumors with a *BRAF* or *NRAS* mutation had readily detectable CD117. Strong expression was also observed in many of the wild-type tumors (21.4%), particularly among the choroidal melanomas (35.7%). Thus, CD117 expression is not a reliable indicator of *KIT* genotype.

## Discussion

Went and colleagues (7) were the first to discover an activating *KIT* mutation (L576P) in a case of melanoma—one of two CD117-positive tumors that they sequenced. Willmore-Payne et al. (9, 29) followed up on this observation in two studies using high-resolution melting curve analysis to screen melanomas for mutations in *KIT* exons 9, 11, 13, and 17. There were 153 melanomas (nonmucosal but otherwise of unspecified origin) described in the two studies; however, mutation screening was actually limited to 35 cases that were CD117-positive. Among these, there were three cases with an L576P substitution. In 2006, Curtin and coworkers (4) surveyed 95 melanomas of various types and found that *KIT* mutations were

**Table 2.** Summary of *KIT* mutations in melanoma

| Exon | Mutation | This study | Other studies | Total | Imatinib sensitive? | Refs |
|---|---|---|---|---|---|---|
| 11 | Del 554-559 | 1 | | 1 | Yes | |
| | Y553N | | 1 | 1 | ? | (4) |
| | W557R | 1 | 1 | 2 | Yes | (30) |
| | K558N | 1 | | 1 | Yes | |
| | V559A | 1 | 2 | 3 | Yes | (4) |
| | V559D | 1 | | 1 | Yes | |
| | N566D | | 1 (with K642E) | 1 | ? | (4) |
| | V569G | | 1 | 1 | ? | (30) |
| | L576P | 5 | 7 | 12 | Yes | (4, 7, 9, 29) |
| | Ins at 583 | | 1 | 1 | Yes | (15) |
| 13 | R634W | | 1 | 1 | ? | |
| | K642E | | 9 (1 with N566D) | 9 | Yes | (4, 6, 30) |
| 17 | D816H | | 2 | 2 | No | |
| | Y823D | 1 | | 1 | No | |
| 18 | A829P | | 1 | 1 | No | (4) |
| | All Mutations | 11 | 27 | 38 | | |

**Table 1.** Summary of mutations in melanoma subtypes (cont'd)

| Conjunctival | | Cutaneous | | Choroidal | |
|---|---|---|---|---|---|
| This study | Other studies | This study | Other studies | This study | Other studies |
| 7.7% (1/13) | No reports | 1.7% (1/58) | 0% (0/50; ref. 31)<br>16.7% (3/18; ref. 4)*<br>16.7% (1/6; ref. 29)†<br>6.9% (2/29; ref. 9)† | 0% (0/60) | 0% (0/10; ref. 41) |
| 26.7% (4/15) | 14.3% (3/21; ref. 43)<br>22.7% (5/22; ref. 44)<br>40% (2/5; ref. 45) | | | | |
| 0% (0/11) | No reports | | | | |

most common among the acral 11% (3 of 28) and mucosal 21% (8 of 38) tumors. Subsequent studies by Antonescu et al. (6) and Rivera et al. (30) have confirmed that *KIT* mutations are more common among rectal and head and neck melanomas, respectively (Table 1). In this study, we used denaturing high performance liquid chromatography to screen a total of 189 melanomas from all sites for mutations in three exons of *KIT* previously reported to harbor mutations (exons 11, 13, and 17). Included were 45 mucosal melanomas (36 head and neck and 9 anorectal/vulvar/vaginal tumors). Our results, summarized in Table 1, build substantially on the earlier reports. Combining our data with the previous studies, *KIT* mutations are present in 14.6% of acral, 19% of mucosal, 4.3% of cutaneous, and 7.7% of conjunctival melanomas, and are absent from choroidal melanomas. A subset of our tumors was also screened for mutations in *KIT* exon 8 and/or exon 9,

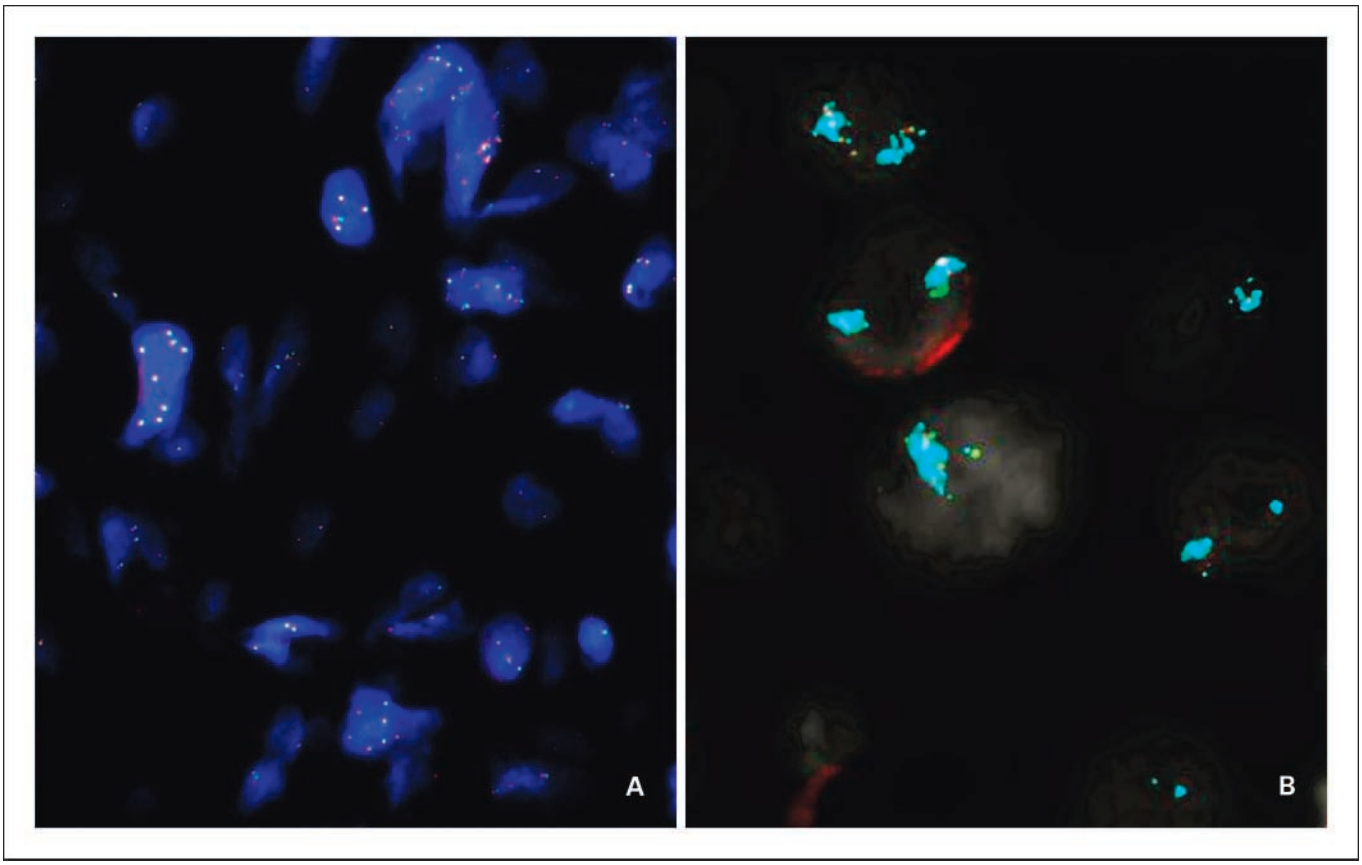


**Fig. 1.** FISH for *KIT. A,* acral melanoma with *KIT* V559D showing increased FISH signals (fused) for probes to 4q12. Signals ranged from 6 to 8 per nucleus; the copy number was 7.8 by reverse transcription-PCR. *B,* a sinonasal melanoma that was wild-type for *KIT* showed a large increase in *KIT* signals, but not for the nearby loci *LNX* (*orange signal*) and *SCFD2* (*green signal*), suggesting a localized amplification. In one of the nuclei, only one *KIT* allele showed amplification, whereas a neighboring nucleus displayed two clusters of *KIT* signals. By reverse transcription-PCR this case had the highest *KIT* copy number (28.8) in the study.

**Table 3.** Summary of *KIT* gene copy number

| | % increased *KIT* number | Increased *KIT* number with *KIT* mutation | Increased *KIT* number without *KIT* mutation |
|---|---|---|---|
| Acral | 27.3% (3/11) | 1 | 2 |
| Mucosal | 26.3% (10/38) | 3 | 7 |
| Cutaneous | 6.7% (3/45) | 1 | 2 |
| Conjunctival | 7.1% (1/14) | 0 | 1 |
| Choroidal | 0% (0/28) | 0 | 0 |

but no mutations were found. Of course, it remains possible that there are mutations in other exons of the *KIT* gene.

We observed that *BRAF* mutations were relatively more common than *KIT* mutations in conjunctival tumors, although the number of tumors available for study was limited. The frequency of *BRAF* mutations that we observed is similar to other studies and parallels the well-established dominance of *BRAF* mutations in cutaneous melanomas (1–3). Among the cutaneous melanomas in our series, there was only one *KIT* mutation. Woenckhaus et al. (31) reported none among 50 cutaneous tumors. On the other hand, Curtin et al. (4) observed *KIT* mutations at a frequency of 16.7% among melanomas arising in chronically sun-damaged skin, in which *BRAF* mutations are comparatively uncommon (3). Although the relationship between sun damage and the likelihood of a *KIT* mutation in cutaneous melanomas deserves further investigation, the cutaneous tumors in our study were not selected on this basis. Rather, they are simply representative of cases referred to a tertiary care center, including patients with advanced disease.

Targeting KIT is a proven strategy in the management of advanced GIST (32–34). Several observations suggest that KIT kinase inhibitors might also serve to control the growth of selected melanomas. First, KIT kinase activity is necessary for the development of melanocytes (10), just as it is for the development of the interstitial cells of Cajal, to which GISTs are thought to be related. As in GIST, oncogenic activation of KIT may serve as an early signal for neoplastic growth. Second, Antonescu and colleagues (6) observed KIT phosphorylation on an immunoblot of a rectal melanoma extract. Third, the great majority of the *KIT* mutations observed in melanoma are of the type known to be sensitive to imatinib and other KIT inhibitors (Table 3). For example, Antonescu et al. (6) showed that the V559D and L576P mutant forms of KIT are inhibited both by imatinib and dasatinib when expressed in Ba/F3 cells. Fourth, a melanoma cell line harboring a *KIT* exon 11 mutation was highly sensitive to growth inhibition by imatinib, whereas nonmutant cell lines were not.[11] Finally, we recently reported the dramatic response to imatinib therapy of a mucosal melanoma patient with a *KIT* exon 11 insertion/duplication (15). Of note, in the current study, we found that the tumor in that case also had an increased *KIT* copy number (12.6).

Imatinib has been essentially ineffective in trials of unselected melanoma patients (11, 13, 14, 35). Likewise, another KIT inhibitor, PKC412, showed little activity in melanoma (12). However, Becker et al. (36) observed in a recent editorial that their imatinib trial was underpowered to detect activity in the subtypes of melanomas most likely to harbor a *KIT* mutation. It is also notable that in the imatinib trial reported by Eton et al. (35), there was an acral melanoma patient who had a nearly complete response. As noted above, the statistical chance of a *KIT* mutation in this patient's tumor was 14.6%. Thus, the responding patient that we have reported may not be unique and routine screening of *KIT* may become important for patients with advanced melanoma.

As *KIT* mutations are much less common in melanoma than in GIST, the question arises as to what would be the most effective screening approach. After the logic that KIT should be highly expressed in *KIT*-mutant tumors, Willmore-Payne and colleagues (9, 29) focused their screening only on CD117-positive cases. Correspondingly, Antonescu et al. (6) observed that all three of their *KIT*-mutant melanomas were 4+ positive for CD117, whereas nearly all of the other tumors in their series were either 1+ or negative. There is, however, evidence from the GIST literature that CD117 expression and *KIT* genotype do not always correlate. For example, *KIT* mutations are detected in 16% of CD117-negative GISTs (37), yet such tumors are still responsive to imatinib. Chirieac and coworkers (38) confirmed that there is no relationship between CD117 expression and imatinib response among GIST tumors. It is perhaps not surprising then that several of our *KIT*-mutant melanomas lacked detectable expression of CD117 (Table 4). Using the identical antibody, the same was true of 1 of 4 *KIT*-mutant melanomas studied by Rivera et al. (30), and of several *KIT*-mutant melanomas examined by Curtin and colleagues (4). The latter group indicated that most of their cases became CD117 "positive" when a 10-fold higher concentration of the antibody was used, but false positive staining at such high concentrations of this antibody is well-documented in the literature (7, 39). Like Rivera et al. and Curtin et al., we observed strong CD117 staining in some melanomas that were *KIT* wild-type (Table 4). We also observed it in one case with

**Table 4.** Genotype versus CD117 expression in nonchoroidal melanomas

| Genotype | CD117 staining | | | |
|---|---|---|---|---|
| | 0 | 1+ | 2+ | Total |
| *KIT* mutation | 3 | 1 | 2 | 6 |
| Increased *KIT* copy number | 6 | 2 | 1 | 9 |
| Normal *KIT* copy number | 40 | 16 | 14 | 70 |
| *BRAF* mutation | 10 | 2 | 1 | 13 |
| *NRAS* mutation | 5 | 2 | 0 | 7 |
| No mutation: | | | | |
| Nonchoroidal | 18 | 4 | 6 | 28 |
| Choroidal | 18 | 9 | 15 | 42 |

[11] F.S. Hodi et al., manuscript submitted.

a *BRAF* mutation. We conclude that CD117 immunohistochemistry is not a reliable indicator of *KIT* genotype, and that restricting mutation screening to CD117-positive melanomas would risk missing mutant cases.

In analyzing three *KIT*-mutant melanomas, Willmore-Payne et al. (9) found that one tumor had a modest increase in *KIT* copy number, as assessed by FISH (2.88-fold). The remaining tumors were homozygous mutants based on comparison of FISH and DNA sequence. Similarly, Antonescu and coworkers (6) observed increased *KIT* copy number (2.5-fold) in 1 of 2 mutant cases examined by FISH. Selective overrepresentation of chromosome 4q12, which includes *KIT*, was measured by array CGH in a subset of cutaneous melanomas evaluated by Bastian et al. (27), and in acral, mucosal, and cutaneous melanomas (from chronically sun-damaged skin) studied by Curtin et al. (4). To simplify the assessment of *KIT* copy number, we developed a quantitative real-time PCR assay for use on formalin-fixed, paraffin-derived tumor DNA. This assay has the advantage that it can be done on the same material used to screen for *KIT* mutations; however, it may be less sensitive than other approaches because the copy number of the reference gene (*GAPDH*) may itself vary in some cases. With this in mind, we identified a number of melanomas with increases in *KIT*, in one case up to 28.8 copies. Included were cases with *KIT* mutations, but most were wild-type tumors. It is intriguing that the mucosal melanoma in the patient that we previously reported as responding dramatically to imatinib therapy had both a *KIT* mutation and increased *KIT* copy number. The usefulness of our assay will be examined in ongoing trials of KIT kinase inhibitors (imatinib or sunitinib) for patients with acral or mucosal melanoma.

Although the significance of increased *KIT* copy number with regard to the treatment of melanoma remains to be determined, the great majority of *KIT* mutations that have been identified in this cancer are potentially sensitive to imatinib and other KIT inhibitors. Table 2 summarizes all of the mutations reported to date, including the current study. The two most common mutations (L576P and K642E) together account for 55% of the cases; both are inhibited by imatinib *in vitro* and predict for good imatinib response in GIST (32–34). The only mutations likely to be insensitive to available inhibitors are those affecting the activation loop encoded by exons 17 and 18. Fortunately, only 4 of 38 cases (10.5%) reported thus far had a mutation in this domain (Table 2). An alternative treatment strategy for such cases might be a MEK1 inhibitor. *BRAF*-mutant melanoma cell lines are highly sensitive to MEK1 inhibition (40) but some *RAS*-mutant and *FGFR1*-mutant melanoma cell lines show similar sensitivities (28). Molecular correlation studies in the ongoing trials with BRAF and MEK1 inhibitors will prove very interesting in this regard.

In summary, we have conducted the largest survey to date of *KIT* genotypes across various melanoma subtypes. Our findings confirm that acral and mucosal melanomas are the subtypes most likely to harbor *KIT* mutations and/or increased *KIT* copy number. However, our results also indicate that this oncogene may play a role in occasional cases of conjunctival and cutaneous melanoma. The mutations do not overlap with mutations in *BRAF* or *NRAS*, but they are sometimes associated with increased *KIT* copy number. We also observed that *NRAS* mutations are even more common in mucosal melanomas than *KIT* mutations, and this should be borne in mind in future efforts to match patient tumors with targeted therapeutics.

## Disclosure of Potential Conflicts of Interest

C. Corless has received a commercial research grant and is a member of the speakers' bureau of Novartis and Pfizer. The other authors disclosed no potential conflicts of interest.

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