Clinical Cancer Research

KIT Pathway Alterations in Mucosal Melanomas of the Vulva and Other Sites

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Abstract

Purpose: A significant proportion of mucosal melanomas contain alterations in *KIT*. The aim of this study was to characterize the pattern of *KIT*, *NRAS*, and *BRAF* mutations in mucosal melanomas at specific sites and to assess activation of the KIT downstream RAF/MEK/extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K)/AKT pathways in mucosal melanoma specimens.

Experimental Design: Seventy-one primary mucosal melanomas from various sites were studied. Mutation analysis was done by DNA sequencing. Expression of KIT, phosphorylated (p)-ERK, and p-AKT was evaluated by immunohistochemistry.

Results: *KIT* mutations were detected in 35% (8 of 23) of vulvar, 9% (2 of 22) of anorectal, 7% (1 of 14) of nasal cavity, and 20% (1 of 5) of penile melanomas. No *KIT* mutations were found in 7 vaginal melanomas. The difference in *KIT* mutation frequency between vulvar and nonvulvar cases was statistically significant ($P = 0.014$). The overall frequencies of *NRAS* and *BRAF* mutations were 10% and 6%, respectively. Notably, vaginal melanomas showed a *NRAS* mutation rate of 43%. *KIT* gene amplification ($\geq$4 copies), as assessed by quantitative real-time PCR, was observed in 19% of cases. KIT expression was associated with *KIT* mutation status ($P < 0.001$) and was more common in vulvar than nonvulvar tumors ($P = 0.016$). Expression of p-ERK and p-AKT was observed in 42% and 59% of tumors, respectively, and occurred irrespective of *KIT*/*NRAS*/*BRAF* mutation status. *NRAS* mutation was associated with worse overall survival in univariate analysis.

Conclusions: Results show that *KIT* mutations are more common in vulvar melanomas than other types of mucosal melanomas and that both the RAF/MEK/ERK and PI3K/AKT pathways are activated in mucosal melanoma specimens. *Clin Cancer Res; 17(12); 3933–42. ©2011 AACR.*

Introduction

Mucosal melanoma is a very rare type of tumor accounting for less than 2% of all melanomas in humans (1). The most common site of origin for 1,089 cases of mucosal melanoma reported to the Swedish National Cancer Registry between 1960 and 2004 was the vulva (37.1%), followed by the anorectal tract (26.2%), and nasal cavity (17.7%; ref. 2). Other sites of occurrence included the oral cavity (6.5%), vagina (7.4%), penis (3.3%), and urethra (1.8%). Patients diagnosed with mucosal melanoma have a poor prognosis, with 5-year survival rates of only 18% and 35% to 61% for anorectal and vulvar melanoma, respectively (3–5).

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Recently, mutations and/or increased copy numbers of the gene encoding the receptor tyrosine kinase KIT have been described in up to 40% of mucosal melanomas (6–8). This makes *KIT* the most frequently altered oncogene identified so far in mucosal melanomas. Activating mutations in the *NRAS* and *BRAF* oncogenes, which characterize cutaneous melanomas arising on sun-exposed body sites, are rare in mucosal melanomas (9–12). The finding of *KIT* mutations in mucosal melanoma has opened the door for targeted therapy as a treatment option for this subtype of melanoma. Indeed, an increasing number of case studies have shown clinical responses of *KIT*-mutated mucosal melanomas to tyrosine kinase inhibitors such as imatinib (13–16), sorafenib (16, 17), and dasatinib (18). Preliminary data from an ongoing phase II study also show promising results, with varying degrees of response to imatinib in *KIT*-mutated melanomas (19).

As a receptor tyrosine kinase, KIT activates multiple downstream signaling cascades, including the RAF/MEK/extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K)/AKT pathways. These pathways are key regulators of cellular processes such as proliferation, apoptosis, and survival. In cutaneous melanoma, the involvement of both ERK and AKT signaling is well established (20, 21), and expression of activated AKT in primary

Translational Relevance

Mucosal melanomas are rare tumors with an aggressive clinical behavior. A significant proportion of mucosal melanomas contain mutations and/or increased copy numbers of the *KIT* gene. Of clinical importance, patients with *KIT*-mutated melanomas have been reported to respond to KIT-directed therapies. In this study, we found that the frequency of *KIT* mutations is significantly higher in vulvar melanomas than other types of mucosal melanomas (35% vs. 10%). The high frequency of *KIT* mutations in vulvar melanomas suggests that a large proportion of patients with melanomas of the vulva are likely to benefit from therapies directed against activated KIT. We also found that the RAF/MEK/ERK and PI3K/AKT signaling pathways are frequently activated in clinical specimens of mucosal melanomas. These pathways may represent promising alternative therapeutic targets in mucosal melanoma, especially in the subset of tumors lacking activating *KIT* mutations.

cutaneous melanomas has been associated with worse patient survival (22). In contrast to the plethora of data on these pathways in cutaneous melanomas, little is known about the role of ERK and AKT signaling in mucosal melanomas. For example, no study has so far examined the activation status of ERK and AKT in mucosal melanoma tissue specimens.

The purpose of this study was to better characterize the pattern of *KIT*, *NRAS*, and *BRAF* mutations in mucosal melanomas and to study signaling downstream of KIT. We specifically focused on the RAF/MEK/ERK and PI3K/AKT pathways and used immunohistochemistry (IHC) to assess tumor lesions for expression of activated ERK and AKT. A large series of primary mucosal melanomas from various anatomical sites was studied. Mutation data and IHC results were related to various clinicopathological variables and patient survival.

Materials and Methods

Tumor samples

Formalin-fixed paraffin-embedded mucosal melanomas from a total of 90 patients were used for this study. Patients were diagnosed between 1982 and 2008 at hospitals throughout Sweden and were reported to the Swedish National Cancer Registry. The tumors consisted of primary melanomas from 5 different mucosal sites: vulva, vagina, anorectum, nasal cavity, and penis. No metastatic lesions were studied. The 90 tumors were part of a larger series of 223 tumors from which paraffin blocks were available. Histological slides from all tumors were reviewed. Many of the lesions were small, sometimes consisting only of punch biopsies of the tumors, and had been used for establishing the primary diagnosis. The 90 samples used for the study represented cases that contained sufficient tumor tissue for both mutation analysis and IHC. A total of 19 tumors (21%) contained no amplifiable DNA and were excluded from the study. Survival times and outcome data were available for all patients. End point of follow-up was September 1, 2009. The study was approved by the Ethics Review Board, Karolinska Institutet, Solna, Stockholm, Sweden.

DNA extraction

Sections of 5-μm thickness were cut from paraffin blocks and placed on plain slides. Sections were deparaffinized and stained with hematoxylin; then tumor cells were isolated by laser capture microdissection (LCM) using the Arcturus Pix Cell II LCM system (Arcturus Molecular Devices). In some instances, tumor cells were isolated by manual dissection. DNA was extracted from the dissected cells by using the Pico Pure DNA Extraction Kit (Arcturus) according to the manufacturer's instructions.

Mutation analysis

Screening for mutations in *KIT* (exons 9, 11, 13, 17, and 18), *NRAS* (exons 1 and 2), and *BRAF* (exon 15) was carried out by PCR and DNA sequencing. Genomic DNA (5 μL LCM extract) was amplified in 10 μL reaction volumes containing 1×PCR buffer, 2.5 mmol/L $MgCl_2$, 200 μmol/L of each deoxynucleotide triphosphate, 500 nmol/L of each primer, 0.2 μg/μL bovine serum albumin (New England BioLabs), and 0.05 units of Platinum *Taq* DNA Polymerase (Invitrogen). The KIT primers used were as follows: *KIT* exon 9 forward 5′-CCCAAGTGTTTTATGTATTTA-3′ and reverse 5′-AGACAGAGCCTAAACATCC-3′; *KIT* exon 11 forward 5′-GATCTATTTTTCCCTTTCTC-3′ and reverse 5′-TTATGTGTACCCAAAAAGG-3′; *KIT* exon 13 forward 5′-GCGTAAGTTCCTGTATGGTA-3′ and reverse 5′-AACCTGACAGACAATAAAAG-3′; *KIT* exon 17 forward 5′-TGATTTTTATTTTTGGTGTACTGA-3′ and reverse 5′-ACTGTCAAGCAGAGAATGGGT-3′; and KIT exon 18 forward 5′-CATTATTGACTCTGTTGTGC-3′ and reverse 5′-GCAGGACACCAATGAAACTT-3′. The *NRAS* and *BRAF* primers were as previously described (23). The PCR conditions were 95°C for 3 minutes, 35 cycles of 94°C for 30 seconds, 54°C to 58°C for 30 seconds, and 72°C for 30 seconds followed by an extension step of 72°C for 10 minutes. A total of 2 μL of the PCR product were amplified in a second PCR consisting of 20 cycles. PCR products were separated in 2% agarose gels, excised, and purified using the QIAquick Gel Extraction Kit (Qiagen). Bidirectional sequencing was carried out using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) on a 3130*xl* Genetic Analyzer (Applied Biosystems). Nucleotide changes were reported as mutations only if observed in 2 independent PCR sequencing reactions.

Quantitative real-time PCR

KIT gene (Chr 4q11-q12) copy numbers were assessed by quantitative real-time PCR using the ABI 7500 instrument

(Applied Biosystems). *Aldolase B* (Chr 9q21-q22) was used as a reference gene. The primer sequences for *KIT* were as follows: 5′-CGCAGCATGTAGGCAGAAAG-3′ (forward) and 5′-CAGAGGGAAAGGACAGATGGA-3′ (reverse). The *KIT* probe (FAM-labeled) was 5′-TCTGTAAGCATGAAGGACGGCTCCC-3′. Primer and probe sequences for *aldolase B* were previously described (24). Amplification was performed in a 25-μL reaction volume containing 5 μL LCM extract, 300 nmol/L of each primer, 150 nmol/L probe, and 1× Platinum Quantitative PCR SuperMix-UDG (Invitrogen). The PCR conditions were 1 cycle of 50°C for 2 minutes followed by 1 cycle of 95°C for 2 minutes, and 50 cycles of 95°C for 15 seconds and 60°C for 60 seconds. All samples were analyzed in duplicate. Relative copy numbers were calculated by the ΔΔCt method. Human genomic control DNA CEPH 1347-02 (Applied Biosystems) was used as a calibrator.

IHC

p-ERK and p-AKT. IHC was done on paraffin sections of 4 μm thickness by using rabbit monoclonal anti-phospho-p44/42 mitogen-activated protein kinase (Thr202/Tyr204; #4376; 1:100 dilution) and anti-phospho-Akt (Ser473; #4060; 1:65 dilution) antibodies from Cell Signaling Technology. In brief, sections were deparaffinized in xylene and rehydrated in series of alcohol. To unmask antigens, sections were heated in 10 mmol/L sodium citrate buffer (pH 6.0) for 10 minutes in a pressure cooker. Endogenous peroxidase was quenched by incubation in 3% H_2O_2 for 10 minutes. To minimize nonspecific staining, sections were blocked with 5% normal goat serum at room temperature for 45 minutes. Primary antibodies were incubated overnight at 4°C. For detection of primary antibodies, the Vectastain Elite ABC-peroxidase Rabbit IgG Kit (Vector Laboratories) was used. Samples were developed with 3, 3′-diaminobenzidine (Vector Laboratories), counterstained with hematoxylin, and mounted. A metastatic melanoma sample with known positive phosphorylated (p)-ERK and p-AKT staining was used as positive control. Negative controls were incubations omitting the primary antibodies.

The p-ERK and p-AKT stains were examined by 2 observers blinded to the clinical data. Both the intensity of staining and the percentage of stained cells were recorded. Staining intensities were scored as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). The percentage of stained cells was also scored in 4 categories: 0 (<5%), 1 (5%–25%), 2 (26%–75%), and 3 (>75%). The intensity score and percentage score were then summed into a total score, in which a score of 0 to 3 was regarded as negative staining and a score of 4 to 6 regarded as positive staining.

KIT. IHC for KIT was done by using a polyclonal rabbit antibody (A4502; dilution 1:100) from Dako. For samples from 51 of the patients, staining was done on a Bond-max automated stainer (Leica Microsystem) according to the manufacturer's instructions, using 3,3′-diaminobenzidine as chromogen. For the remaining 20 patient samples, staining was done on a Ventana BenchMark (Ventana Medical Systems) stainer. Because the BenchMark instrument yielded stains of somewhat lower intensities than the Bond-max instrument, KIT expression was evaluated by scoring the percentage of stained cells as follows: less than 5% of stained cells, 5% to 50% of stained cells, and greater than 50% of stained cells. Only the invasive parts of the tumors were evaluated.

Statistical analysis

Statistical analyses were done with the SPSS version 17.0 software (SPSS, Inc.). The Chi-square test was used to compare distributions among groups, and student's *t*-test was used to compare differences in means among groups. Fisher's exact test was used to compare the distributions of *KIT* mutations in vulvar melanomas and *NRAS* mutations in vaginal melanomas, respectively, versus other groups. Kaplan–Meier method and Gehan–Wilcoxon test were used to compare overall survival (defined as the time between date of diagnosis and date of death or last follow-up) among groups. Multivariate analysis was done by the Cox proportional hazards regression model. A value of $P < 0.05$ was regarded as statistically significant. All statistical analyses were double sided. No correlations for multiple testing were carried out because in the Fisher's exact test analyses, only single sites were compared with the remaining tumors; moreover, this was an exploratory analysis, the results of which should be validated in independent tumor samples.

Results

Clinicopathologic characteristics

Clinicopathologic characteristics of cases are summarized in Table 1. The patients' median age was 76 years (range 41–95). A total of 50 patients were women and 21 were men. Of the 71 tumors included in the study, 23 were from the vulva, 22 from the anorectal tract, 14 from the nasal cavity, 7 from the vagina, and 5 from the penis. The penile melanomas were located on the glans penis or between the glans penis and prepuce. Median time from diagnosis to death/last follow-up was 28 months (range 2–236).

Mutation analysis

KIT. A total of 71 primary mucosal melanomas were screened for mutations in exons 11, 13, and 17 of *KIT* by using direct DNA sequencing. A subset of tumors (60 and 48, respectively) was also screened for mutations in exons 9 and 18. *KIT* mutations were detected in 12 tumors (17%; Table 2). With respect to anatomical site, *KIT* mutations were observed in 8 of 23 vulvar (35%), 2 of 22 anorectal (9%), 1 of 14 nasal cavity (7%), and 1 of 5 penile melanomas (20%). No *KIT* mutations were found in the 7 vaginal melanomas analyzed. Since vulvar melanomas was the largest group of tumors and also showed an abundance of *KIT* mutated lesions, we compared the presence of *KIT* mutations in melanomas of the vulva versus the nonvulvar tumors (i.e., all other mucosal melanomas). We found that *KIT* mutations were significantly more

Table 1. Clinicopathologic characteristics of 71 primary mucosal melanomas

Variable	No. of patients (%)
Median age (range), y	76 (41–95)
Median overall survival (range), mo	28 (2–236)
Gender	
Male	21 (29.6)
Female	50 (70.4)
Anatomical site	
Vulva	23 (32.4)
Anorectum	22 (31.0)
Nasal cavity	14 (19.7)
Vagina	7 (9.9)
Penis	5 (7.0)
Clinical stage	
I	41 (57.7)
II	6 (8.5)
III	5 (7.0)
Data missing	19 (26.8)
Thickness	
<10 mm	26 (36.6)
≥10 mm	29 (40.8)
Data missing[a]	16 (22.5)
Histogenetic type	
Mucosal lentiginous melanoma	23 (32.4)
Nodular melanoma	17 (23.9)
Superficial spreading melanoma	3 (4.2)
Mixed[b]	2 (2.8)
Unspecified[c]	26 (36.6)
Histology	
Epithelioid	36 (50.7)
Spindle	6 (8.5)
Mixed[d]	29 (40.8)
Mitoses (10 high-power fields)	
1–10	35 (49.3)
>10	35 (49.3)
Data missing	1 (1.4)
Ulceration	
Present	63 (88.7)
Absent	3 (4.2)
Data missing	5 (7.0)
Pigmentation	
Present	49 (69.0)
Absent	22 (31.0)

[a]Tumor thickness was sometimes not possible to evaluate because a free deep surgical margin was lacking.
[b]Combination of mucosal lentiginous melanoma and superficial spreading melanoma.
[c]Tumors technically not classifiable because they were located in biopsies without any free surgical margins, which precluded adequate assessment of RGP.
[d]Mixture of epithelioid and spindle cells.

frequent in vulvar than nonvulvar cases ($P = 0.014$, Fisher's exact test).

Of the 12 *KIT* mutations identified, 10 mutations were in exon 11, whereas the other 2 were in exon 17. The most common *KIT* mutation observed in melanomas so far, L576P, was detected in 5 cases. Other single amino acid substitutions identified were W557R, V559D, V560D P573L, D820Y, and N822K. P573L represents a novel mutation not previously described in melanoma or any other tumor type, whereas all other mutations have been reported before in melanoma (25, 26). In addition to the single amino acid substitutions, 1 small insertion/deletion affecting codons 574 to 577 of exon 11 was also identified (Table 2).

Of the 12 tumors with *KIT* mutations, 3 contained radial growth phase (RGP) cells. To better determine the timing of *KIT* mutations in these tumors, RGP cells were isolated by LCM and subjected to mutation analysis. These analyses were successful in 2 tumors. In one, a vulvar melanoma, the V559D mutation identified in the invasive parts of the tumor was readily detected in corresponding RGP cells, whereas in the other tumor, also a vulvar melanoma, the V560D change in the invasive cells was not detectable in matched RGP cells. Thus, in at least 1 of the tested tumors, the *KIT* mutation represented an early genetic event.

Mutations in *KIT* showed no correlation with patient age at diagnosis, clinical stage, tumor thickness, histogenetic type, histology, mitotic count, or pigmentation.

NRAS and BRAF. *NRAS* and *BRAF* mutations were found in 7 of 71 (10%) and 4 of 71 (6%) tumors, respectively (Table 2). Of the 7 *NRAS* mutations identified, 4 involved codon 61 and 3 involved codons 12 and 13. Of the *BRAF* mutations, 2 (D594G and L597V) were outside codon 600, the position where more than 90% of mutations occur in cutaneous melanomas. Table 3 summarizes the frequencies of *NRAS* and *BRAF* mutations with respect to anatomical site. Worth noting is that 3 of the 7 vaginal melanomas (43%) analyzed contained an activating *NRAS* mutation. Thus, vaginal melanomas had a significantly higher proportion of *NRAS* mutated lesions than tumors of the other sites ($P = 0.018$, Fisher's exact test). Mutations in *NRAS* and *BRAF* were mutually exclusive and occurred only in tumors wild-type for *KIT*, confirming previous results that *KIT*, *NRAS*, and *BRAF* mutations do not coexist in mucosal melanomas (6–8).

Quantitative real-time PCR

By using quantitative real-time PCR, we also analyzed some tumors for *KIT* gene amplification. Accordingly, increased *KIT* copy numbers (≥3 copies) were observed in 16 of 43 cases tested (37%), whereas 4 or more copies were seen in 8 cases (19%). Of the 8 tumors with ≥ 4 copies of *KIT*, 4 had a concurrent *KIT* mutation (Table 2). The observation that *KIT* amplification and *KIT* mutation coexist in the same lesion is in agreement with previous studies of mucosal melanomas (6–8).

Table 2. *KIT*, *NRAS*, and *BRAF* mutations identified in primary mucosal melanomas

Case	Site	Gene	Exon	Nucleotide Change	Amino Acid Change	KIT copy Number	IHC		
							KIT[a]	p-ERK[b]	p-AKT[c]
1	Vulva	KIT	11	T1669C	W557R	≥4	>50	–	–
2	Vulva	KIT	11	T1676A	V559D	3 to <4	>50	+	+
3	Vulva	KIT	11	T1679A	V560D	nd[d]	>50	+	+
4	Vulva	KIT	11	C1718T	P573L	<3	<5	–	–
5	Anorectum	KIT	11	T1727C	L576P	<3	>50	–	–
6	Anorectum	KIT	11	T1727C	L576P	≥4	>50	–	+
7	Nasal cavity	KIT	11	T1727C	L576P	≥4	>50	–	+
8	Vulva	KIT	11	T1727C	L576P	<3	>50	+	+
9	Vulva	KIT	11	T1727C	L576P	nd	>50	–	–
10	Penis	KIT	11	1722insACA, 1723_1731del9	T574insT, Q575_P577del	3 to <4	>50	+	+
11	Vulva	KIT	17	G2458T	D820Y	nd	>50	+	+
12	Vulva	KIT	17	T2466G	N822K	≥4	>50	–	+
13	Vagina	NRAS	1	G35A	G12D	3 to <4	<5	+	+
14	Anorectum	NRAS	1	G38A	G13D	3 to <4	5–50	+	+
15	Penis	NRAS	1	G38A	G13D	nd	<5	–	–
16	Nasal cavity	NRAS	2	C181A	Q61K	nd	<5	–	–
17	Nasal cavity	NRAS	2	A182G	Q61R	nd	5–50	–	+
18	Vagina	NRAS	2	A182T	Q61L	nd	5–50	+	+
19	Vagina	NRAS	2	A183T	Q61H	<3	<5	–	+
20	Penis	BRAF	15	A1781G	D594G	3 to <4	<5	–	–
21	Anorectum	BRAF	15	C1789G	L597V	≥4	>50	+	+
22	Vulva	BRAF	15	T1799A	V600E	nd	<5	–	+
23	Vulva	BRAF	15	T1799A	V600E	<3	<5	–	–

[a]Percent positive tumor cells.
[b]+ = positive p-ERK staining, – = negative p-ERK staining.
[c]+ = positive p-AKT staining, – = negative p-AKT staining.
[d]Not determined.

IHC

KIT. To correlate *KIT* mutation status with KIT expression levels, tumors were analyzed by IHC using an antibody against KIT. Of the 12 tumors with *KIT* mutations, 11 (92%) expressed KIT in more than 50% of tumor cells (Table 2 and Fig. 1A). In contrast, of the 59 cases without detectable *KIT* mutations, only 14 (24%) were positive for KIT in more than 50% of tumor cells. This difference was statistically significant ($P < 0.001$). An additional 22% (13 of 59) of the *KIT* wild-type cases expressed KIT in 5% to 50% of tumor cells. Notably, of the 11 tumors with *NRAS* or *BRAF* mutations, only 1 (a *BRAF*-mutated tumor) expressed KIT in more than 50% of tumor cells (Table 2 and Fig. 1B). With respect to site, KIT staining in more than

Table 3. KIT pathway alterations in mucosal melanomas of different anatomical sites

Site	*KIT* mutation *n* (%)	*NRAS* mutation *n* (%)	*BRAF* mutation *n* (%)	KIT IHC *n* (%)	p-ERK IHC *n* (%)	p-AKT IHC *n* (%)
Vulva	8/23 (35)	0/23 (0)	2/23 (9)	13/23 (56)	7/21 (33)	13/21 (62)
Anorectum	2/22 (9)	1/22 (4.5)	1/22 (4.5)	6/22 (27)	8/20 (40)	11/20 (55)
Nasal cavity	1/14 (7)	2/14 (14)	0/14 (0)	4/14 (28)	7/11 (64)	9/11 (82)
Vagina	0/7 (0)	3/7 (43)	0/7 (0)	1/7 (14)	4/7 (57)	4/7 (57)
Penis	1/5 (20)	1/5 (20)	1/5 (20)	1/5 (20)	1/5 (20)	1/5 (20)
Total	12/71 (17)	7/71 (10)	4/71 (6)	25/71 (35)	27/64 (42)	38/64 (59)

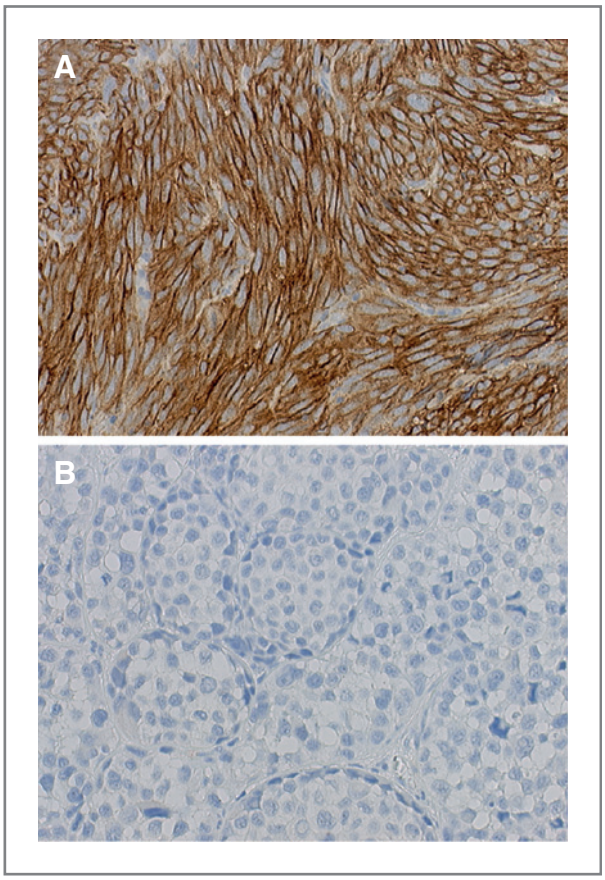

Figure 1. KIT immunohistochemistry in primary mucosal melanomas. (A) Strong membraneous expression of KIT in a vulvar melanoma harboring a KIT D820Y mutation. (B) Absence of KIT expression in a vaginal melanoma harboring a NRAS G12D mutation. Original magnification ×200 for A and B.

50% of tumor cells was found in 56% (13 of 23) of vulvar, 27% (6 of 22) of anorectal, 28% (4 of 14) of nasal cavity, 14% (1 of 7) of vaginal, and 20% (1 of 5) of penile melanomas (Table 3). The difference in KIT staining between vulvar and nonvulvar tumors was statistically significant ($P = 0.016$).

KIT expression associated significantly with a spindle-type histology ($P = 0.035$) and tended to be more common in tumors with a thickness less than 10 mm, although statistical significance was not reached in our analysis ($P = 0.056$). No associations were observed between KIT expression and age, clinical stage, histogenetic type, mitotic count, or pigmentation.

p-ERK and p-AKT. To examine the activation status of ERK and AKT in mucosal melanoma tissue, 64 tumors were immunostained with antibodies to p-ERK and p-AKT. Seven tumors were excluded from analysis because of heavy pigmentation or insufficient material. Overall, p-ERK and p-AKT staining was positive in 27 (42%) and 38 (59%) tumors, respectively. As shown in Table 3, expression of p-ERK and p-AKT was not restricted to a specific anatomical site but occurred in tumors at all locations. There was no difference in p-ERK expression among *KIT* mutated tumors (5 of 12; 42%), *NRAS*/*BRAF* mutated tumors (4 of 11; 36%), and *KIT*/*NRAS*/*BRAF* wild-type tumors (18 of 41; 44%). Similarly, expression of p-AKT did not differ markedly when *KIT* mutated (8 of 12; 67%), *NRAS* mutated (5 of 7; 71%), and *KIT*/*NRAS* wild-type tumors (25 of 45; 56%) were compared. Representative staining examples are shown in Figure 2.

Expression of p-ERK and p-AKT was more frequent in tumors that were relatively small than large tumors. Large tumors (≥10 mm) sometimes completely lacked staining in the central parts with positivity limited to the most peripheral parts. A possible technical explanation for this staining pattern is that proteins in the core of large specimens are dephosphorylated as an artifact, due to slow fixation (27, 28).

Survival analysis

Of the clinicopathological variables tested, advanced clinical stage, tumor thickness ≥ 10 mm, and high mitotic counts showed an association with worse overall survival ($P = 0.002$, 0.004, and 0.012, respectively; Supplementary Fig. S1A–C). The impact of *KIT* and *NRAS* mutations on clinical outcome was also tested. As depicted in Figure 3A, patients whose tumors contained *NRAS* mutations had a significantly shorter survival than patients whose tumors were wild-type for *NRAS* (median overall survival 9 vs. 31 months, $P = 0.039$). The correlation between *NRAS* mutations and overall survival did not, however, remain significant after adjusting for clinical stage or tumor thickness in multivariate analyses (data not shown). No significant associations were observed between *KIT* mutation status (Fig. 3B) or KIT expression levels and overall survival. Similarly, no associations were found between p-ERK or p-AKT expression and overall survival.

Discussion

In this study, we show that the frequency of *KIT* mutations in primary mucosal melanomas varies significantly with anatomical site. The highest *KIT* mutation rate (35%) was detected in melanomas of the vulva. We also report on activation of the KIT downstream RAF/MEK/ERK and PI3K/AKT signaling pathways in clinical specimens of mucosal melanoma. Our findings may have implications for the choice of targeted therapy in mucosal melanoma.

In support of our results, Beadling and colleagues recently reported a higher *KIT* mutation frequency in melanomas of the anorectum/vulva/vagina (4 of 9, 44%) than melanomas of the head/neck (3 of 36, 8.3%; ref. 8). Another recent study analyzing mucosal melanomas from several sites found *KIT* mutations in 30% (3 of 10) of those from the genital tract, 17% (2 of 12) from the head/neck, and 12.5% (1 of 8) from anorectal sites (29). Curtin and colleagues were the first to show that *KIT* is genetically altered in mucosal melanomas (6). Without specifying the anatomical origin of the tumors, they identified *KIT* mutations in 21% (8 of 38) of

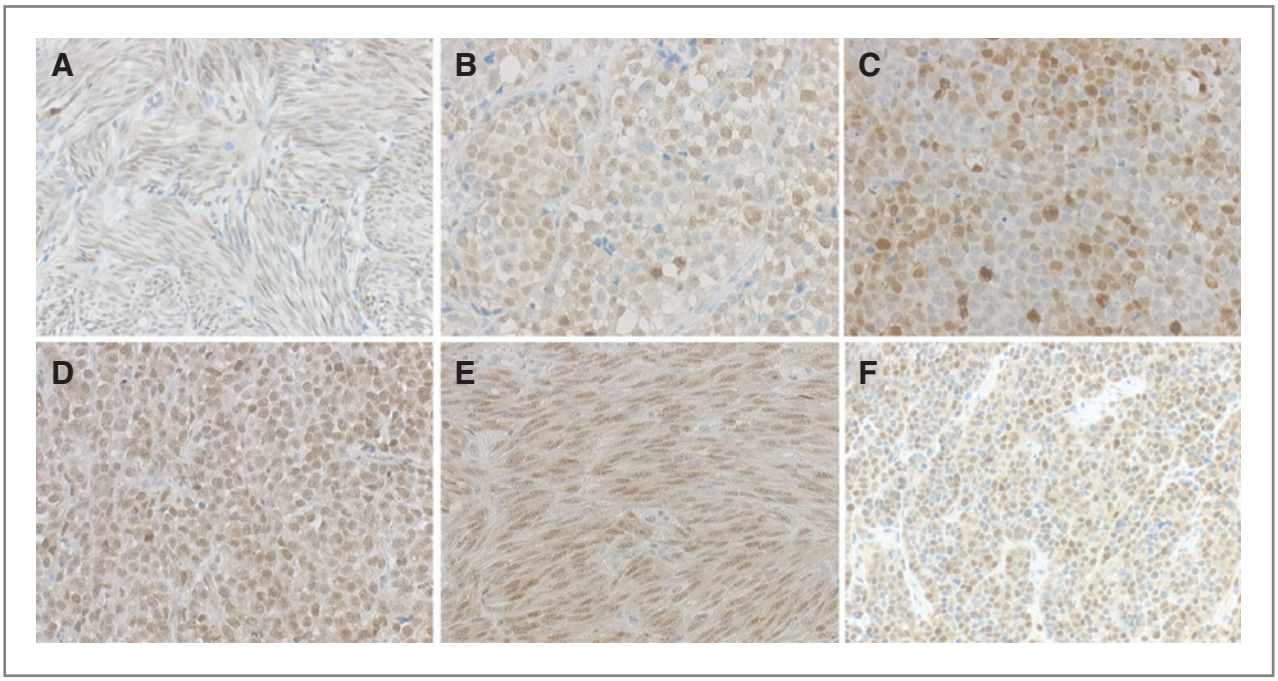

Figure 2. Immunohistochemical analysis of p-ERK and p-AKT in primary mucosal melanomas. Positive p-ERK staining in (A) a vulvar melanoma with a KIT V560D mutation, (B) a vaginal melanoma with an NRAS G12D mutation, and (C) a nasal cavity melanoma negative for mutations in the *KIT*, *NRAS*, and *BRAF* genes. Positive p-AKT staining in (D) a penile melanoma with a insertion/deletion affecting codons 574 to 577 of exon 11 of KIT, (E) an anorectal melanoma with an NRAS G13D mutation, and (F) an anorectal melanoma without mutations in the *KIT*, *NRAS*, and *BRAF* genes. Original magnification ×200 for (A) to (F).

cases (6). Authors of subsequent studies described *KIT* mutations in 15% (3 of 20) of anorectal (7) and 27% (4 of 15) of oral melanomas (30).

In line with previous studies, a majority (10 of 12) of the *KIT* mutations identified here occurred within exon 11, encoding the juxtamembrane domain of the KIT receptor.

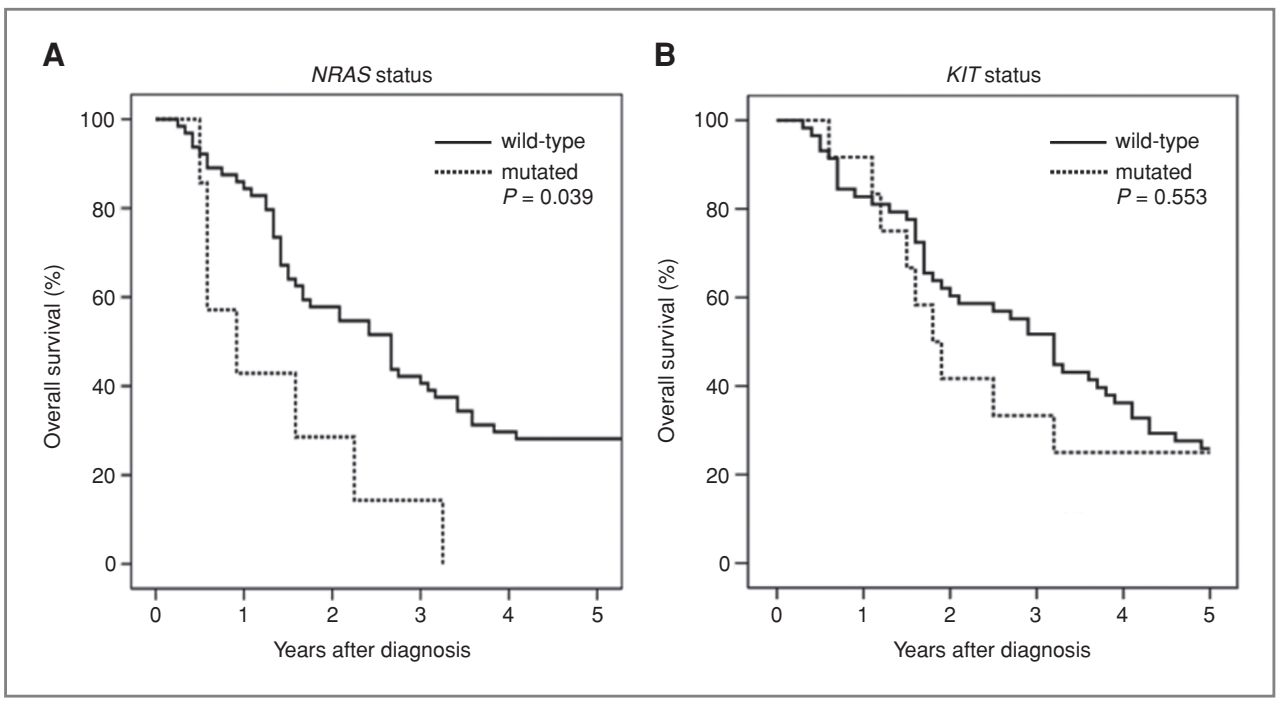

Figure 3. Overall survival of mucosal melanoma patients according to (A) *NRAS* and (B) *KIT* mutation status.

In addition, a minority of mutations were found in exon 17, which encodes the tyrosine kinase-2 domain of KIT. Single amino acid substitutions were the most common type of alterations identified. It is interesting to note that of the 8 vulvar melanomas with *KIT* mutations, 6 had a mutation other than L576P. In gastrointestinal stromal tumor (GIST), patients with *KIT* exon 11 mutations are known to respond better to imatinib treatment than do patients without such alterations (31). In melanoma, cells with the exon 11 V559A mutation show sensitivity to imatinib *in vitro* (32). However, cells with the L576P mutation seem to be relatively resistant to imatinib, but are sensitive to dasatinib (18). Clinically, mucosal melanoma patients with *KIT* mutations located outside position L576 have been responsive to imatinib (13, 14, 16) and sorafenib (16, 17), whereas patients with L576P mutant tumors responded to dasatinib (18). More recently, those with L576P mutated mucosal melanomas were also described as responsive to imatinib (15, 16).

Our results showed a significant association between expression of KIT by IHC and *KIT* mutation status. Such an association in melanoma tumors has been described in several previous studies, the largest one being a recent report by Torres-Cabala and colleagues (33). The correlation between KIT expression levels and mutation status was, however, far from perfect with 1 of the 12 *KIT*-mutant tumors being negative for KIT expression and one quarter of the *KIT* wild-type cases showing positive KIT staining in more than 50% of tumor cells. Our recommendation is that IHC should not replace mutation analysis in the identification of patients who may benefit from KIT targeting therapies. The observation of more frequent KIT expression in vulvar melanomas than other types of mucosal melanomas has not been previously reported. This finding, together with our mutation results, points to KIT having a more pronounced role in vulvar melanomas than melanomas of other mucosal sites.

As expected, the overall frequency of *NRAS* and *BRAF* mutations was low in our series of tumors. With respect to site, however, it is of interest to note that melanomas of the vagina showed a *NRAS* mutation rate of 43%. Another site of mucosal melanoma where *NRAS* mutations also seem to be common is the esophagus. In a recent study of 16 esophageal melanomas, Sekine and colleagues identified *NRAS* mutations in 6 cases (37.5%; ref. 34). Interestingly, the type of *NRAS* mutations that we observed in vaginal melanomas (G12D, Q61L, and Q61H) and the type of mutations described in melanomas of the esophagus (34) differ from the type of mutations that predominate in cutaneous melanoma on sun-exposed skin (35), probably denoting that the mutagenic agents involved differ between mucosal and cutaneous melanomas. Together, the results of our study and that of Sekine and colleagues suggest that the pattern of *KIT* and *NRAS* mutations in mucosal melanomas is more complex than initially thought. Also, our study implies that despite the close anatomical proximity, mucosal melanomas of the vulva and vagina have different biology and also may require different systemic therapies in the metastatic setting.

This study is the first to report on expression of p-ERK and p-AKT in clinical samples of mucosal melanoma. A previous study has shown increased phosphorylation of ERK and AKT in mucosal melanoma cell cultures (32). Of 12 tumors with *KIT* mutations, 5 (42%) were positive for p-ERK and 8 (67%) showed positivity for p-AKT. Similar results have been observed in *KIT*-mutated GISTs (36) and a *KIT*-mutated acral melanoma (37). Importantly, treatment of *KIT*-mutant mucosal melanoma cells *in vitro* with imatinib results in decreased levels of ERK and AKT phosphorylation (32). Decreased AKT phosphorylation has also been described in a metastatic melanoma lesion from a patient responding to imatinib treatment, although neither the type of primary tumor nor the *KIT* mutation status was reported (38). We observed no association between p-ERK or p-AKT expression and *KIT*/*NRAS*/*BRAF* mutation status in our series of tumors. A total of 44% of *KIT*/*NRAS*/*BRAF* wild-type tumors were positive for p-ERK and 56% of *KIT*/*NRAS* wild-type tumors showed positivity for p-AKT. These results suggest that other mechanisms than activating mutations in the *KIT*, *NRAS*, and *BRAF* genes may lead to activation of the ERK and AKT pathways in mucosal melanoma. Two other such mechanisms by which AKT is activated in human cancer are (1) through mutation of the *PIK3CA* oncogene (encoding the catalytic subunit p110α of phosphatidylinositol 3′-kinases) and (2) inactivation of the *PTEN* tumor suppressor gene. In an analysis of 57 mucosal melanomas, however, we identified only 1 case with a *PIK3CA* mutation, suggesting that *PIK3CA* plays no major role in the development of mucosal melanoma (K. Omholt, B. Ragnarsson-Olding, and J. Hansson, unpublished data). The role for *PTEN* in mucosal melanoma remains to be determined.

Our study suggests that *KIT* mutations lack prognostic significance in mucosal melanoma. This finding is in contrast to results of a recent study of Chinese patients with melanoma in which *KIT* mutations adversely affected survival (26). *NRAS* mutation was associated with worse overall survival in univariate analysis but lost its predictive value in multivariate analysis. This is not surprising, considering the small number of patients with *NRAS*-mutant tumors in the present study. Our results are analogous to findings in lung cancer, showing that patients with *KRAS* mutations have a worse survival compared with patients with mutations in the epidermal growth factor receptor (39). We found no evidence that p-AKT is a negative prognostic marker in mucosal melanoma, which is the case in many other tumor types, including cutaneous melanoma (22). Given the problem with phospho-specific antibodies on formalin-fixed tissue specimens, however, it cannot be excluded that false-negative staining might have contributed to the lack of association between p-AKT expression and survival (27, 28).

In summary, we have shown that activating mutations of *KIT* are found in a high percentage of vulvar melanomas.

Vulvar melanomas thus represent a subgroup of mucosal melanomas that is likely to respond to therapies directed against KIT. We also report that the RAF/MEK/ERK and PI3K/AKT signaling pathways are activated in a significant proportion of mucosal melanomas. Activation of these pathways occurs irrespective of the *KIT*/*NRAS*/*BRAF* mutation status of the tumors. Both the RAF/MEK/ERK and PI3K/AKT pathways may represent promising alternative therapeutic targets in mucosal melanoma, especially in the subset of tumors lacking activating *KIT* mutations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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