Identification of recurrent mutational events in anorectal melanoma

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Anorectal melanoma is a rare disease that carries a poor prognosis. To date, limited genetic analyses confirmed *KIT* mutations as a recurrent genetic event similar to other mucosal melanomas, occurring in up to 30% of anorectal melanomas. Importantly, a subset of tumors harboring activating *KIT* mutations have been found to respond to c-Kit inhibitor-based therapy, with improved patient survival at advanced tumor stages. We performed comprehensive targeted exon sequencing analysis of 467 cancer-related genes in a larger series of 15 anorectal melanomas, focusing on potentially actionable variants based on gain- and loss-of-function mutations. We report the identification of oncogenic driver events in the majority (93%) of anorectal melanomas. These included variants in canonical MAPK pathway effectors rarely observed in cutaneous melanomas (including *an HRAS* mutation, as well as *a BRAF* mutation resulting in duplication of threonine 599), and recurrent mutations in the tumor suppressor *NF1* in 20% of cases, which represented the second-most frequently mutated gene after *KIT* in our series. Furthermore, we identify *SF3B1* mutations as a recurrent genetic event in mucosal melanomas. Our findings provide an insight into the genetic diversity of anorectal melanomas, and suggest significant potential for alternative targeted therapeutics in addition to c-Kit inhibitors for this melanoma subtype.
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Anorectal melanoma is a rare and highly lethal malignant neoplasm, comprising ~1% of all melanomas and <2% of anal tumors.[1–3] The annual incidence in the United States is ~0.3 per million with a male to female ratio of 2:3, whereas other large population-based studies report higher incidence rates of 1.0 per million.[1,4] The anal canal is best defined *in vivo*, extending from the rectal pubalis sling to the anal verge. As such, the anal canal has three epithelial zones: rectal/colonic mucosal zone, the transitional zone, and the squamous mucosal zone. The anal transitional zone varies in length and is comprised of variable mucosa. Although anorectal melanoma was historically thought to arise from anal squamous epithelium, it was recognized that melanocytes are present within the anal transitional zone, as well as above the dentate line in the proximal anal canal/distal rectum within colorectal mucosa.[5–7] Approximately 60% of melanomas are diagnosed in the anal canal and up to 40% in the rectum.[1] Of note, anorectal melanoma in the United States shows a rising incidence.[1]

A unifying staging system for mucosal melanoma, including anorectal melanoma, is currently lacking, partially owing to rarity of the disease. A simplified three-tiered system for melanomas arising on the head and neck[8] categorizes disease extent into clinically localized (Stage I), regional lymph node involvement (Stage II), and distant metastasis (Stage III), and was shown to correlate with outcome in a recent large retrospective series of anorectal melanomas.[9] Surgical treatment appears effective for localized disease.[2,10] However, overall survival for patients suffering from anorectal melanoma remains dismal, with 5-year survival rates for Stage I, Stage II, and Stage III disease of 26%, 9.8%, and 0%, respectively.[9]

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To date, analyses of few genetic loci in anorectal melanoma revealed that, similar to melanomas at other mucosal sites, mutations in *BRAF* and *NRAS* are significantly less frequent as compared with cutaneous melanomas, whereas activating *KIT* mutations represent a recurrent mutational event.[11,12] One of the largest studies comprising 31 primary anorectal melanomas reported *KIT* mutations in >30% of cases.[13] Importantly, whereas radical surgery and radiotherapy failed to improve survival,[2,14] several prospective trials demonstrated clinical benefit of imatinib in patients with metastatic mucosal melanomas harboring activating *KIT* mutations, with response rates between 16 and 25% and some responses lasting for longer than one year.[15–17] However, secondary resistance eventually develops, and although alternative kinase inhibitors such as nilotinib have shown limited efficacy in c-Kit inhibitor-refractory disease, the overall prognosis for these patients remains poor.[18] Furthermore, a majority of mucosal melanomas lack *KIT* mutations. Exploration of additional actionable mutational events is therefore crucial to refine molecular therapy for this subset of tumors, and to expand treatment options with the potential of improving survival in this devastating disease.

The development of targeted therapies in cancer has accelerated the development of molecular diagnosis, with the emergence of next-generation sequencing technologies as useful new tools in oncology and personalized medicine. In light of the limited data that includes mutation status of select oncogenes and tumor suppressors,[11,13,19–21] we performed expanded molecular profiling of a larger series of anorectal melanomas.

We report the identification of annotated oncogenic driver events in the majority of anorectal melanomas (14 of 15 cases), with potential implications for targeted therapy.

Materials and methods

Case Selection

Fifteen cases of anorectal melanoma diagnosed between 1 January 1990 and 1 January 2015 with sufficient residual material for analysis were retrieved from the surgical pathology archives of the Columbia University Medical Center, New York, NY the Vancouver General Hospital, Vancouver, BC, and the Ludwig Maximillian University Tumor bank, Munich, Germany, with approval of respective Institutional Review Boards. Original diagnosis was based on clinical (anatomic site) and histologic features, and melanocytic lineage of tumors confirmed by immunohistochemistry. Clinical data were reviewed to confirm the absence of a prior history of melanoma. H&E-stained sections and immunohistochemical stains of all study cases were reviewed to verify anatomic site, relationship to the anal transitional zone, as well as for assessment of an intraepithelial/in situ component. Where available, follow-up information was obtained from review of medical records. Survival time was defined as the time from initial diagnosis until last follow-up.

DNA Extraction, Targeted Sequencing, and Data Analysis

To enrich for lesional tissue, representative tumor areas were manually microdissected from formalin fixed, paraffin-embedded tissue sections. DNA was extracted using QIAcube (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNA was analyzed by the Columbia Molecular Pathology Combined Cancer Panel.[22] In total, 200 ng of DNA was sheared to a median length of 200 bp using a Covaris S2 Sonication system (Covaris, Woburn, MA, USA), and exonic sequence from 467 cancer-related genes was captured using custom Agilent SureSelect reagents (Santa Clara, CA, USA). Sequencing was performed on the Illumina HiSeq2500 (San Diego, CA) using Illumina TruSeq v3 chemistry and as 100-bp paired-end reads (up to nine indexed samples per run). Demultiplexing was performed with CASAVA and alignment and variant calling was performed using NextGENe software (Softgenetics, State College, PA, USA), with the following parameters: 0 allowable ambiguous alignments, at least 90% of reads matching the reference genome, at least 10% variant allelic fraction and at least three variant reads required to call a variant. Single-nucleotide variants as well as small insertions and deletions were annotated and filtered by an in-house developed pipeline and evaluated by a molecular pathologist. In brief, variants were cross-referenced with the 1000 Genomes Project, OMIM, dbSNP and the Exome Variant Server. Variants with >1% allele frequency, common variants present in our departmental database of variants identified in prior constitutional exome analysis, non-pathogenic variants reported in dbSNP as well as low-quality calls were filtered out. Variants were annotated with dsSNP, ClinVar, HGMD, OMIM, and COSMIC databases, as well as by predicted protein effect (using *in silico* predictors Provean and SIFT). Potential variants were manually curated and classified by literature review to evaluate for pathogenic changes consistent with protein function.

Immunohistochemistry

Immunohistochemical analysis was performed using a Ventana automated slide stainer and Ventana ultraView universal DAB detection kit, as we described previously.[23] The following pre-diluted antibodies (Ventana, Tucson, AZ, USA) were used: MLH1 (clone G168-15), MSH2 (clone FE11), PMS2 (clone MRQ-28) and MSH6 (clone 44).

Table 1 Clinical and pathologic features of anorectal melanomas

Case no.	*Age, sex*	*Relationship to ATZ*	*Thickness (mm)*	*MIS*	*Mitotic rate/mm²*	*Histology*	*Metastases*	*Alive/dead, durvival (m)*	*Stage**
1	73 M	Above	4.5	ND	10	Epithelioid	Liver, spleen, LN	DOD, 21	III
2	83 M	At	9.2	yes	8	Epithelioid/ spindled	Lung	DOD, 13	III
3	48 F	Below	4.5	yes	5	Epithelioid	Adrenal, lung	DOD, 12	III
4	64 F	Below/At	5.1	yes	7	Epithelioid	LN	Dead, 18	II
5	68 F	Below	≥5	yes	33	Epithelioid	Lung, liver, spleen, LN	DOD, 7	III
6	45 M	Above	≥2.5	ND	4	Epithelioid	Unknown	Alive, 1	–
7	81 M	At	7	yes	38	Epithelioid	Negative	Alive, 30	I
8	83 M	Above/At	46	yes	16	Epithelioid/ spindled	LN	DOD, 9	II
9	79 F	Below	≥3	yes	3	Epithelioid	Liver, peritoneum, LN	Unknown	III
10	65 F	Above	14	ND	20	Epithelioid/ spindled	Liver	DOD, 12	III
11	57 F	Below	2.5	yes	4	Epithelioid	Liver, LN	DOD, 16	III
12	73 F	At	4.5	yes	16	Epithelioid	Liver, LN	DOD, 5	III
13	77 M	Below	≥2.2	yes	19	Epithelioid	Negative	Dead, 3	I
14	74 M	Below/At	3.9	yes	3	Epithelioid	Unknown	Dead, 5	–
15	57 F	Below	≥11	ND	8	Spindled	Brain, liver, LN	DOD, 8	III

Abbreviations: M, male; F, female; ATZ, anal transitional zone; MIS, melanoma in situ; ND, not determined due to colonic mucosal localization and/or extensive ulceration; LN, lymph node; DOD, dead of disease; *Ballantyne staging system.

Microsatellite Instability Testing

Microsatellite instability (MSI) testing was performed using 1–2 ng of DNA extracted from formalin fixed, paraffin-embedded tissue using the Promega MSI Analysis System (Promega, Madison, WI, USA), according to the manufacturer's instructions. Analysis of PCR products was performed on an ABI PRISM 3100-Avant genetic analyzer (Thermo Fisher Scientific, Waltham, MA, USA).

Results

Clinical and Histologic Characteristics

The clinical and histologic findings of fifteen cases of primary anorectal melanoma are summarized in Table 1. Patient age ranged from 45 to 83 years, with a mean age of 68.5 years and median age of 73 years. Cases included seven men and eight women. In four cases, the tumor was centered above the anal transitional zone within colonic mucosa and in 11 cases at or below the anal transitional zone (transitional type mucosa/squamous mucosa). Melanoma *in situ* was identified in all tumors occurring at or below the anal transitional zone, except in one case which showed extensive ulceration. Careful review of clinical data revealed no history of melanoma at other sites in any of the patients. Clinical follow-up data was available for 14 patients and ranged from 1 to 30 months after initial diagnosis. Two patients were alive at last follow-up, with no documented evidence of metastasis (Table 1).

A representative case of invasive anorectal melanoma is depicted in Figure 1.

Molecular Findings

Next-generation sequencing analysis of exonic sequence from 467 cancer-related genes (Columbia Combined Cancer Panel) was successfully performed in all 15 cases of anorectal melanoma, with a mean depth-of-coverage of the region of interest of 731×. On average, 14.1 non-synonymous or small insertion/deletion variants were detected per case (range 4–33 variants/case), the majority of which represented variants with unknown significance (Supplementary Table 1). Of note, driver mutations, defined as pathogenic alterations recurrent in human cancers and conferring a growth advantage,[24] were identified in 14 of 15 cases (93%) (Figure 2). Furthermore, a majority of these driver events represent actionable mutations, with significant potential to enhance targeted therapy for this melanoma subtype (Table 2). In most cases (11 of 15), a single driver mutation was identified, whereas three cases showed two and one case showed three driver mutations (Figure 2).

The most frequently mutated gene in our series of anorectal melanomas was *KIT*, with mutations identified in 5 of 15 tumors (33%). Three mutations (W557R, V560D, V559A), previously reported as oncogenic, were found in exon 11 (juxtamembrane domain), and are expected to predict sensitivity to tyrosine kinase inhibitors such as imatinib, nilotinib, or sorafenib (Table 2). In addition, we identified two *KIT* mutations in exon 17 involving the distal tyrosine kinase domain (Y823D, D820Y), which are known to correlate with acquired imatinib resistance in gastrointestinal stromal tumors but

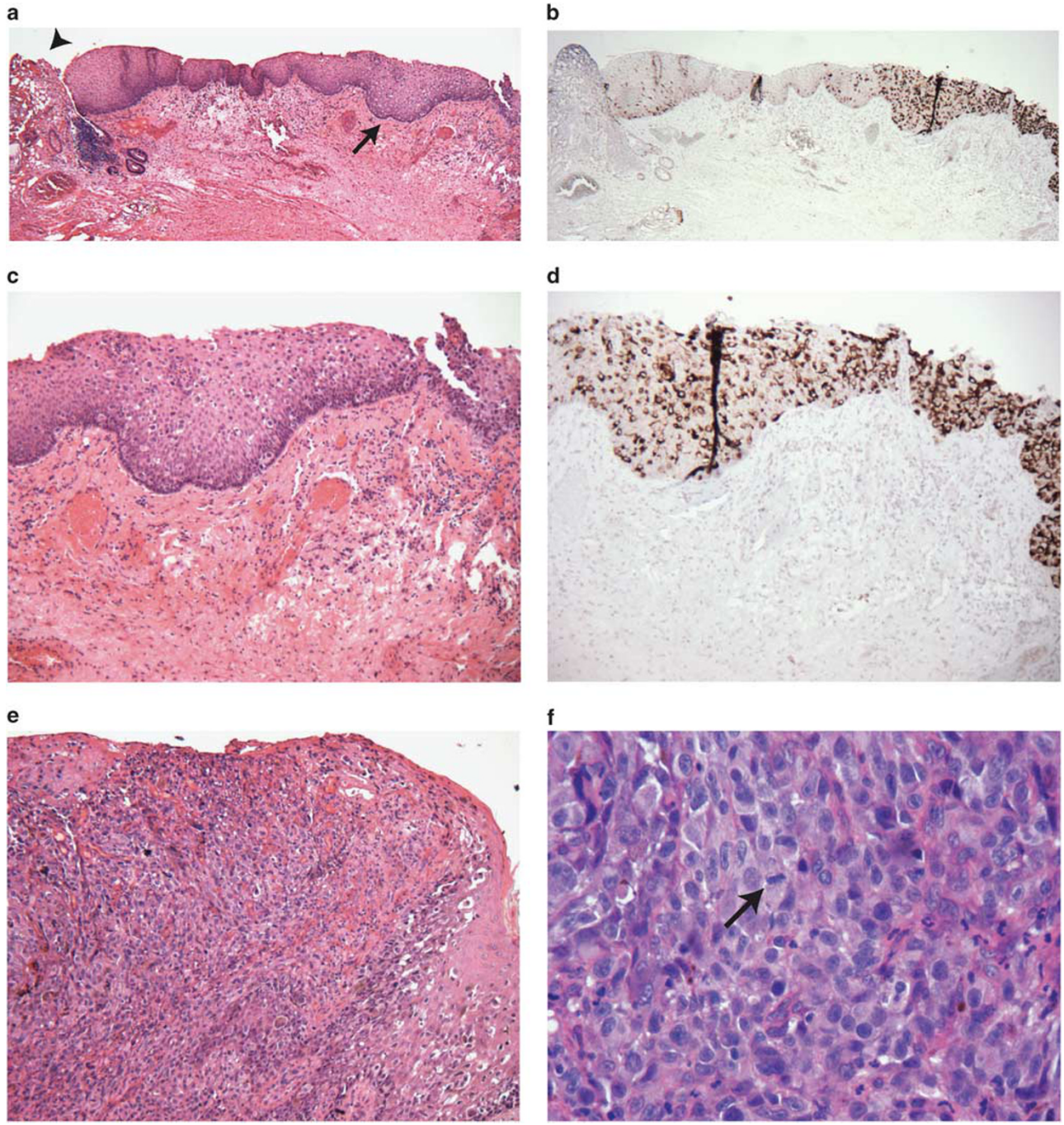

Figure 1 Representative case of anorectal melanoma, histologic features. (**a**) Atypical intraepithelial melanocytic proliferation within squamous mucosa of the anal transitional zone (arrow), adjacent colonic mucosa (arrowhead) (original magnification × 40) (**b**) Atypical intraepithelial melanocytes stain strongly positive for Melan-A (original magnification × 40). (**c**, **d**) Melanoma *in situ* with prominent pagetoid scatter of atypical melanocytes (**c**), highlighted by Melan-A immunohistochemical stain (**d**) (original magnification × 100). (**e**) Invasive melanoma with nests and sheets of atypical melanocytes. Note overlying ulceration and pigment production (original magnification × 100). (**f**) Invasive melanoma. Proliferation of atypical melanocytes with pleomorphic nuclei and mitotic activity (arrow) (original magnification × 400).

described in one case of mucosal melanoma showing a partial treatment response,[17] (Table 2). Overall, these results are in agreement with previous studies and confirm *KIT* mutations as a predominant mutational event in anorectal melanomas.

Anorectal Melanomas Harbor Recurrent Mutations in *NF1*, as well as Mutations in MAPK Pathway Effectors Distinct from Cutaneous Melanomas

Oncogenic mutations in genes affecting *RAS* and its canonical downstream effectors were seen in 3 of 15

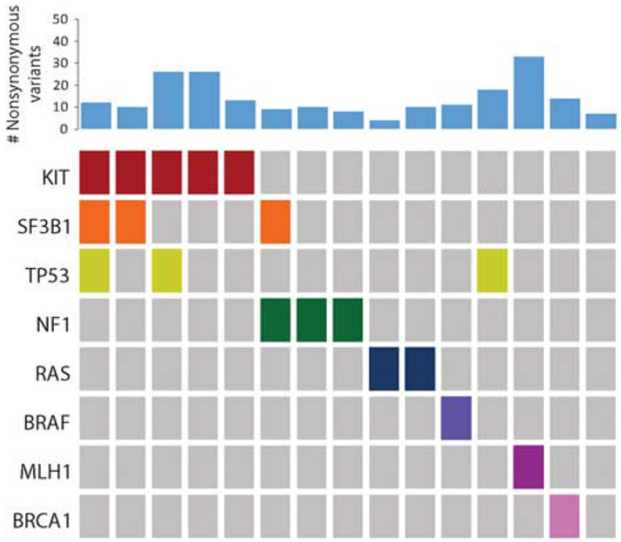

Figure 2 Overview of genetic alterations detected in anorectal melanoma. Number of non-synonymous and insertion/deletion variants detected per case (top panel). Pathogenic driver mutations, defined as recurrent oncogenic events reported in the COSMIC database, are indicated by colored boxes (lower panel).

Table 2 Driver mutations, affected pathways, and potential inhibitors

Case no.	*Gene*	*Mutation*	*Affected pathway*	*Potential inhibitors*
1	***NF1***	**p.K1844fs**	MAPK/PI3K	MEKi
2	*KIT*	Y823D	MAPK/PI3K	—
3	*KIT*	p.V560D	MAPK/PI3K	RTKi
	SF3B1	**p.R625C**		
	TP53	p.W53X		
4	*KIT*	p.W557R	MAPK/PI3K	RTKi
	SF3B1	**p.R625H**		
5	*KIT*	p.V599A	MAPK/PI3K	RTKi
6	*KIT*	p.D820Y	MAPK/PI3K	RTKi
	TP53	p.Y220C		
7	***MLH1***	**p.G67R**	DNA repair	—
8	***BRCA1***	**p.T557fs**	DNA repair	—
9	—	—	—	—
10	***NF1***	**p.H1251fs**	MAPK/PI3K	MEKi
	NF1	**p.Y2629X**		
	SF3B1	**p.R625C**		
11	***BRAF***	**p.T599dup**	MAPK	MEKi
12	***NF1***	**p.W571X**	MAPK/PI3K	MEKi
	NF1	**c.204+1G>C**		
13	*TP53*	p.C242Y	DNA repair	—
14	*HRAS*	p.Q61R	MAPK/PI3K	MEKi
15	*NRAS*	p.G12A	MAPK/PI3K	MEKi

Abbreviations: Dup, duplication; fs, frameshift; MAPK, mitogen-activated protein kinase (Erk); MEKi, MAPK kinase inhibitor (eg, selumetinib, trametinib, binimetinib);[38] RTKi, inhibitors with activity against receptor tyrosine kinases (eg, imatinib, nilotinib); bold, mutations newly discovered in mucosal melanomas.

cases of anorectal melanoma (20%, Table 2). Interestingly, in addition to one *NRAS* (G12A) mutation, we also identified one case each carrying an *HRAS* (Q61R) mutation, more typically seen in Spitz nevi,[25] as well as a rare three-base-pair insertion resulting in duplication of threonine at codon 599 in the *BRAF* activation loop (p.T599dup). This mutation was previously described to display *in vitro* kinase activity comparable to *BRAF* (V600E),[26] the predominant mutation in cutaneous melanomas. Significantly, we furthermore detected recurrent deleterious mutations in *NF1*, a tumor suppressor and negative regulator of *RAS* in cutaneous melanoma (Figure 3).[27] *NF1* mutations were present in 3 of 15 cases (20%), thereby representing the second most frequent recurrent single-gene mutation in our series, after *KIT* mutations (Figure 2,Table 2). We identified one frameshift in *NF1* in one case, and two tumors carried two *NF1* variants each, all of which constitute putative loss-of-function mutations (Figure 3).[28,29] As expected, oncogenic mutations in *KIT*, *RAS* isoforms and *BRAF* were mutually exclusive (Figure 2). Furthermore, these mutations were also mutually exclusive with *NF1* mutations, indicating significance of *NF1* loss as a newly identified oncogenic event in anorectal melanoma.

Recurrent *SF3B1* Mutations in Anorectal Melanoma

Three cases showed mutations in *SF3B1*, previously described in uveal melanoma and at very low frequency in cutaneous melanoma, but not, to our knowledge, in mucosal melanoma[30,31] (Figure 4a). In all three cases, mutations occurred at codon 625, located in the fifth HEAT (Huntingtin, elongation factor 3, protein phosphatase 2A subunit PR65/A, *TOR1*) domain repeat,[32] and comprised one R625H and two R625C substitutions (Table 2). An overwhelming majority of *SF3B1* mutations in uveal melanomas occur at this residue (Figure 4a), and our findings identify *SF3B1* (R625) mutations as a recurrent event also in mucosal melanomas. Interestingly, codon 625 mutations do not predominate in hematological malignancies, as mutations in codons 622, 662, 666, and 700 are frequent in myelodysplastic syndrome as well as chronic lymphocytic leukemia/small lymphocytic leukemia (http://cancer.sanger.ac.uk/cosmic, Figure 4b). In our series of anorectal melanomas, *SF3B1* (R625) mutations were seen in combination with other driver events, as two tumors carried activating *KIT* mutations and one tumor showed a deleterious mutation in *NF1* (Figure 2).

A subset of Anorectal Melanomas Carry Mutations in Genes Affecting Genomic Stability

Five cases in our series demonstrated mutations in genes involved in DNA damage repair (*TP53*, *BRCA1*, and *MLH1*). Missense mutations in *TP53*

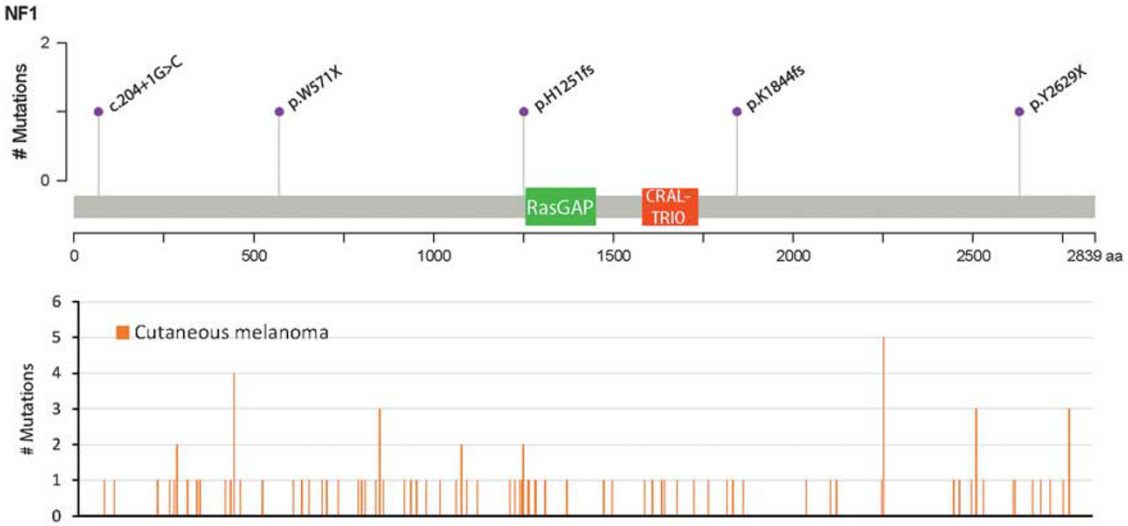

Figure 3 NF1 mutations in anorectal melanoma, schematic representation (top). Mutations are distributed throughout the protein. RasGAP: GTPase-activator protein for Ras-like GTPase domain (AA 1256–1451); CRAL-TRIO, C-terminal CRAL-TRIO phospholipid binding domain (AA 1591–1736). Frequency of NF1 mutations in cutaneous melanoma, as reported in the COSMIC database (bottom).

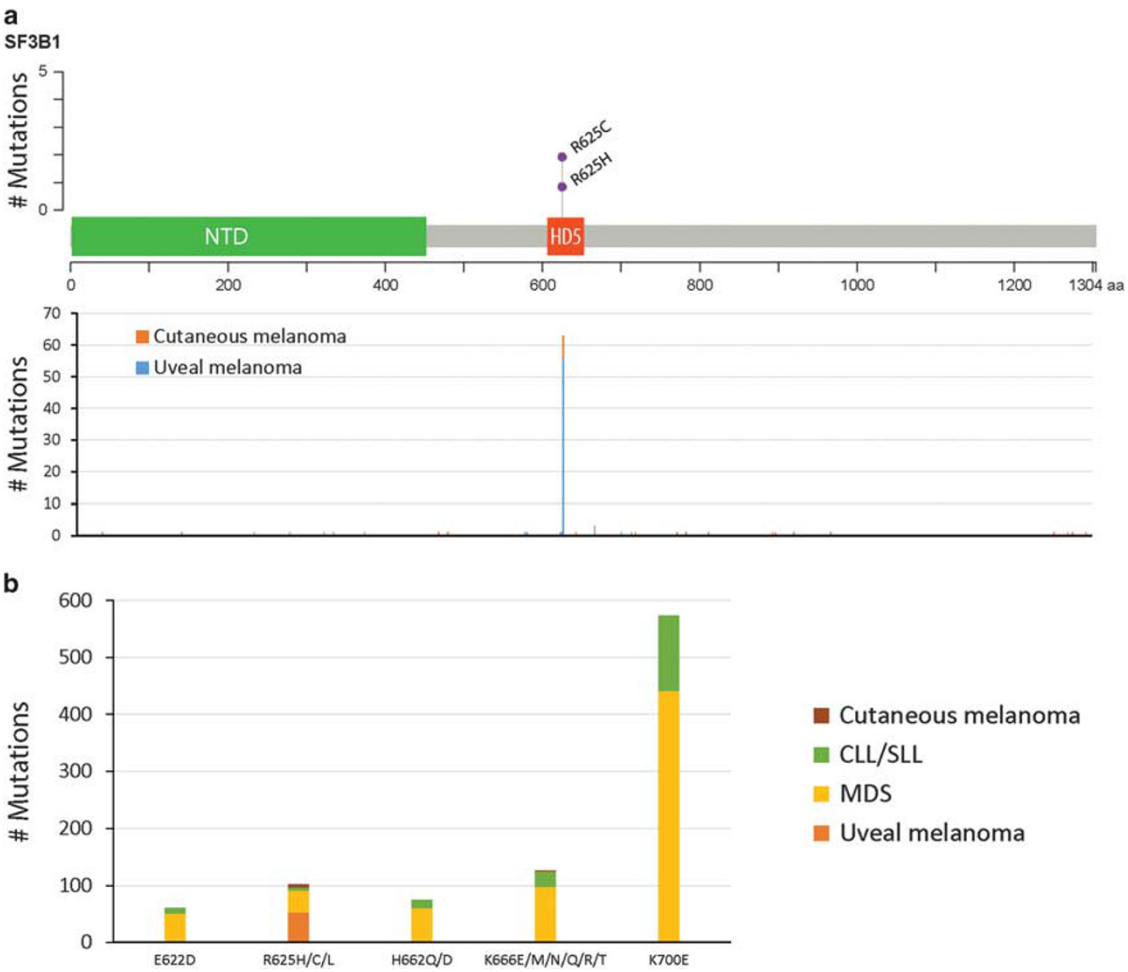

Figure 4 Distribution of SF3B1 mutations. (**a**) SF3B1 mutations in anorectal melanoma, schematic representation (top). Recurrent mutations are clustered in the region encoding HEAT domain 5. HD, HEAT domain; NTD, N-terminal domain (AA 1–450). Frequency of SF3B1 mutations in cutaneous and uveal melanomas, as reported in the COSMIC database (bottom). (**b**) Frequency of SF3B1 hotspot mutations by the major tumor types harboring these variants, as reported in the COSMIC database. CLL/SLL, chronic lymphocytic leukemia/small lymphocytic leukemia; MDS, myelodysplastic syndrome.

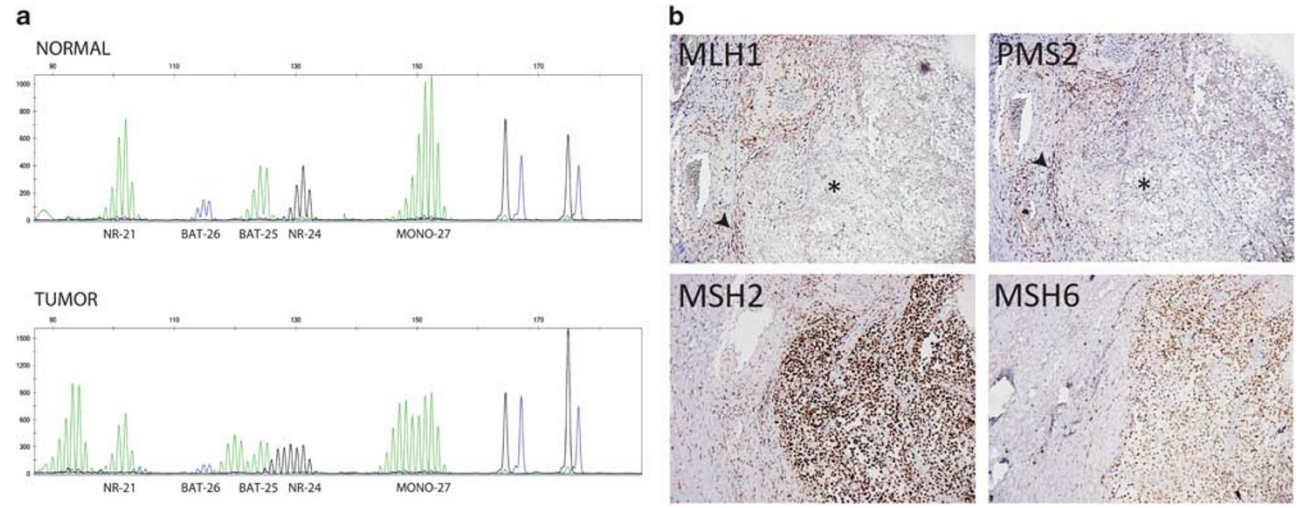

Figure 5 Evidence of genetic instability in anorectal melanoma with MLH1 (G67R) mutation (case 7). (**a**) Microsatellite instability testing by PCR shows altered alleles at 5/5 tested loci in the tumor (bottom panel), but not in normal control tissue (top panel). (**b**) Immunohistochemistry. Loss of nuclear expression of MLH1 and PMS2 in melanoma cells (asterisk). Expression of MSH2 and MSH6 is retained. Note retained expression of all mismatch repair proteins in peripheral stromal tissue (arrowhead) (original magnification × 100).

were found in three of 15 cases (20%), two of which were previously reported as pathogenic mutations involving the DNA binding domain (Y220C, C242Y), as well as one nonsense mutation involving the N-terminal/transactivation domain (Table 2). Furthermore, whereas one tumor showed a deleterious frameshift mutation in *BRCA1* (T557fs), a tumor suppressor gene involved in DNA repair and homologous recombination, in one case we detected a pathogenic *MLH1* (G67R) mutation, reported previously in individuals with hereditary non-polyposis colorectal cancer syndrome (Lynch syndrome).[33] The variant allele frequency was 53%, suggesting a heterozygous *MLH1* mutation (Supplementary Table 1). This case showed a higher mutation rate (33 variants) compared to the average number (14.1) of variants detected in our panel of 467 genes tested, and furthermore showed multiple frameshift mutations at mononucleotide repeat sequences involving additional cancer-related genes, consistent with a mutator phenotype (Figure 2, Supplementary Table 1). PCR testing in this tumor confirmed MSI in five of five loci ('MSI-High', Figure 5a), whereas immunohistochemical analysis for mismatch repair proteins MLH1, MSH2, PMS2, and MSH6 demonstrated loss of MLH1 protein expression, as well as loss of dimerization partner PMS2, as expected [34] (Figure 5b). Interestingly, although this patient was elderly (81y) with a reported history of prostate cancer, clinical features were not typical of Lynch syndrome, as a personal and family history of colon cancer was lacking.

Discussion

Melanoma is a heterogeneous disease, characterized by distinct molecular subsets based on anatomic site as well as level of sun-exposure.[35] Here, we describe the mutational landscape in a series of 15 anorectal melanomas, a rare melanoma subtype with an annual incidence of <1.0 per million population in the United States, which nevertheless shows a rising incidence.[1] Importantly, we identify driver events in 14 of 15 anorectal melanomas, a majority of which representing potentially actionable mutations.

Primary oncogenic events previously described in melanomas arising in the oral mucosa and anogenital region include activating *KIT* mutations in 15–30% of cases,[11–13] whereas oncogenic *BRAF* mutations are infrequent (~6%).[36] In a large case series analyzing 26 anorectal melanomas for *BRAF*, *NRAS*, *KIT*, and *PDGFRA* mutations, *KIT* mutations were detected in three cases (15%), one tumor (5%) harbored an *NRAS* mutation and *BRAF* point mutations were absent.[11] Our findings confirm recurrent activating *KIT* mutations in anorectal melanomas, which represented the predominant single-gene mutation in our series (5/15 cases, 33%). Whereas three of five *KIT* mutations affected the juxtamembrane domain (exon 11) and are expected to predict response to c-Kit inhibitors,[15,17,37] imatinib may be less effective in melanomas with mutations involving the distal kinase domain.[16] A partial response to imatinib was reported in one patient with metastatic mucosal melanoma with a *KIT* exon 17 mutation (D820Y, present in one case of our anorectal melanoma series),[17] but disease progression observed in another patient with metastatic acral melanoma harboring the same mutation.[15] In addition to imatinib, multikinase inhibitor sorafenib and newer-generation tyrosine kinase inhibitors such as nilotinib, dasatinib, and masitinib have shown some efficacy in metastatic mucosal melanomas harboring *KIT* exon 11 or 13 mutations.[38,39] However, development of resistance to c-Kit

inhibition is common,[40] and although nilotinib may achieve temporary disease control in some imatinib-resistant mucosal melanomas,[18] these examples illustrate limitations for c-Kit inhibitor-based therapy.

We did not assess copy number variation in the present study, and *KIT* amplifications, which can occur in *KIT*-mutant as well as *KIT*-wt tumors, may represent an additional mechanism of elevated c-Kit activity in our series of anorectal melanomas. However, mucosal melanomas harboring only *KIT* amplifications alone were found to be largely insensitive to tyrosine kinase inhibitors.[17,37]

Mutation frequency in *RAS* and its canonical downstream effectors was low in our series as expected, and interestingly, revealed variants that are rarely observed in cutaneous melanomas: *HRAS* hotspot mutations, present in one case of anorectal melanoma, occur in 10–15% of Spitz nevi but in $<1\%$ of cutaneous melanomas.[25,41] Furthermore, we identified a duplication of threonine in the *BRAF* activation loop (p.T599dup), a rare mutation with only one case reported in melanoma,[42,43] and previously not described in mucosal melanomas. Interestingly, *BRAF* (T599dup) mutations are also seen in rare cases of colorectal adenocarcinomas,[44] and display *in vitro* kinase activity and cellular MEK/ERK activation potential comparable to *BRAF* (V600E).[26] Of note, *BRAF* (T599dup) is not detected by immunohistochemistry using BRAF-antibody VE1,[45] and our findings therefore demonstrate that more extensive sequencing is warranted in anorectal melanomas negative for *NRAS/BRAF* hotspot mutations. Consistent with previous case reports in mucosal melanomas, activating mutations in *KIT*, *BRAF*, and *RAS* isoforms were mutually exclusive in our series of anorectal melanomas.

To our knowledge, this is the first study to identify *NF1* loss-of-function mutations as a recurrent mutational event in melanomas of the anal canal. *NF1* mutations showing C>T transitions are known to occur in cutaneous melanomas lacking *BRAF/NRAS* hotspot mutations, and are also prevalent in pure desmoplastic melanomas, implicating UV-radiation as an oncogenic factor in these melanoma subtypes.[28,29,41,46] However, our findings of *NF1* mutations in 20% of anorectal melanomas in which sunlight as an etiologic factor can be excluded suggest alternative mechanisms of mutagenesis. As a RAS-specific GTPase-activating protein, NF1 negatively regulates RAS and thereby MAPK pathway activity. Importantly, NF1 loss-of-function was associated with RAS activation as well as with MEK dependence in melanoma cell lines, although other studies indicated that NF1 suppression is not always associated with MEK-inhibitor response *in vitro*.[27,47] Nevertheless, MEK inhibition is an attractive strategy for tumors with *NF1* loss-of-function mutations,[38] and phase II clinical trials for Selumetinib-based therapy of inoperable neurofibromas in patients with germline *NF1* loss-of-function mutations are currently underway (clinicaltrials.gov). As a group, we identified oncogenic events leading to MAPK pathway hyperactivation in 6 of 15 cases (40%), either by rare activating mutations in *BRAF* or *RAS* isoforms, or by *NF1* loss-of-function mutations, implicating opportunities for MEK inhibitor-based therapy in a significant subset of anorectal melanomas.

Interestingly, three cases in our series demonstrated mutations in *SF3B1* at codon 625. In uveal melanoma, *SF3B1* mutations occur exclusively at codon 625 and correlate with absence of adverse prognostic factors, such as monosomy 3 and mutations in *BAP1*.[30] In cutaneous melanoma, *SF3B1* (R625) mutations occur at very low frequency ($<1\%$),[31] and to our knowledge our study is the first to identify recurrent *SF3B1* codon 625 mutations in mucosal melanomas (20%). Interestingly, *SF3B1* mutation pattern and correlation with outcome appear to be tumor-specific: mutations at codon 700 predominate in hematological malignancies as well as in breast cancer, and are associated with favorable outcome in myelodysplastic syndrome, but with poor survival in chronic lymphocytic leukemia.[48] Functionally, loss of *SF3B1*, which encodes subunit 1 of the RNA splicing factor 3b protein complex, leads to missplicing of critical neural crest transcription factors in a zebrafish model.[49] *SF3B1* mutations identified in uveal melanoma, however, do not appear to be associated with missplicing, and their functional consequences are unknown.[30] Whether *SF3B1* (R625) mutations are prognostically significant in mucosal melanoma remains to be determined—in our series, all three patients whose tumors carried *SF3B1* mutations developed visceral or nodal metastases. Although our case numbers are limited, these findings contrast with previous studies showing that *SF3B1* (R625) mutations are rare in metastatic uveal melanoma.[30]

Similar to findings in cutaneous melanoma,[29,41] mutations in tumor suppressor genes *NF1* and *BRCA1* in our series of anorectal melanomas occurred in tumors lacking hotspot mutations in *RAS* or *KIT*. However, two tumors showed concurrent mutations in *KIT* and *TP53*. *TP53* mutations have been reported in up to 19% of cutaneous melanomas overall, with increased mutation frequencies detected in desmoplastic melanomas and melanomas occurring on chronically sun-damaged skin.[28,29,50] Interestingly, a previous study identified non-silent *TP53* mutations in 28% of mucosal melanomas, including in three anal melanomas.[51] Similarly, *TP53* mutations were observed at a frequency of 18% in a recent study of melanomas of the female genital tract.[52] Our study therefore confirms *TP53* mutations as a recurrent mutational event in anorectal melanomas (20%). We furthermore identified pathogenic mutations in one case each for *BRCA1* and *MLH1*. Although carriers of *BRCA2* germline mutations have an increased risk for development of cutaneous melanoma,[53]

associations with *BRCA1* mutations are less well established, and to our knowledge the deleterious *BRCA1* (T557fs) mutation described here has not been previously identified in melanoma tissue.

The pathogenic *MLH1* (G67R) mutation identified in our anorectal melanoma series was previously reported in several Lynch syndrome families, and deleteriously affects protein function.[33,54] Accordingly, this tumor demonstrated MSI and loss of mismatch repair protein expression, and furthermore showed the highest mutational load of all cases. Interestingly, this tumor carried several frameshift mutations at mononucleotide repeat sequences involving additional cancer-related genes, suggesting that these mutational events occurred secondary to genomic instability. As allele frequency estimates suggested a heterozygous *MLH1* mutation (variant allele frequency 53%), inactivation of the second allele in this case was likely epigenetic. Notably, the clinical history did not support a diagnosis of Lynch syndrome in this elderly patient (81 years of age), making an *MLH1* germline mutation unlikely. These findings furthermore contrast with the low frequency of mismatch repair gene mutations in sporadic MSI-High colon cancers.[55] In cutaneous melanoma, lack of comprehensive mutation analysis in the majority of studies to date precludes distinction between epigenetic inactivation versus primary mutational events.[56] Biallelic somatic inactivation of *MLH1* through chromosomal deletion and splice site mutation, respectively, has been described in one case of primary cutaneous melanoma, and deletions of entire exons of the *MLH1* gene occur in a significant proportion of cutaneous melanomas and correlate with decreased patient survival.[57,58] Taken together, our findings implicate MSI in the pathogenesis of a subset of anorectal melanomas, and together with the identification of deleterious *BRCA1* as well as *TP53* mutations are in agreement with the hypothesis that genomic instability has a role in tumor development.[59]

Finally, we note that activating mutations in *GNAQ/GNA11*–seen in blue nevi and related melanocytic neoplasms, as well as in uveal melanomas[60] —are absent in anorectal melanomas, in accordance with studies reporting absence of *GNAQ/GNA11* mutations in melanomas of the female genital tract.[52,61] No mutations were furthermore identified in beta-catenin, a gene infrequently mutated in so-called 'triple-wild type' cutaneous melanomas, which lack *BRAF*, *NRAS*, and *NF1* mutations.[41]

In summary, whereas previous reports describe driver events such as activating *KIT* mutations in up to 30% of mucosal melanomas, our study reveals that comprehensive molecular analysis could identify melanoma-associated mutational events in the majority of anorectal melanomas. Furthermore, we show that anorectal melanomas are genetically heterogeneous. Predominant molecular subgroups include *KIT*-mutant tumors (33%), as well as tumors carrying mutations expected to result in MAPK pathway hyperactivation (40%). The latter group includes recurrent loss-of-function mutations in *NF1* (20% of cases), a novel finding in mucosal melanomas, as well as rare mutations targeting *BRAF* and *RAS* isoforms, and extended sequence analysis in anorectal melanomas negative for *BRAF/NRAS* hotspot mutations is warranted. Importantly, as these mutations as well as *NF1* mutations appear to be mutually exclusive with *KIT* mutations in anorectal melanoma, our findings raise the possibility that MEKi may expand the armamentarium of effective targeted therapeutics for patients whose tumors lack actionable *KIT* mutations. In this regard, although evidence for efficacy of MEKi in *BRAF/NRAS*-wt melanomas is still sparse, patients with rare tumors such as anorectal melanoma may benefit from novel clinical trial designs ('basket' studies), which aim to exploit putative predictive biomarkers to increase eligibility for patients with rare heterogeneous diseases, rather than focusing on cancer entities.[62] A third molecular group identified in our series of anorectal melanomas represents tumors with a spectrum of mutations potentially affecting genomic stability (*BRCA1*, *MLH1*, *TP53*). The significance of recurrent *SF3B1* (R625) mutations in mucosal melanoma, also a novel finding of this study, remains to be determined.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol)