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## Kinase Gene Fusions in Defined Subsets of Melanoma

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### Summary

Genomic rearrangements resulting in activating kinase fusions have been increasingly described in a number of cancers including malignant melanoma, but their frequency in specific melanoma subtypes has not been reported. We used break-apart fluorescence *in-situ* hybridization (FISH) to identify genomic rearrangements in tissues from 59 patients with various types of malignant melanoma including acral lentiginous, mucosal, superficial spreading, and nodular. We identified four genomic rearrangements involving the genes *BRAF*, *RET*, and *ROS1*. Of these, three were confirmed by IHC or sequencing and one was found to be an *ARMC10-BRAF* fusion that has not been previously reported in melanoma. These fusions occurred in different subtypes of melanoma but all in tumors lacking known driver mutations. Our data suggest gene fusions are more common than previously thought-and should be further explored particularly in melanomas lacking known driver mutations.

### Keywords

Melanoma; rearrangement; pan-negative; acral; kinase

### Introduction

Metastatic melanoma is an aggressive cancer which until recently had a 5-year survival rate of less than 15% when spread to distant sites (Leung et al., 2012, Chang et al., 1998). Approximately 90% of all melanomas arise on chronically sun-damaged skin or intermittently sun-exposed skin, traditionally classified into superficial spreading, lentigo maligna, and nodular subtypes (Bulliard et al., 2007, Whiteman et al., 2006, Shain and Bastian, 2016). The second most common type of melanoma (4%) is uveal melanoma,

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which occurs in the pigmented areas of the eye and is also partially related to UV exposure (Singh et al., 2011, Pane and Hirst, 2000). Although less common, other types can arise on non-sun-exposed areas such as acral lentiginous melanomas and mucosal melanomas. Acral lentiginous melanomas account for 2-3% of all melanomas and occur on non-glabrous skin such as the palms of the hands, soles of the feet, and underneath fingernails and toenails (Phan et al., 2007, Bradford et al., 2009). Mucosal melanomas (1%) arise in mucosa of the sinonasal, oral, anorectal, or vulvovaginal regions (Chang et al., 2015). While these melanoma subtypes differ in their location and molecular characteristics, they are all aggressive once they have metastasized and typically respond poorly to currently available therapy (Bhatia et al., 2009).

Nearly 50% of patients with cutaneous melanoma have an activating mutation in *BRAF* of which V600E is the most common mutation, which can be treated with inhibitors specific for the *BRAF*<sup>V600E</sup> mutation or its downstream kinase MEK (Davies et al., 2002 Davies et al., 2015, McArthur et al., 2014, Holderfield et al., 2014). However, *BRAF*<sup>V600E</sup> mutations are less common in other melanoma subtypes (approximately 10-15% in acral lentiginous and mucosal melanomas) (Yamazaki et al., 2015, Menzies et al., 2012, Lee et al., 2011, Sasaki et al., 2004). Other oncogenic driver mutations in genes such as *NRAS*, *KIT*, *GNAQ*, and *GNA11* have been identified in different melanoma subtypes but at a much lower frequency (Hodis et al., 2012). Overall, approximately 25% of cutaneous melanomas and nearly 40-50% of non-cutaneous melanomas lack these common driver mutations (“pan-negative”) and have no targeted therapy options available (Xia et al., 2014).

An alternate mechanism of BRAF activation, gene fusion, has been identified across several cancer types and has been found in a small percentage of cutaneous melanomas (Hutchinson et al., 2013, Menzies et al., 2015, Ross et al., 2015, Palanisamy et al., 2010, Botton et al., 2013, Stransky et al., 2014). Genomic alterations include structural changes in chromosomes. Gene fusions are the result of genomic rearrangements that join two previously separated genes. Oncogenic fusions in common kinases such as *BRAF*, *ALK*, *MET*, *ROS1*, *RET*, and *NTRK1* have been found in Spitz nevi and Spitzoid melanomas (Wiesner et al., 2014, Yeh et al., 2015). Analysis of the TCGA database containing 328 cutaneous melanomas identified 223 different fusion events including *BRAF* and *RAF1* kinases, however no fusions in *ALK*, *ROS1*, *RET*, or *NTRK1* were identified in this set of cutaneous melanomas (Cancer Genome Atlas, 2015). In a recent study, *ALK* fusions were reported in 4/30 (13%) of acral lentiginous melanomas, and none were identified in the mucosal melanomas (Niu et al., 2013). These studies suggest that the genes involved in oncogenic fusion events in melanoma may be subtype-specific, however the frequency of *BRAF*, *ALK*, *ROS1*, *RET*, and *NTRK1* fusions has not been systematically studied across different melanoma subtypes. Characterizing kinase fusions in specific melanoma subtypes is critical in understanding the development of these melanomas and devising new treatment strategies.

In this study, we used break-apart fluorescence *in situ* hybridization (FISH) to screen for kinase rearrangements in *BRAF*, *ALK*, *ROS1*, *RET*, and *NTRK1* in different subtypes of melanoma from 59 patients. We identified genomic rearrangements in tumors from four patients involving *BRAF*, *RET*, and *ROS1*. These rearrangements occurred in superficial

spreading, acral lentiginous, and unknown primary subtypes, and we demonstrated three out of four rearrangements result in gene fusions by IHC or targeted RNA sequencing. These findings highlight the differences in gene rearrangements between specific melanoma subtypes, and prompt further characterization of the gene fusion landscape in all types of melanomas.

## Results

### Break-apart FISH identifies genomic alterations in *RET*, *ROS1*, *NTRK1*, and *BRAF*

In order to identify genomic rearrangements in *BRAF*, *ALK*, *RET*, *ROS1*, and *NTRK1* kinase genes, we used break-apart FISH to screen 98 samples from 59 patient tumors (27 primary and 32 metastatic) across multiple subtypes including superficial spreading, nodular, acral lentiginous, mucosal, lentigo maligna, desmoplastic, and unknown primary (Table 1). We identified 4 samples that had genomic rearrangements (Figure 1A, D-F) and 2 samples that showed an atypical hybridization pattern of *NTRK1* (Figure 1B-C).

One sample from a patient with acral lentiginous melanoma of the plantar surface of the foot (#30) had a *RET* hybridization pattern showing single copies of the 3' probe consistent with a rearrangement (Figure 1A). Another sample from a patient with acral lentiginous foot melanoma (#29) had an atypical FISH pattern for *NTRK1*. In this sample, there was both clustering of the *NTRK1* signal and separation of the 3' and 5' signals (Figure 1B). The distance between them was, however, below the scoring threshold used to indicate the presence of a break in the gene. Multiple samples from this case were examined by FISH and interestingly the different samples had discordant results. Two samples were classified as *NTRK1* atypical while two other samples from the same tumor were classified as negative for any atypical *NTRK1* patterns suggesting intra-tumoral heterogeneity. H&E staining and *NTRK1* FISH analysis of the original patient specimen showed the negative biopsies were taken from a region which contained only early stage in-situ melanoma cells, whereas the *NTRK1* atypical biopsies were taken from a region of the same sample which contained more advanced vertical growth phase tumor cells (Fig. S1). An atypical FISH pattern for *NTRK1* was also observed in a nasal sinusoid mucosal melanoma sample (#41), where single signals of the 5' portion of *NTRK1* were adjacent to an intact copy of the *NTRK1* gene (Figure 1C). Both *NTRK1* atypical hybridization patterns suggest the presence of an unknown genomic alteration but does not indicate a rearrangement or a gene fusion. These three cases are the first demonstrations of *RET* and *NTRK1* genomic alterations in melanoma subtypes other than Spitz nevus and Spitzoid melanoma.

Samples from eleven superficial spreading cutaneous melanomas were examined and two had rearrangements. A *ROS1* rearrangement was identified in a sample from patient #8 where single signals were observed for the 3' probe (Figure 1D), and in a sample from patient #9 a *BRAF* rearrangement was identified with split 3' and 5' signals (Figure 1F). Samples from four unknown primary melanomas were included in the analysis. A *BRAF* rearrangement with extra copies of single 3' *BRAF* signals was observed in a metastatic lymph node in one patient with an unknown primary melanoma (Figure 1E). Overall, 2 out of 11 superficial spreading melanomas (18.2%), 1 out of 17 acral lentiginous melanomas (5.9%), and 1 out of 3 unknown primary tumors (33%) had genomic rearrangements.

## NTRK1, RET, and ROS1 expression in tumors with genomic rearrangements

Rearranged or atypical hybridization patterns by FISH do not necessarily indicate a functional gene fusion event. We used immunohistochemistry (IHC) with kinase-domain specific antibodies for NTRK1, RET, and ROS1 to determine if protein was expressed in the rearranged or atypical samples. The sample from patient #30 with a *RET* rearrangement from an early-stage primary in-situ acral lentiginous melanoma showed protein expression suggesting the presence of a functional gene fusion (Figure 2A).

The sample from patient #29 with an atypical *NTRK1* FISH hybridization pattern showed scattered expression of the NTRK1 protein suggesting the atypical genomic alteration results in protein expression (Figure 2B). Expression of NTRK1 was observed only in scattered malignant melanocytes in the sample from patient #41 (Figure 2C) and was substantially different from patient #29. The conclusion from IHC is consistent with the FISH analysis which found different genomic events between the two tumors.

No IHC expression of ROS1 was demonstrated for patient #8 (Figure 2D). Real-time qPCR was also performed on tumor tissue and ROS1 transcript expression was undetectable, confirming the negative IHC result (Fig. S3). These data suggest the genomic rearrangement did not result in a functional gene fusion.

## Characterization of *BRAF* rearrangements

*BRAF* fusions in melanoma have been reported to occur frequently in tumor samples with a Spitz-like morphology (Menzies et al., 2015, Botton et al., 2013). Both of our samples with *BRAF* rearrangements were examined by an experienced dermatopathologist, and did not show Spitz cytomorphology (Figure 3A). We performed targeted RNA sequencing for each sample positive for a *BRAF* rearrangement and identified the *BRAF* fusion transcripts and 5' gene partners.

In the metastatic sample from patient #9 with superficial spreading melanoma, an *AGK-BRAF* fusion transcript was found that fused exon 2 of *AGK* with exons 8 through 18 of *BRAF* (Figure 3B). The sample from patient #56, a melanoma of unknown primary, was found to have an *ARMC10-BRAF* transcript with a fusion of exons 1-4 of *ARMC10* and exons 11-18 of *BRAF* (Figure 3B). The fusion and breakpoint were confirmed for both patient samples using RT-PCR and Sanger sequencing (Figure 3C). *AGK-BRAF* fusions have been previously identified in melanomas (Menzies et al., 2015, Botton et al., 2013), however, this is the first report of an *ARMC10-BRAF* fusion occurring in a melanoma.

## Classification of genomic alterations

Break-apart FISH analysis identified genomic rearrangements in tumors from four melanoma patients. Tumor from patient #30 with a *RET* rearrangement also had positive RET IHC staining. Because RET is normally hardly detectable, positive RET staining suggests upregulation due to a gene fusion. There was, however, not sufficient tumor material for confirmation by targeted RNA sequencing (Table 2). Tumor from patient #8 with a *ROS1* rearrangement was negative for ROS1 protein expression by IHC, therefore a functional kinase fusion did not occur. Targeted RNA sequencing was performed on RNA

from this tumor, and as expected, no *ROS1* fusion transcripts were identified (Table 2). This result is consistent with our real-time qPCR data (Fig. S3). *BRAF* rearrangements in tumors from patients #56 and #7 were confirmed using targeted RNA sequencing. Melanomas express endogenous BRAF protein so IHC cannot be used for validation (Table 2). Therefore, we identified a total of three kinase fusions occurring in *BRAF* and *RET* genes.

Tumors from two patients, #29 and #41, were negative for rearrangements in the break-apart FISH analysis but had atypical hybridization patterns for *NTRK1*. Although these tumors did not have rearrangements, they were IHC positive for NTRK1 suggesting unknown genomic alterations led to expression of NTRK1 protein. –p0trrr cccl>

### Kinase fusions occur in pan-negative patient samples

Using next generation whole exome sequencing, we examined all samples for the presence of mutations in seven common melanoma driver genes (*BRAF*, *NRAS*, *KRAS*, *HRAS*, *KIT*, *GNAQ*, and *GNAI1*) as well as *NFI*, which has been recently described as a major subgroup of melanoma

Consistent with previous observations, all 6 samples with genomic alterations detected by FISH, across different subtypes, occurred in pan-negative melanoma patients whose tumor lacked any known hotspot mutations in common driver genes (Figure 4) (Botton et al., 2013, Hutchinson et al., 2013). We observed an *NFI* mutation in the *ROS1* rearranged superficial spreading melanoma tumor, however this rearrangement did not result in a functional gene fusion. The frequency of kinase fusions within the subset of pan-negative melanomas showed strong enrichment in cutaneous, acral lentiginous, and unknown primary subtypes compared to the overall frequency (Table 3). These findings suggest kinase fusions are more frequent across multiple subtypes of pan-negative melanomas.

## Discussion

In this study we report a high frequency of kinase fusions in pan-negative melanoma samples across different subtypes, which lack common driver gene mutations. We identified a *RET* fusion in an acral lentiginous melanoma and unknown genomic alterations in *NTRK1* in acral lentiginous and mucosal melanomas. These genomic alterations have not been previously described in melanoma. We did not find *RET* and *NTRK1* alterations in cutaneous melanoma subtypes. Conversely, we observed *BRAF* fusions in cutaneous melanoma subtypes but not in acral lentiginous or mucosal melanomas. Finally, we did observe a *ROS1* rearrangement in a cutaneous superficial spreading melanoma although lack of expression suggests this may not be a functional gene fusion. Our patient numbers are not large enough to conclude that these events are mutually exclusive.

Kinase fusions in *NTRK1* and *RET* have been previously reported in Spitz neoplasms (Wiesner et al., 2014) but have yet to be described in malignant melanoma. Here, we identified the first case of a *RET* fusion in an acral lentiginous melanoma. Acral lentiginous melanomas are known to have a lower mutational burden, but higher levels of genomic instability and more DNA copy number alterations (Curtin et al., 2005). We also found an atypical FISH pattern in an acral lentiginous melanoma which corresponded with NTRK1

protein expression. Interestingly, the atypical *NTRK1* FISH pattern was observed in more advanced vertical growth phase cells but not early stage melanoma in-situ tumor cells, suggesting the development of the atypical *NTRK1* genomic alteration may be associated with melanoma progression. Further investigation of the genomic *NTRK1* region is required to fully understand our observations and the tumorigenic potential and significance of *NTRK1* alterations in melanoma.

In our cohort, we also observed a *ROS1* rearrangement by FISH which did not express protein by IHC. To rule out the possibility of a false negative IHC result, we ran the samples with positive and negative controls that were stained within two hours of being cut (Fig. S2). The results were unchanged. Hence, the probability for a false negative outcome by IHC is unlikely. We also performed real-time qPCR and did not detect any *ROS1* transcript expression (Fig. S3). Alternate explanations for the inconsistency between the *ROS1* rearrangement FISH and IHC results include a 5' gene partner that is not expressed or an intergenic rearrangement where *ROS1* does not have a 5' gene partner.

Detection of activating rearrangements in the clinical arena can be complex and time-consuming. Structural genomic alterations such as potential gene fusions can be identified using FISH, but their expression must be confirmed by other methods since these alterations are not always functional. Using break-apart FISH as the only molecular diagnostic technique may result in false negatives when the target genes are in the same chromosome and are located less than 10 Mbp apart, unless the fusion is generated by a chromosomal deletion which encompasses the sequences of one of the probes. Examples of this have been observed with the *EML4-ALK* rearrangements in lung adenocarcinoma (Ying et al., 2013). IHC can generate false positives when protein expression is a consequence of pathway crosstalk (Sullivan et al., 2015). Multiplex analysis, such as joint FISH and IHC, increases the likelihood of detection and has been shown to increase efficacy of detection in contrast to single screening techniques. Nonetheless, there may still be discordant outcomes which could be a causal result of tumor heterogeneity, out-of-frame rearrangements, or minimally expressed fusion transcripts. Lambros et al. suggested using a massive parallel sequencing technique (MassARRAY) in conjunction with FISH and IHC to decrease the possibility of false positive or false negative results (Lambros et al., 2011). Overall there are methodological advantages and drawbacks to both FISH and IHC. In addition, FISH is of limited value in detecting new and previously undescribed rearrangements. This is obviously best done using RNA techniques as have been previously described (Lambros et al., 2011). We think multiplex analysis using a combination of FISH, RNA analysis, IHC, and other expression techniques will be needed to uncover a multitude of gene rearrangements important in the pathophysiology and treatment of melanoma and other cancers.

Altogether, our findings from FISH and IHC analyses may have clinical significance. We observed a high frequency of kinase fusions in pan-negative melanomas across most clinical subtypes. Kinase fusions are actionable therapeutic targets in other cancers and the same may be true for melanoma (Downing, 2008, Shaw and Engelman, 2014, Shaw et al., 2014, Shaw et al., 2013, Ross et al., 2015, Palanisamy et al., 2010). The demonstration here of activating kinase fusions in melanoma, particularly in pan-negative patient tumors, suggests expanded screening for fusions in patients with metastatic malignant melanoma should be



considered. The data presented likely provides only a limited scope of the frequency of fusions in melanoma. Further work in this area is warranted and could uncover other gene fusions that are important in the development, growth and treatment of melanoma.

## Materials and Methods

### Melanoma patient samples

Ninety-eight samples from 59 melanoma patients from the University of Colorado Denver Hospital, from 2008-2015, were included in the analysis. These were obtained as part of the International Melanoma Biorepository and Research Laboratory at the University of Colorado Cancer Center, with patient consent and approval from the Colorado Institutional Review Board (IRB# 05-0309). A total of 98 samples were randomly selected from superficial spreading, nodular, acral lentiginous, mucosal, desmoplastic, and unknown primary melanoma subtypes. Formalin-fixed, paraffin-embedded (FFPE) tissue microarrays (TMAs) were made from 1.5 mm punches of melanoma patient tumor FFPE blocks.

### Break-apart fluorescence *In Situ* hybridization

This technique uses two different colored fluorescent FISH probes designed in the 5' and 3' portion of a gene to identify potential gene fusions. The two signals will overlap in normal cells, but there will be only one signal or split apart signals in cells where a genomic rearrangement in the gene has occurred. Dual-colored FISH assays were performed using break-apart (BA) probes for *ALK*, *BRAF*, *RET*, and *ROS1* genes, using commercial reagents (Abbott Molecular). The *NTRK1* probe was developed in our laboratory as previously described (Vaishnavi et al., 2013). The *BRAF*BA probe set was developed for this study using BAC clones RP11-837G3 and RP11-948O19 containing human DNA inserts from regions homologous to the 3' and 5' end of *BRAF*, respectively. The signals from these clones were overlapped or very close in the native copy of the gene. Assays in the tissue microarrays (TMAs) were performed as previously described (Toschi et al., 2014).

The BA FISH analyses were conducted as previously described with minor modifications (Davies et al., 2012). Duplicate tissue cores for one or more specimens per patient were analyzed. A total of 50 cells were scored in each core. For each probe set, 3' and 5' signals physically separated by 1 signal diameter were considered split. Specimens were considered positive for specific gene rearrangement if >15% of the cells showed split signals or single 3' or 5' signals.

### Immunohistochemistry

Archival FFPE specimens were stained as previously described (Narita et al., 2009, Zhang et al., 2005), with the following modifications and antibodies: NTRK1 staining was performed with citrate buffer target retrieval solution (TRS pH 6) and a one hour room temperature incubation with primary rabbit monoclonal [EP1058Y] TrkA antibody (ab76291, dilution: 1:500), followed by secondary antibody (Dako Envision+ Anti-Rabbit K4003, pre-prepared standard dilution (Elias et al., 1989)). RET staining was performed with citrate buffer target retrieval solution (TRS pH 6) and a one hour room temperature incubation with primary rabbit monoclonal [EPR2871] anti-RET antibody (ab134100, dilution: 1:50), followed by

secondary antibody (Dako Envision+ Anti-Rabbit K4003, pre-prepared standard dilution (Elias et al., 1989)). ROS1 staining was performed using ROS1 monoclonal antibody (Cell Signaling D4D6®, dilution: 1:25) under previously reported conditions (Boyle et al., 2015).

### Next-generation whole exome sequencing

Whole exome sequencing was done on samples from 51/59 melanoma patients. Blood samples were collected in PAXGene DNA tubes and stored at 4°C until processed. Depending on material, tissue genomic DNA was isolated using the DNeasy Blood and Tissue kit or the QiaAmp DNA FFPE kit. DNA concentration and purity was determined using Qubit (Thermo Fisher Scientific) and Agilent 2100 bioanalyzer analysis. 200 ng of genomic DNA was sheared using Covaris S220 at 150bp. Sheared DNA was end-repaired and used to construct the exome library following Agilent SureSelect XT Target Enrichment System for Illumina Paired End Multiplexed Sequencing Library (cat# G9641B). Exome capture was done through hybridization using XT5 probe. Resulting captured libraries were indexed and purified. The cDNA library was validated on the Agilent 2100 Bioanalyzer using DNA-1000 chip. Libraries were sequenced on the Illumina HiSeq 2000 with 125 bp pair-end reads. We obtained an average of 400× and 200× sequencing coverage for the cancer and normal exomes, respectively. Data for blood and tissue samples were analyzed using the data analysis pipeline IMPACT (Hintzsche et al., 2016), and germ line variants identified in normal blood were removed.

### Targeted RNA sequencing

RNA was isolated from fresh frozen patient tumor tissue using the RNeasy Plus Mini Kit (Qiagen). Tissues were homogenized with the TissueLyser II (2 × 2 minutes at 30 Hz) and on-column DNase I digest was performed per protocol using the RNase-free DNase I set (Qiagen). Library preparation and sequencing were performed by the University of Colorado Denver Genomics and Microarray Core. Targeted libraries were generated using the Ovation cDNA module and Ovation Fusion Panel Target Enrichment System (NuGEN, Inc.) with 250 ng - 750 ng RNA input as previously described (Scolnick et al., 2015). Paired-end sequencing (2 × 75 bp) was performed on a MiSeq instrument (Illumina) and approximately 1.5 million total reads were collected for each sample. Fusions were detected by analyzing FASTQ files on the BaseSpace data analysis hosting platform (Illumina) using the NuGEN Ovation Fusion Target App (NuGEN).

### RT- PCR, Sanger sequencing

RNA was isolated from fresh frozen patient tumor tissue using the RNeasy Plus Mini Kit (Qiagen). Tissues were homogenized with the TissueLyser II (2 × 2 minutes at 30 Hz) and on-column DNase I digest was performed per protocol using the RNase-free DNase I set (Qiagen). RNA was reverse transcribed into cDNA using the Verso cDNA Synthesis Kit (Thermo Scientific). PCR was performed using primers specific for the *AGK-BRAF* fusion (CGCTTCGAAATCACTGGAAGAA (sense); CCACGAAATCCTTGGTCTCTAATC (anti-sense)) and *ARMC10-BRAF* fusion (TCGCAGCCTGAAGACTTAAC (sense); GTGGAATAGCCCATGAAGAGTAG (anti-sense)). PCR products were purified using the QIAquick PCR purification kit (Qiagen) and submitted to the Barbara Davis Center for



sequencing using the BigDye Terminator Cycle Sequencing Ready Reaction kit version 3.1 (Applied Biosystems) with both forward and reverse primers used in the PCR reactions.

### Quantitative Real-Time PCR

The real-time PCR reaction was done in triplicate using PowerUp SYBER Green master mix (Thermo Scientific) and was analyzed on the StepOne Plus real-time PCR system (Applied Biosystems). Primer sequences are as follow: exons 1-2 (TCCGAAGCTTGCAATTTTGC (sense); TGTGCCAAGGTCAAGCTG (anti-sense)), exons 3-4 (AGGATGTCACTTTTGGAAGCTCTG (sense); TCTTCATATGCACCTTCCGC (anti-sense)), exons 36-37 (AAACTGACTCTGCGTCTCTTG (sense); CTTCTCCTGGTCTGTGGAAC (anti-sense)), exons 40-41 (GATGGCTCCAGAAAGTTTGATG (sense); TGCACATAGTTTAAACACATCAAGG (anti-sense)).

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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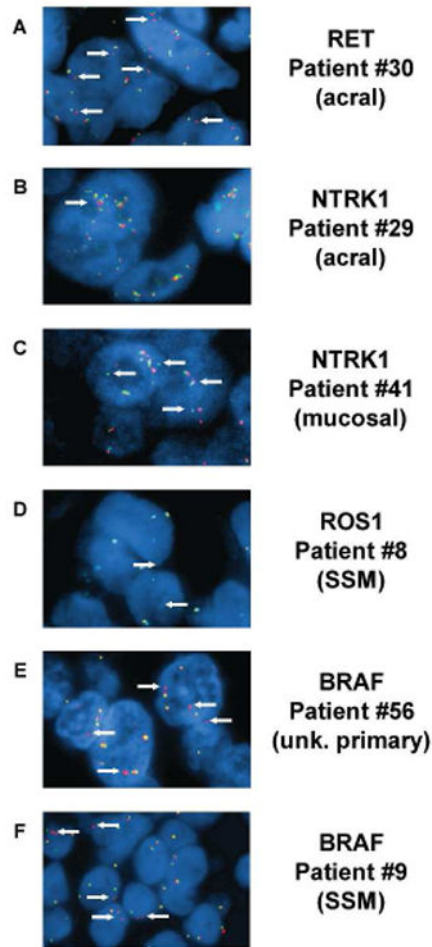
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### Significance

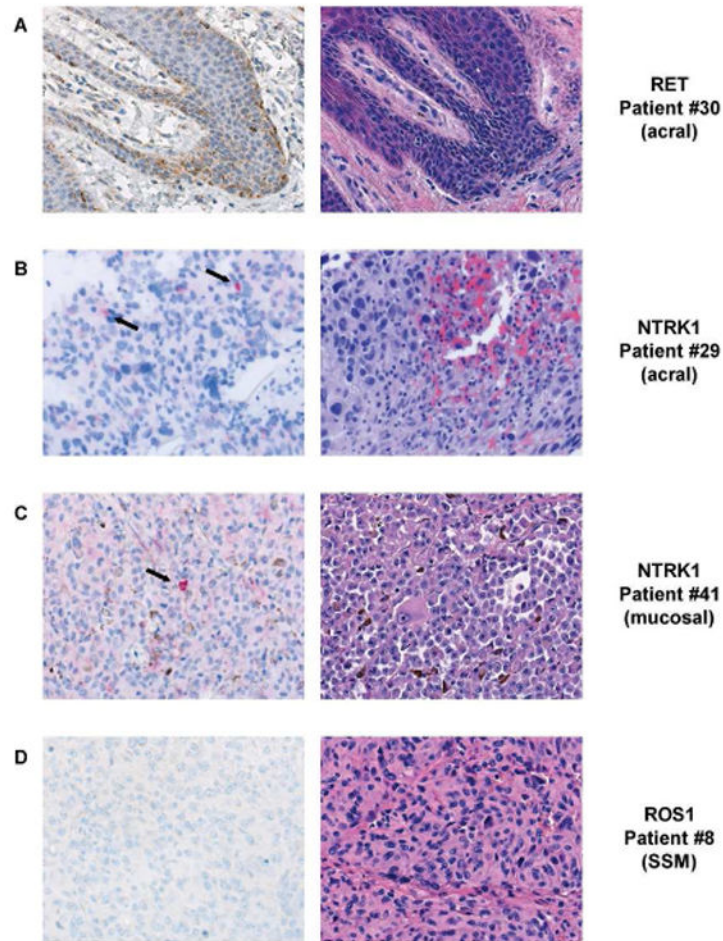
Driver mutations, which can be targeted therapeutically, occur in approximately 50% of patients with malignant melanoma. We show here that 3/59 (5.1%) of all melanoma patients and 3/33 (9.1%) of melanoma patients with tumors lacking hotspot mutations in common driver genes have kinase fusions that may serve as targets for therapeutic intervention. Our data suggest that broader screening for kinase fusions in melanoma should become part of clinical practice.



**Figure 1.**

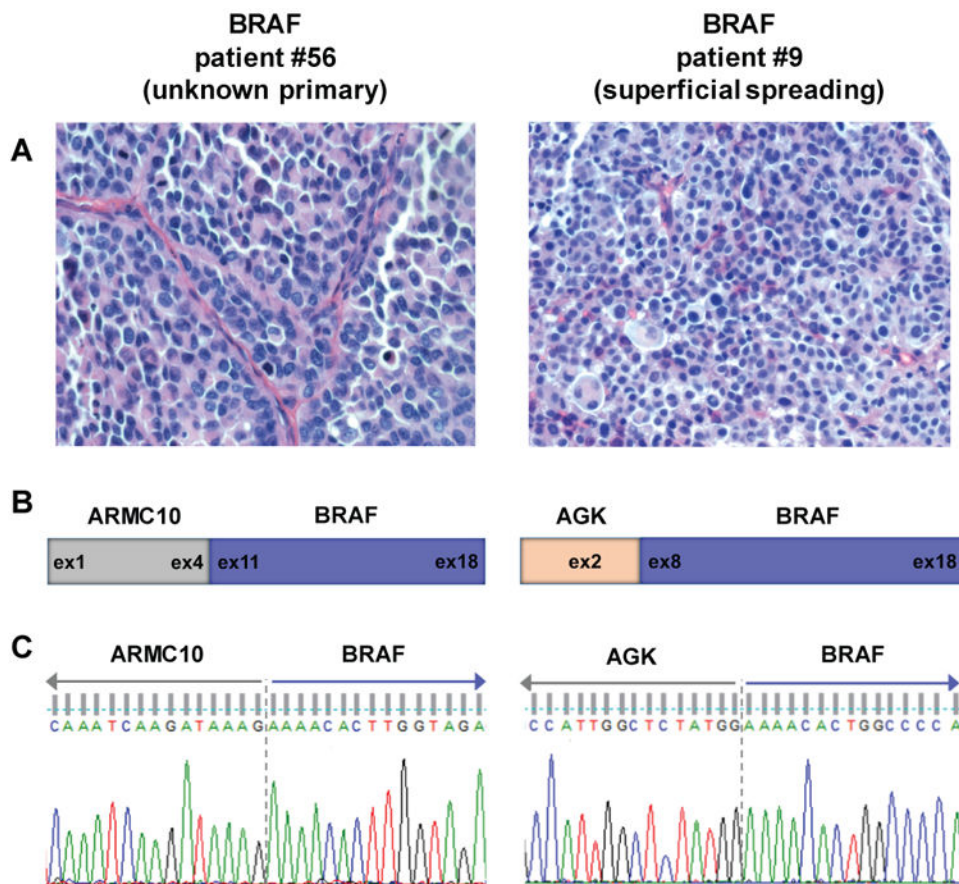
FISH images show rearrangements and alterations in different melanoma subtypes. Representative FISH images from tumors with rearrangement or atypical staining patterns. (A) An acral lentiginous melanoma from patient #30 harboring a RET rearrangement where single copies of the 3' probes are observed (white arrows). This sample is classified as a rearrangement. (B) An acral lentiginous melanoma from patient #29 shows an NTRK1 atypical FISH pattern including mini clusters of both probe signals (white arrows). This sample is classified as an unknown genomic alteration. (C) A mucosal melanoma with NTRK1 atypical FISH pattern where single copies of the 5' probes are seen adjacent to intact copies of NTRK1 (white arrows). This sample is classified as an unknown genomic alteration. (D) A superficial spreading melanoma from patient #8 shows single copies of the 3' ROS1 probe (white arrows). This sample is classified as a rearrangement. (E) An unknown primary melanoma from patient #56 shows single copies of the 3' BRAF probe (white arrows). This sample is classified as a rearrangement. (F) A superficial spreading melanoma from patient #7 shows single copies of the 3' BRAF probe. This sample is classified as a rearrangement.



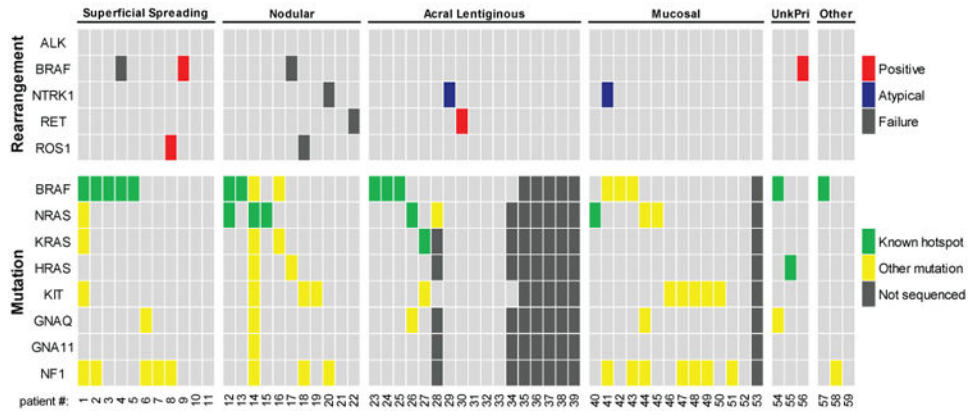


**Figure 2.**

Immunohistochemistry analysis of tumor samples with RET, and ROS1 genomic rearrangements and NTRK1 unknown genomic alterations. IHC analysis of tumors with rearrangement or atypical FISH hybridization pattern. (A) Tumor from patient #30, identified with a RET rearrangement by FISH, shows staining with the brown DAB chromagen (left panel) indicating expression of the RET protein. The H&E section of this tumor (right panel) shows large, pleomorphic tumor cells with some blood. (B) NTRK1 staining (black arrows) of a red chromagen in of an acral lentiginous melanoma from patient #29 with an unknown genomic alteration is shown (left panel) and the H&E stain of malignant tumor cells is shown (right panel). (C) Lower protein expression for NTRK1 is seen in scattered tumor cells precipitating the red chromagen (black arrow) in a sample from patient #41 (left panel) that was identified with an unknown genomic alteration by FISH. The matched H&E section (right panel) shows small amounts of melanin (brown pigment) are scattered throughout the section. (D) ROS1 IHC (left panel) of a superficial spreading melanoma from patient #8 found to have a ROS1 genomic rearrangement by FISH. The ROS1 IHC does not show any staining of the brown DAB chromagen (left panel) and thus is negative for ROS1 protein expression. The H&E stain of the tumor is shown (right panel).



**Figure 3.** Characterization and validation of BRAF fusions in rearranged samples. (A) Representative H&E stained images from each BRAF fusion tumor show lack of Spitz associated morphology in these tumors. (B) Schematic of exonic breakpoints in the BRAF fusions. Targeted RNA sequencing was used to identify the breakpoints and 5' partners for each BRAF fusion. (C) Sanger sequencing confirmation of BRAF fusion transcripts. RT-PCR products for each fusion were sequenced and chromatograms confirm in-frame transcripts for ARMC10-BRAF and AGK-BRAF fusions with the expected breakpoints.



**Figure 4.** Genomic alterations occur in melanomas without hotspot mutations in common driver genes in all subtypes. Results from the FISH analysis and whole exome sequencing are presented for each melanoma patient sample. Samples were classified positive if > 15% of cells exhibited rearrangement patterns. Samples were classified as atypical if they had a consistent atypical FISH hybridization pattern and were not positive for rearrangement. Patients within each subtype are grouped by the presence of driver mutations, followed by the presence of genomic rearrangements or alterations. Dark grey bars indicate missing data for the FISH analysis (failure of hybridization for that specific gene) and the whole exome sequencing analysis (failure of library preparation for sequencing or insufficient material to use for sequencing analysis). Clinical information was analyzed for mutation testing in patients where samples were unable to be sequenced, and resulted in only partially missing driver gene mutation status for some patients.

Table 1

## Clinical Characteristics of Melanoma Patients

No.	Subtype	Gender	Age	Sample Source	Tissue Source
1	Superficial spreading	M	75	Metastatic	Lymph node
2	Superficial spreading	M	46	Metastatic	GI
3	Superficial spreading	M	50	Metastatic	Lymph node
4	Superficial spreading	M	73	Metastatic	Subcutaneous
5	Superficial spreading	F	58	Metastatic	Brain
6	Superficial spreading	F	61	Metastatic	Thyroid
7	Superficial spreading	M	75	Metastatic	Subcutaneous
8	Superficial spreading	F	66	Metastatic	Lymph node
9	Superficial spreading	F	34	Metastatic	Subcutaneous
10	Superficial spreading	F	54	Primary	Right upper chest
11	Superficial spreading	F	62	Metastatic	Subcutaneous
12	Nodular	M	68	Metastatic	Bone
13	Nodular	M	66	Metastatic	Subcutaneous
14	Nodular	M	64	Metastatic	GI
15	Nodular	F	60	Metastatic	Subcutaneous
16	Nodular	M	88	Metastatic	Subcutaneous
17	Nodular	M	26	Metastatic	Subcutaneous
18	Nodular	M	78	Metastatic	Subcutaneous
19	Nodular	F	69	Metastatic	Brain
20	Nodular	M	74	Metastatic	Subcutaneous
21	Nodular	M	78	Primary	Left upper arm
22	Nodular	M	72	Metastatic	Brain
23	Acral lentiginous	M	60	Primary	Right ankle
24	Acral lentiginous	F	64	Primary	Bottom of right foot
25	Acral lentiginous	F	64	Metastatic	Breast
26	Acral lentiginous	M	61	Metastatic	Subcutaneous
27	Acral lentiginous	M	54	Primary	Left 4 <sup>th</sup> toe
28	Acral lentiginous	M	62	Primary	Right lateral foot

No.	Subtype	Gender	Age	Sample Source	Tissue Source
29	Acral lentiginous	F	39	Primary	Left thumbnail
30	Acral lentiginous	F	77	Primary	Left foot
31	Acral lentiginous	M	46	Metastatic	Subcutaneous
32	Acral lentiginous	F	56	Metastatic	Lymph node
33	Acral lentiginous	M	38	Primary	Left thumb
34	Acral lentiginous	M	66	Primary	Webspace of 4 <sup>th</sup> and 5 <sup>th</sup> toe
35	Acral lentiginous	F	61	Primary	Bottom of right foot
36	Acral lentiginous	M	87	Primary	Left great toe
37	Acral lentiginous	M	78	Primary	Sole of left foot
38	Acral lentiginous	F	66	Primary	Right third toe
39	Acral lentiginous	F	56	Primary	Left heel
40	Mucosal	F	35	Metastatic	Brain
41	Mucosal	M	75	Primary	Anorectal
42	Mucosal	F	85	Primary	Maxillary sinus
43	Mucosal	M	78	Primary	Pelvic cavity
44	Mucosal	F	97	Primary	Nasopharyngeal
45	Mucosal	F	51	Primary	Vaginal
46	Mucosal	F	88	Primary	Vulvovaginal
47	Mucosal	F	61	Metastatic	Lymph node
48	Mucosal	F	72	Primary	Rectum
49	Mucosal	F	63	Primary	Anal canal
50	Mucosal	F	68	Metastatic	Pancreas
51	Mucosal	F	66	Primary	Anorectal
52	Mucosal	F	78	Primary	Vaginal
53	Mucosal	F	71	Primary	Vulva
54	Unknown primary	F	49	Metastatic	Lymph node
55	Unknown primary	M	59	Metastatic	Subcutaneous
56	Unknown primary	M	54	Metastatic	Lymph node
57	Lentigo	M	68	Metastatic	Subcutaneous
58	Lentigo	M	46	Primary	Mid back
59	Desmoplastic	M	57	Metastatic	Lung

**Table 2**  
**Classification of genomic alterations based on findings from FISH, IHC, and targeted RNA sequencing**

No.	Gene	Technique					Classification		
		FISH	IHC	Targeted RNA seq	Rearrangement	Kinase fusion	Unknown genomic alteration		
Patient #30	RET	Positive	Positive	-*	Yes	Yes	No		
Patient #29	NTRK1	Negative, atypical	Positive	-	No	No	Yes		
Patient #41	NTRK1	Negative, atypical	Positive	-	No	No	Yes		
Patient #8	ROS1	Positive	Negative	Negative	Yes	No	No		
Patient #56	BRAF	Positive	-	Positive	Yes	Yes	No		
Patient #9	BRAF	Positive	-	Positive	Yes	Yes	No		

“-” indicates experiment was not performed

\* Attempted but unsuccessful due to lack of material



**Table 3**  
**Kinase fusion frequency in melanomas**

Subtype	Number of kinase fusions	
	All patients	Pan-negative patients
Superficial spreading	1/11 (9.1%)	1/6 (16.7%)
Nodular	0/11 (0%)	0/7 (0%)
Acral lentiginous	1/17 (5.9%)	1/5 (20%)
Mucosal	0/14 (0%)	0/12 (0%)
Unknown primary	1/3 (33.3%)	1/1 (100%)
Lentigo	0/2 (0%)	0/1 (0%)
Desmoplastic	0/1 (0%)	0/1 (0%)
<b>Total:</b>	<b>3/59 (5.1%)</b>	<b>3/33 (9.1%)</b>

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