



Spitz melanoma is a distinct subset of spitzoid melanoma

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Abstract

Melanomas that have histopathologic features that overlap with those of Spitz nevus are referred to as spitzoid melanomas. However, the diagnostic concept is used inconsistently and genomic analyses suggest it is a heterogeneous category. Spitz tumors, the spectrum of melanocytic neoplasms extending from Spitz nevi to their malignant counterpart Spitz melanoma, are defined in the 2018 WHO classification of skin tumors by the presence of specific genetic alterations, such as kinase fusions or *HRAS* mutations. It is unclear what fraction of “spitzoid melanomas” defined solely by their histopathologic features belong to the category of Spitz melanoma or to other melanoma subtypes. We assembled a cohort of 25 spitzoid melanomas diagnosed at a single institution over an 8-year period and performed high-coverage DNA sequencing of 480 cancer related genes. Transcriptome wide RNA sequencing was performed for select cases. Only nine cases (36%) had genetic alterations characteristic of Spitz melanoma, including *HRAS* mutation or fusion involving *BRAF*, *ALK*, *NTRK1*, or *MAP3K8*. The remaining cases were divided into those with an MAPK activating mutation and those without an MAPK activating mutation. Both Spitz melanoma and spitzoid melanomas in which an MAPK-activating mutation could not be identified tended to occur in younger patients on skin with little solar elastosis, infrequently harbored *TERT* promoter mutations, and had a lower burden of pathogenic mutations than spitzoid melanomas with non-Spitz MAPK-activating mutations. The MAPK-activating mutations identified affected non-V600 residues of *BRAF* as well as *NRAS*, *MAP2K1/2*, *NF1*, and *KIT*, while *BRAF* V600 mutations, the most common mutations in melanomas of the WHO low-CSD category, were entirely absent. While the “spitzoid melanomas” comprising our cohort were enriched for bona fide Spitz melanomas, the majority of melanomas fell outside of the genetically defined category of Spitz melanomas, indicating that histomorphology is an unreliable predictor of Spitz lineage.

Introduction

Spitz nevi are composed of epithelioid to spindled melanocytes with abundant homogenous cytoplasm and large vesicular nuclei, and often display epidermal hyperplasia,

clefts around and between melanocytes, Kamino bodies, and maturation with descent. They can have limited scatter of melanocytes into the upper levels of the epidermis [1]. They were first described by Sophie Spitz in 1948 as melanomas of childhood [2]. Clinically, they are most commonly encountered as rapidly growing lesions in children but can occur at any age [1, 3]. Spitz tumor refers to the spectrum of melanocytic tumors with histopathologic features of Spitz nevus that ranges from benign to malignant. They may be challenging to classify as benign or malignant because histopathologic features that are commonly taken as indicators of malignancy, such as nuclear atypia, scatter of melanocytes in the upper epidermis, poor maturation within the dermis, deep extension, and deep dermal mitoses, are not uncommonly seen in Spitz tumors with benign biologic behavior [4].

Recently, a broad spectrum of MAPK activating alterations has been identified in Spitz nevi that differ from those in other types of melanocytic nevi. These include activating

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point mutations in *HRAS* (often with copy number gain of mutant *HRAS*) and rearrangements involving the serine/threonine kinases *BRAF* and *MAP3K8* or the receptor tyrosine kinases (RTKs) *ROS1*, *ALK*, *NTRK1*, *NTRK3*, *RET*, *MET*, and *MERTK* [5–13] and occur in a mutually exclusive fashion with only one mutation in a given Spitz nevus. Despite comprehensive genetic profiling of many Spitz nevi for other oncogenes and tumor suppressors, additional concomitant oncogenic drivers have not been identified, suggesting that these mutations alone are capable of initiating the clonal expansion that results in a Spitz nevus. Early studies indicated that the histomorphologic features of Spitz nevi correspond with their initiating mutations to some degree [14–16]. Thus, the diverse spectrum of initiating mutations in Spitz tumors and the resulting variation in the clinical phenotype likely contributes to the diagnostic difficulty of Spitz tumors. In contrast, the initiating mutations in common acquired nevi are much more homogeneous with over 80% harboring *BRAF* V600E mutations [17, 18]. While there have been a number of iterations of criteria to distinguish Spitz nevi from melanoma [19], even their authors acknowledge their limitations.

The term “spitzoid melanoma” refers to histopathologically malignant tumors comprised of large epithelioid melanocytes resembling those found in Spitz nevi. Spitzoid melanoma is thus a purely morphology-based diagnosis, and prior studies have shown that many tumors classified as such have genetic hallmarks of melanomas of skin with low-cumulative sun-induced damage (low-CSD melanomas), such as *BRAF* and *NRAS* mutations, and a high mutation burden with a UV signature. As Spitz nevi do not show activating *BRAF* or *NRAS* point mutations, spitzoid melanomas with these mutations are not genetically related to Spitz nevi. In one study, fusions involving *BRAF* or RTKs were identified in 41% of Spitz nevi, but only 13% of spitzoid melanomas [7]. In another study of six pediatric spitzoid melanomas, kinase fusions typical of Spitz nevi were identified in five cases [20]. Activating *BRAF* or *NRAS* mutations, which are typical of other melanomas were identified in 20–86% of spitzoid melanomas across various studies [21–24]. De Forno et al. did not identify *HRAS* mutation, found in a subset of Spitz tumors, in a cohort of 21 spitzoid melanomas and on this basis argued that spitzoid melanomas are not related to *HRAS*-mutated Spitz nevi [23]. Lazova et al. performed exome sequencing of 27 spitzoid melanomas and *BRAF* V600 mutations were found in 37% and *NRAS* mutations in 15% of cases. Only one case (4%) had an *HRAS* mutation indicative of a Spitz lineage [24]. Gene fusions are not detectable by exome sequencing and thus could not be analyzed in this study. The majority of cases in this series also had a mutation burden comparable with low- and high-CSD melanomas.

Based on the observation that a considerable portion of spitzoid melanomas have the genetic hallmarks of other types of cutaneous melanomas, the 2018 WHO classification of skin tumors introduced the concept of Spitz melanoma (malignant Spitz tumor) as a melanoma subtype that not only has the morphologic features of Spitz tumors, but also has their genetic hallmarks, namely the distinct spectrum of MAPK activating alterations described above [25]. This is likely an important distinction, as clinical evidence suggests that tumors at the malignant end of the spectrum of Spitz tumors have a better prognosis than other types of cutaneous melanomas. This applies particularly to children, in whom spitzoid tumors with malignant histopathologic features less commonly metastasize with lethal outcome as compared with conventional melanomas [26]. Therefore, we have designated spitzoid melanomas in children as “spitzoid melanoma, childhood type” in our practice and provide a comment that although they have histopathologic features of malignancy, their risk for recurrence and death appears lower than for conventional (or “adult-type”) cutaneous melanoma.

Here, we investigate what fraction of spitzoid melanomas in our practice fall into the newly defined entity Spitz melanoma, and analyze their clinical, histomorphologic and genetic features.

Materials and methods

Case selection and histopathologic analysis

After approval from the institutional review board at the University of California, San Francisco, we searched our archives for melanomas diagnosed from 2010 to 2017 and annotated as having spitzoid features. Each case was rereviewed by three pathologists (SP, SR, and IY) and only cases that were determined to have spitzoid cytomorphologic features by consensus and had archival tissue available for molecular analysis were included. Pathologic features such as epidermal effacement and hyperplasia, ulceration, Kamino body formation, dermal mitoses, and cytology/pleomorphism were recorded for each case. Pigmentation was graded on a 0–4 scale as previously described [27]. Similarly, the extent of solar elastosis was graded on a previously developed scale [28]. Clinical follow-up data were obtained from a combination of chart review and patient outreach.

DNA and RNA extraction

Hematoxylin and eosin stained sections were used to guide tumor microdissection from formalin-fixed paraffin embedded (FFPE) sections of 10 μm in thickness. DNA and

RNA extraction were performed using the QIAGEN All-prep DNA/RNA FFPE extraction kit (QIAGEN, Germantown, MD, p/n 80204).

DNA sequencing and analysis

A total of 50–250 ng of DNA was prepared for sequencing using the KAPA Hyper Prep Kit (KAPA Biosciences, Wilmington, MA p/n KK8504) according to the manufacturer's instructions. Custom-designed bait libraries were used to target the coding regions of 480 genes (Supplementary Table 1). Select introns of kinases rearranged in melanocytic tumors (*ALK*, *BRAF*, *MET*, *NTRK1*, *NTRK3*, *RET*, *ROS1*, *PRKCA*) and the promoter region of *TERT* were also targeted. Sequencing was performed as paired-end 100 bp reads on a HiSeq2500 (Illumina).

Sequence reads were mapped to the human genome (hg19) using the Burrows–Wheeler aligner [29]. Recalibration of reads and variant calling were performed using the Genome Analysis Toolkit [30] and FreeBayes [31]. Quality metrics were determined using Picard [32] (Supplementary Table 2). Three samples with coverage below 25× tumor-specific coverage were excluded from the study. Variant annotation was performed with Annovar [33]. Structural variant detection was performed with Delly [34] and reviewed by visual inspection in the Integrative Genomics Viewer [35]. Resulting fusion transcripts were predicted by joining the exons upstream and downstream from the genomic breakpoint. Genomic copy number assessment was performed with CNVkit [36] which uses off-target reads with adjustment of copy ratios for tumor fraction as estimated by THetA [37].

We reviewed all alterations annotated as pathogenic or likely pathogenic in ClinVar [38]. We excluded variants with >1% prevalence in the NHLBI GO Exome Sequencing Project [39] or 1000 Genomes Project [40] from further review as they likely represent benign germline polymorphisms. We classified truncating mutations in known tumor suppressors as pathogenic. For genes in which a cancer related pathogenic alteration was identified, we manually annotated all remaining variants of that gene as pathogenic, likely pathogenic, uncertain significance, and likely benign by literature review, using various databases as annotation resources (OMIM [41], MyCancerGenome [42]). We determined the number of pathogenic copy number alterations in each case. Shallow deletions of a tumor suppressor accompanied by pathogenic alteration of that tumor suppressor was considered a pathogenic copy number alteration. Deep deletions of tumor suppressors were counted as two pathogenic alterations. Copy number increase of an oncogene was counted as one pathogenic alteration, whereas amplification was counted as two pathogenic alterations. We also counted copy loss of

chromosome 9p without mutation in the remaining allele of *CDKN2A* as a pathogenic alteration, given the importance of this tumor suppressor in melanoma and reports of gene silencing by promoter hypermethylation.

RNA sequencing and analysis

For the nine cases in which a pathogenic MAPK activating alteration was not identified by DNA sequencing, whole transcriptome RNA sequencing was performed. A total of 170–400 ng of total RNA was prepared for sequencing using the KAPA stranded RNA-seq kit (KAPA Biosciences, Wilmington, MA, p/n KK8400) according to the manufacturer's instructions. Subsequently, exome capture was carried out using NimbleGen SeqCap EZ MedExome target enrichment probes (Roche, Basel, Switzerland, p/n 07681330001) following the manufacturer's protocol. Sequencing was performed on a NovaSeq 6000 system (Illumina Inc, San Diego, CA) with 150-bp paired-end reads. Sequence reads were mapped to the reference human genome (hg19) using STAR [43] and fusions were identified by FusionCatcher [44] and verified by manual review.

Statistical analyses

A genetic dataset for cutaneous melanoma provided by the Cancer Genome Atlas (*Skin Cutaneous melanoma, TCGA*) [45] was downloaded from cBioPortal [46, 47], providing mutational data for 448 cases of cutaneous melanomas. Previous studies have identified kinase fusions in the TCGA [48, 49]. The prevalence of mutations in the TCGA dataset was compared with that of our cohort by Fisher's exact test.

Pearson correlation was calculated between age, number of pathogenic mutations, and extent of solar elastosis (converting the scale from Landi et al. which ranges from 0 to 3+ to an numeric scale ranging from 1 to 11). The statistical significance of the differences between each group was calculated by two-sided t-test.

Results

A total of 25 patients with spitzoid melanoma were included in this study (see Table 1 for summary of clinical and histologic features). For 19 patients, we received biopsies in formalin, and for the remaining six patients we received slides sent in consultation. Their average age was 43.8 years (range 4–80 years), including six patients 16 years of age or younger. Males and females were evenly represented. The spitzoid melanomas most commonly involved the extremities (64%, 16/25) and less commonly the head and neck (20%, 5/25) or the trunk (16%, 4/25). The average Breslow thickness was 2.1 mm (range

Table 1 Clinical and Histopathologic features of Spitzoid Melanomas.

Case	Age	Sex	Site	Epidermal hyperplasia	Epidermal effacement	Ulceration	Kamino-bodies	Pigmentation (score 0-4)	Solar elastosis	Dermal mitoses (per mm ²)	Cytology	Plomorphism	Breslow depth (mm)	Diameter(-) (mm)	Clinical follow-up
1	4	Male	Lower leg	Mild	No	No	No	1	0	2	Epithelioid	Moderate to severe	6	2.9	
2	11	Female	Right arm	Yes	No	No	No	3	0	1	Epithelioid, spindled	Moderate	1.8	7.5	
3	11	Female	Left ear	Yes	Yes	No	No	1	0	3	Epithelioid, spindled	Mild to moderate	1.1	≥2.2	No recurrence at 34 months
4	13	Female	Left lateral calf	No	No	No	No	3	0	0	Epithelioid, spindled	Mild	N/A	N/A	
5	13	Female	Left cheek	Yes	No	No	No	0	0	2	Epithelioid, spindled	Mild	8.4	9.2	
6	16	Male	Left posterior upper ear rim	Yes	Yes	No	No	2	0+	1	Epithelioid, spindled	Mild	1.3	≥3.3	
7	23	Female	Left medial thigh	Yes	Yes	No	No	1	0	3	Epithelioid, spindled	Moderate	2.2	≥8.9	
8	26	Female	Left calf	Yes	Yes	No	No	1	1	1	Epithelioid, spindled	Mild to moderate	1.2	5.3	
9	27	Male	Right buttock	Yes	No	No	No	2	0	0	Epithelioid, spindled	Mild	0.6	5.9	No recurrence at 48 months
10	34	Male	Right lateral leg	Yes	No	No	Yes	3	0+	2	Epithelioid	Moderate to severe	1.3	5.5	No recurrence at 36 months
11	39	Male	Right thigh	Yes	No	No	No	0	2	0	Epithelioid	Severe	3.7	6.9	
12	47	Female	Left knee	Yes	Yes	No	No	0	0+	8	Spindled	Mild	3.8	9.9	No recurrence at 31 months
13	49	Male	Right lower leg	Yes	No	No	Yes	4	1	0	Epithelioid	Severe	0.7	5.5	Died after 60 months (unknown reason)
14	50	Female	Left leg	No	No	No	No	0	1	2	Epithelioid, spindled	Moderate	1.3	≥5.7	
15	52	Female	Right proximal forearm	Yes	Yes	No	No	1	2	0	Epithelioid	Moderate	0.4	6.7	
16	55	Male	Left lateral back	Yes	No	No	No	0	1+	0	Epithelioid, spindled	Moderate	0.7	7.9	
17	57	Male	Right chest	Yes	Yes	Yes	No	4	1	1	Epithelioid	Severe	0.9	≥8.5	No recurrence at 36 months, sentinel node negative
18	61	Male	Right thigh	Yes	Yes	No	No	1	2-	2	Epithelioid, spindled	Moderate	1.9	4.4	
19	68	Male	Left forearm	Yes	Yes	No	No	0	2+	5	Epithelioid	Severe	1.9	≥5.7	No recurrence at 57 months
20	68	Male	Right cheek	Yes	Yes	Yes	No	1	3	1	Epithelioid, spindled	Moderate	1.9	4.4	No recurrence at 34 months
21	69	Male	Left posterior shoulder	Yes	Yes	No	No	4	1+	0	Epithelioid, spindled	Mild to moderate	1.1	5.5	Multiple cutaneous recurrences beginning 26 months after diagnosis
22	70	Female	Right anterior thigh	Yes	No	No	No	4	1+	0	Epithelioid, spindled	Moderate	0.5	5.8	No recurrence at 54 months
23	75	Female	Right triceps	No	No	No	No	3	2-	2	Spindled	Mild	1.1	3.8	
24	78	Female	Left lateral thigh	No	Yes	No	No	2	1-	4	Epithelioid	Moderate	1.1	2.6	No recurrence at 13 months
25	80	Female	Right cheek	No	Yes	No	No	2	3	9	Epithelioid	Moderate	5.2	9.8	Local lymph node metastasis detected after 1 month, underwent left neck dissection; No recurrence after 60 months

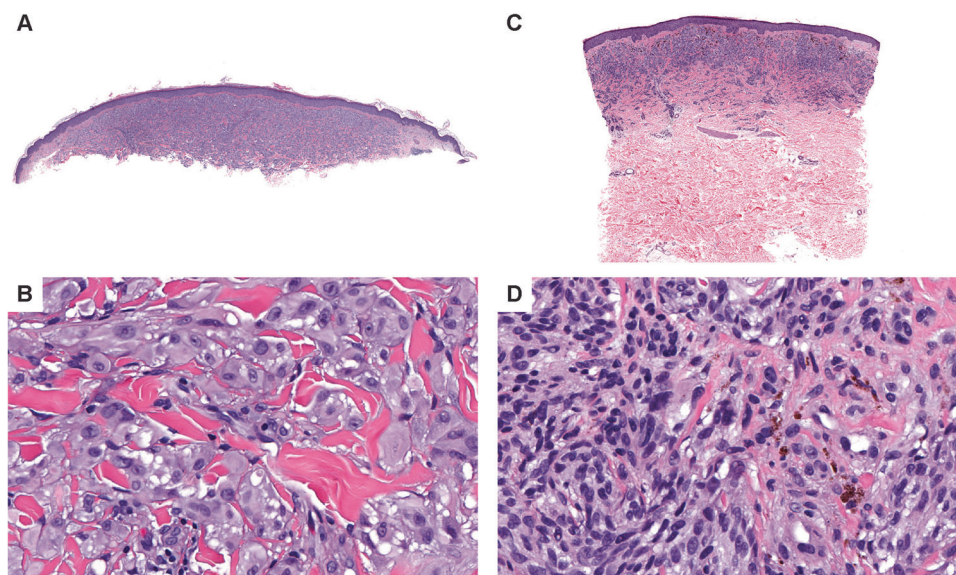


Fig. 1 Spitz melanomas with *HRAS* mutations share features with *HRAS* mutant Spitz nevi. **a** Low power view of case 14 which contained an *HRAS*^{Q61R} mutation. The melanoma is predominantly intradermal and has a broad distribution. **b** On higher power view, the melanocytes are epithelioid and pleomorphic and distributed as single cells and elongated nests in between dense sclerotic collagen bundles.

0.4–8.4 mm) and the mean diameter was 5.9 mm (range 2.2–9.8 mm). It should be noted that some melanomas were transected at the base of the biopsy, so the Breslow thickness could be underestimated.

The majority of tumors ($n = 23$, 92%) were predominantly composed of large epithelioid melanocytes with abundant cytoplasm with a minority of spindled melanocytes. Two cases (8%) were composed exclusively of spindled melanocytes. Cytoplasmic melanin was identified in 19 cases (76%), with eight tumors (32%) demonstrating strong (grade 3–4) pigmentation. Epidermal hyperplasia was present in 80% of cases. Kamino bodies were uncommon, identified in only two (8%) cases.

Spitz melanomas

Two spitzoid melanomas contained *HRAS* hotspot mutations with gain of the mutant allele (8%). One occurred on the leg of a 50-year-old woman and there was also loss of chromosome 9 on which *CDKN2A* resides (case 14). In this case, p16 expression was absent by immunohistochemistry, suggesting inactivation of the remaining allele of *CDKN2A*. The other melanoma occurred on the arm of a 75 year-old woman, and in addition to the *HRAS* mutation there was a three-codon deletion in *MAP2K1* (p.102_104del), a known gain-of-function mutation [50, 51] with a copy number gain of the mutant allele, a hemizygous truncating mutation in *ARID1A*, an amplification of *NOTCH2*, and homozygous deletion of *CDKN2A* (case 23). Both *HRAS*-mutated Spitz

Occasional intra-nuclear inclusions are noted. **c** Low power view of case 23 which contained an *HRAS*^{G12D} mutation. The melanoma is predominantly intradermal and broader than it is deep. **d** On higher power, superficial melanocytes are irregularly pigmented and deeper within the dermis. Dermal melanocytes have scant cytoplasm and hyperchromatic nuclei.

melanomas were predominantly intradermal with a broad, horizontal silhouette and haphazardly arranged, thickened collagen bundles (Fig. 1), similar to *HRAS*-mutated Spitz nevi [5].

BRAF fusions were identified in two cases (8%). Case 12 occurred on the knee of a 47-year-old woman and had an *NRF1-BRAF* fusion, similar to those reported in pleomorphic xanthoastrocytoma [52], a *TERT* promoter hotspot mutation [53, 54], and a hemizygous *PTEN*^{G165R} mutation [55] (Fig. 2). Case 4 (included in a previous study of *BRAF* fusions in melanocytic tumors [6]) harbored a *SOX6-BRAF* fusion with focal amplification of the fusion gene. While additional pathogenic point mutations were not identified, there were multiple copy number aberrations, including gains of distal chromosome 1p, chromosomes 2, 12, 13, and 15 as well as copy number neutral loss of heterozygosity of chromosome 3.

Rearrangements affected the RTKs *ALK* in two cases (8%) and *NTRK1* in one case (4%). Case 7 occurred on the thigh of a 23-year-old-female and contained a *DCTN1-ALK* fusion with loss of chromosome 9 and increased dermal mitoses (3/mm²) (Fig. 3). Case 10 occurred on the leg of a 34-year-old man and harbored a fusion of the 3' portion of *ALK* to an intergenic region of chromosome 11q, with a gain of the distal portion of *ALK* encoding the kinase domain, suggesting that a complex rearrangement resulted in an *ALK* kinase fusion as the oncogene in this case. The tumors with *ALK* rearrangements demonstrated fascicular nests of melanocytes as characteristic of Spitz nevi with

Fig. 2 Histopathology of Spitz melanomas with *BRAF* fusions. **a, b** Case 12—*NRF1-BRAF* fusion. Sections show a highly cellular tumor with a thin overlying epidermis. The growth pattern is predominantly syncytial and the lesion is composed of epithelioid and spindled melanocytes with vesicular nuclei.

c, d Case 4—*SOX6-BRAF* fusion with amplification of the fusion gene. The melanocytes show vesicular nuclei and prominent nucleoli with heterogeneous pigment. There is mild epidermal hyperplasia.

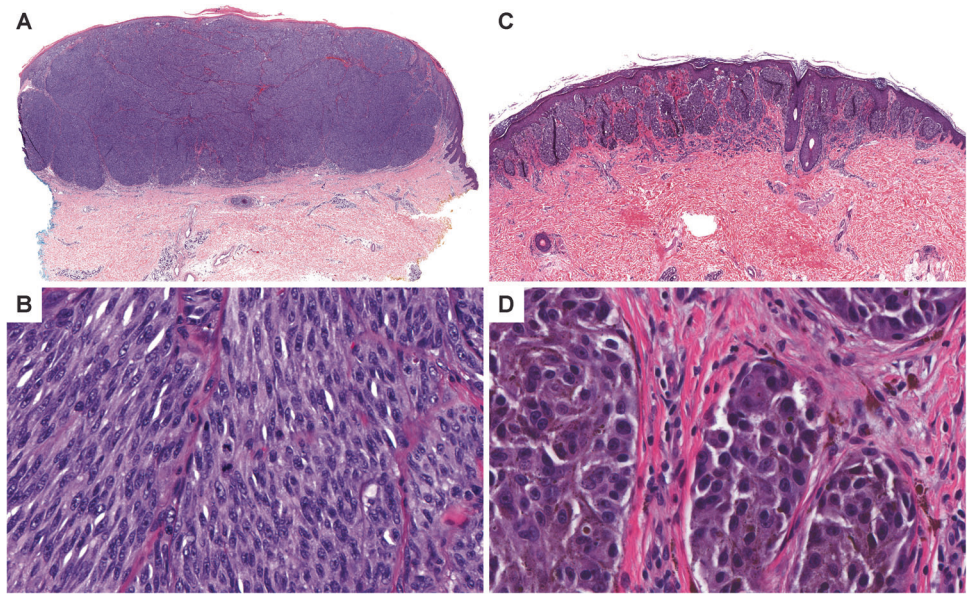
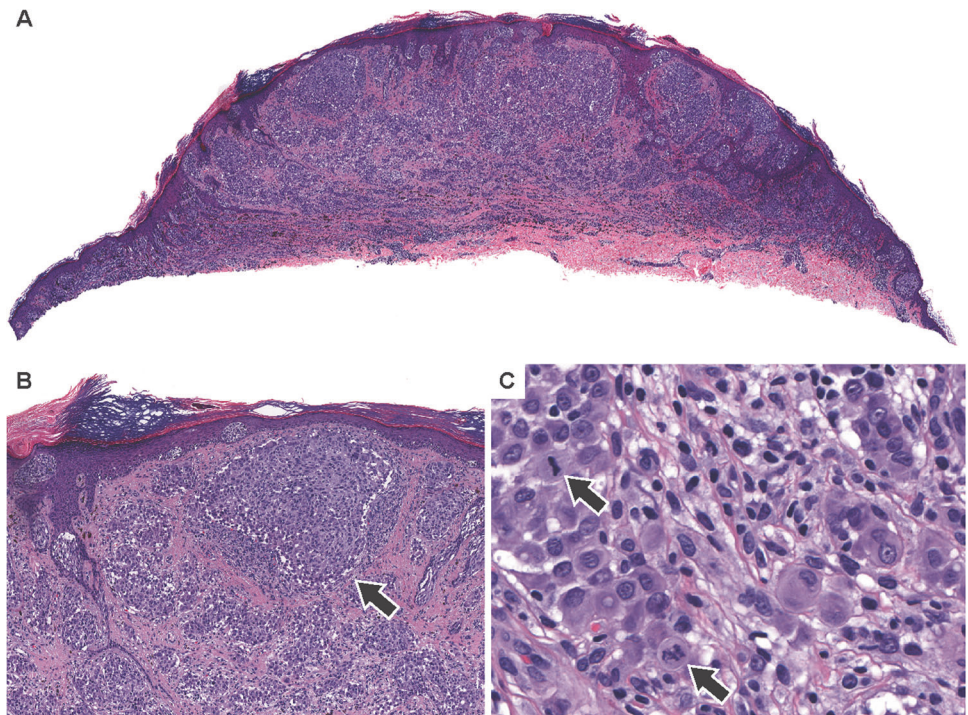


Fig. 3 Spitz melanoma with *ALK* fusion. **a** Low power view of case 7 with a *DCTN1-ALK* fusion. As is characteristic in melanomas with *ALK* fusions, there are large vertically oriented nests in the superficial dermis.

b Medium power view shows fusiform melanocytes arrayed in irregularly sized dermal nests with discohesive melanocytes (arrow). **c** High power view shows epithelioid melanocytes and multiple dermal mitoses.



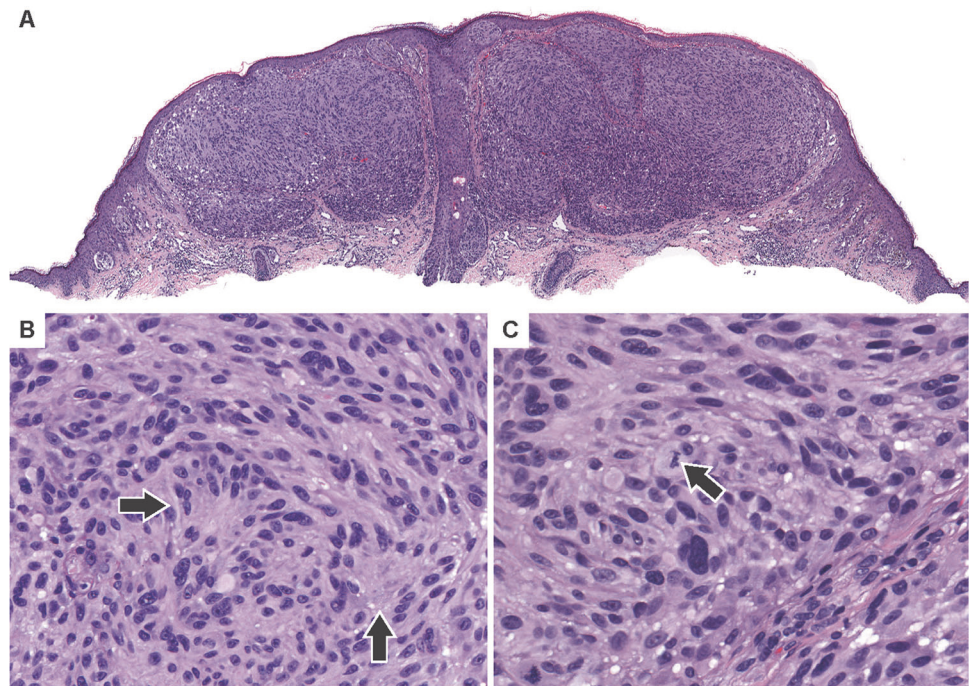
ALK fusions [14, 15]. A *TPM3-NTRK1* fusion was present in one case along with gain of chromosome 17 and loss of chromosome 5p upstream of *TERT* suggestive of a genomic rearrangement which altered *TERT* promoter or enhancer sequences, a mechanism of telomerase reactivation previously described in neuroblastoma [56]. This tumor contained rosette-like structures within the dermal component (Fig. 4), a feature that is more common in Spitz nevi with *NTRK1* fusion [16].

Rearrangements of *MAP3K8* were identified in 2 cases by RNA sequencing (8%). Case 2 demonstrated a

ZFP36L1-MAP3K8 fusion and occurred on the right arm of an 11-year-old girl and was notable for pigment rich epithelioid and spindled cells with some adnexal extension. Case 11 contained an *MAP3K8-SVIL* fusion and presented on the right thigh of a 55-year-old man and contained severely pleomorphic epithelioid cells with mild epidermal hyperplasia. Both cases also demonstrated bi-allelic loss of *CDKN2A* without other pathogenic alterations.

While *HRAS* mutations and *BRAF* or RTK fusions were identified in TCGA skin cutaneous melanoma (SKCM) cohort [45, 48], these alterations occurred at greater

Fig. 4 Rosette-like structures in Spitz melanoma with *NTRK1* fusion. Case 3—*TPM3-NTRK1* fusion. **a** Low power view demonstrates a dome shaped lesion with mild epidermal thinning. **b** High power shows epithelioid to spindled melanocytes with rosette-like structures (arrows in **b**), a feature that can be seen in Spitz tumors with *NTRK1* fusions. **c** Mitotic figures in the deep dermis are easily recognized.



prevalence in our cohort ($P = 0.0479$ for *HRAS* mutation, $P = 0.00041$ for fusions of *BRAF*, *NTRK1*, or *ALK*). The difference in prevalence of *MAP3K8* fusion was not statistically significant ($P = 0.0942$).

Mutations activating the MAP-kinase pathway were present in half of spitzoid melanomas without Spitz nevus associated mutations

Altogether, nine (36%) of the spitzoid melanomas were classified as Spitz melanoma due to the presence of an oncogenic alterations typical of Spitz tumors. Other MAP-kinase pathway activating alterations not associated with Spitz nevi were identified in another nine (36%) spitzoid melanomas. Interestingly, we did not identify any *BRAF*^{V600} mutations in our cohort. *BRAF*^{V600E} is common in melanomas without chronic sun-induced damage (non-CSD) and was significantly underrepresented in our spitzoid melanomas as compared with the TCGA SKCM cohort ($P = 1.3 \times 10^{-6}$). This underrepresentation was not due to excluding cases that were positive by *BRAF* V600E immunohistochemistry. We identified a *BRAF*^{D594G} mutation in a single case (case 19), which is a class 3 *BRAF* mutation that is considered weakly activating [57]. This tumor had a concomitant activating *MAP2K2*^{F57Y} mutation [58–60]. *MAP2K2*, also known as *MEK2*, acts directly downstream of *BRAF* in the MAPK pathway, and the *MAP2K2* mutation likely cooperates with the class 3 *BRAF* mutation. This tumor also had a hotspot mutation in the promoter of *TERT* and an activating *EZH2*^{Y646N}

mutation [61]. Histopathologically, this tumor contained large epithelioid cells with a high mitotic index and severe nuclear pleomorphism (Fig. 5).

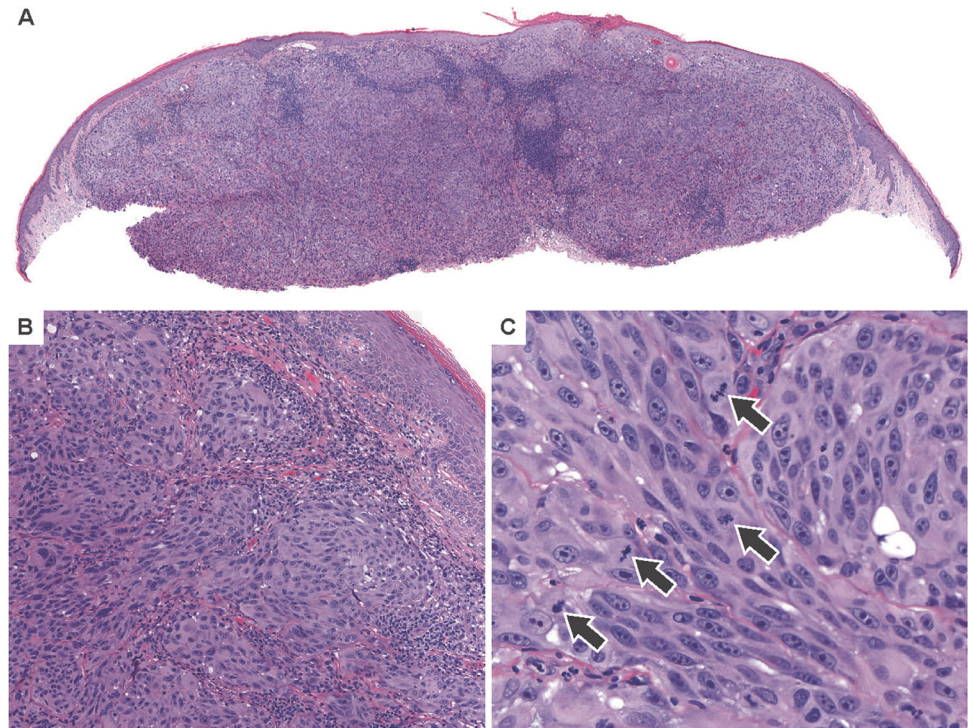
NRAS hotspot mutations were identified in five of cases (20%), at a similar prevalence as in the SKCM cohort of TCGA. It included a melanoma on the helix of the ear of a 16-year-old boy (case 6, Fig. 6) with an *NRAS*^{Q61H} mutation that underwent secondary amplification as the sole detected pathogenic mutation. In case 25, the *NRAS*^{Q61H} mutation occurred in combination with an activating *CTNNB1*^{G34I} mutation, a *TERT* promoter mutation, and *IDH1*^{R132C} and *NFKBIE*^{G41E} mutations [62–64]. This spitzoid melanoma also demonstrated a minor small cell component, which did not demonstrate the strong and uniform beta-catenin staining indicative of *CTNNB1* mutation observed in the majority of the melanoma. This finding suggests that the *CTNNB1* mutation arose later in melanoma progression resulting in the epithelioid cytomorphology present in the majority of the melanoma (Supplementary Fig. 1).

Bi-allelic loss of *NF1* occurred in two (8%) cases, in combination with *NRAS*^{G12D} in one case (case 17). Activating *KIT*^{L576P} and *MAP2K1*^{E203K} mutations were identified in the absence of additional MAPK pathway activating mutations in one case each [65, 66].

In the remaining seven (28%) of cases, no mutations in MAP-kinase pathway were identified. This included an unpigmented epithelioid cell tumor with a *CRTC1-TRIM11* fusion, typical for a new class of tumors that bear resemblance to clear cell sarcoma [67], on the cheek of a 13-year-old girl (case 5, Supplementary Fig. 2).

Fig. 5 Spitzoid melanoma with a class 3 BRAF mutation.

a Low power view of case 19 which harbors *BRAF*^{D594G} mutation along with *EZH2*^{Y646S}, *BLM*^{W803*}, *MAP2K2*^{F57Y}, and *TERT* promoter mutations. The neoplasm is predominantly dermal and is composed of coalescing nests and sheets of large epithelioid melanocytes with a focally dense infiltrate of lymphocytes. **b** Medium power shows a predominantly nested architecture with numerous admixed lymphocytes. **c** Nuclei are pleomorphic with prominent nucleoli and brisk mitotic activity (arrows).

**Subclasses of spitzoid melanoma**

A summary of the pathogenic mutations identified in our series is presented in Fig. 7a. We identified three genetic subcategories in our series: (1) Spitz melanoma, (2) spitzoid melanoma without identified MAPK activating mutations, and (3) spitzoid melanoma non-Spitz MAPK activating mutations. The latter tended to occur in older patients and arose in skin with higher degrees of solar elastosis and had more pathogenic alterations (Fig. 7b and Supplementary Fig. 3).

Mutations in the promoter of *TERT* are present in ~70% of cutaneous melanomas and are associated with worse prognosis in both conventional and spitzoid melanoma [68, 69]. Hotspot *TERT* promoter alterations were identified in eight spitzoid melanomas (32%) and were more common in spitzoid melanomas with a non-Spitz nevus MAPK activating mutation (Fig. 7c).

Inactivation of the tumor suppressor *CDKN2A* is a major driver in melanoma, and homozygous deletion of *CDKN2A* may be associated with poor outcome in atypical Spitz tumors [68, 70]. Bi-allelic loss of function of *CDKN2A* was identified in five (20%) spitzoid melanomas. In four cases, bi-allelic loss occurred by homozygous deletion of *CDKN2A*, and in the remaining case, there was a truncating mutation of *CDKN2A* with loss of the wild-type allele. Four of nine (44%) Spitz melanomas demonstrated bi-allelic inactivation of *CDKN2A*.

Clinical follow-up

We were able to obtain clinical follow-up data for 12 patients, 11 of whom were alive at the time of this study (Table 1, mean follow-up 44 months). One patient died 60 months after excision from unknown causes. One patient (case 21) had multiple cutaneous recurrences beginning 26 months after excision. Another patient had a positive sentinel lymph node biopsy that was followed by completion lymphadenectomy with no record of relapse during follow-up. Nine patients were treated with simple excision all of which had an uneventful follow-up period.

Discussion

The genetic alterations in our series of 25 spitzoid melanomas differed significantly from those reported for cutaneous melanomas. *BRAF* V600 mutations found in the majority of low-CSD cutaneous melanomas were completely absent, indicating that spitzoid melanomas do not typically arise from common acquired nevi. Instead we found an increased incidence (30%) of oncogenic alterations typical for Spitz tumors such as *HRAS* mutation and activating fusions *BRAF*, *MAP3K8*, *ALK*, and *NTRK1*.

The Spitz melanomas with *HRAS* mutation or *ALK* or *NTRK1* kinase fusion retained the some of the distinct

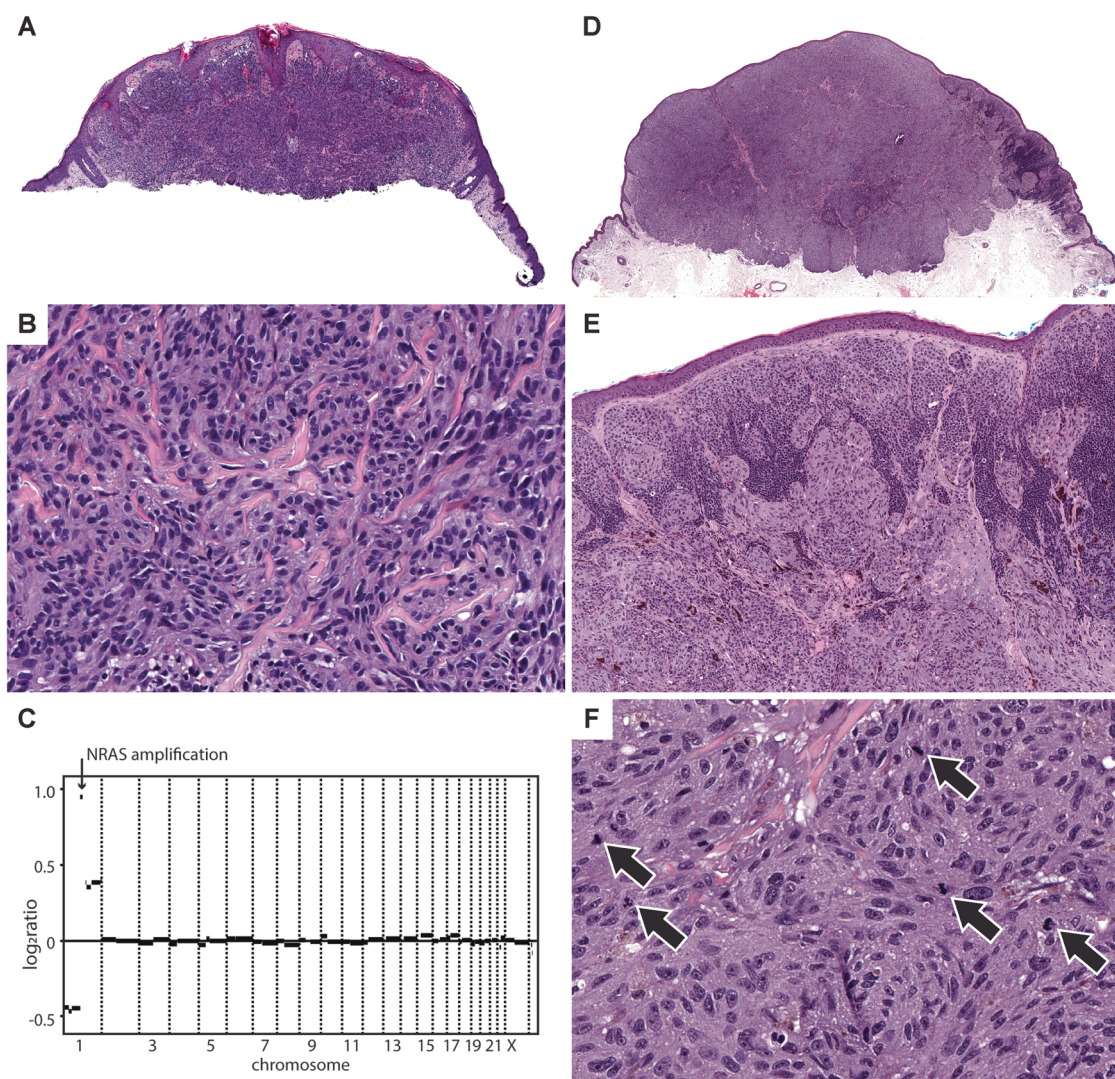


Fig. 6 Spitzoid melanomas with NRAS mutation. **a** Low power view of case 6 which harbors *NRAS*^{Q61H} with high-level focused amplification of the mutant allele shows a dome shaped compound tumor with epidermal hyperplasia and clefting around nests of melanocytes in the superficial dermis. **(b)** The lesion is composed of epithelioid melanocytes with hyperchromatic nuclei, growing as nests and fascicles distributed between thickened sclerotic collagen bundles. **(c)** Whole genome copy number profile demonstrates *NRAS* amplification

as well as loss of chromosome 1p distal to *NRAS* and gain of chromosome 1q. **(d)** Low power view of case 25—which harbors *NRAS*^{Q61K}, *CTNNB1*^{G34I}, *IDH1*^{R132C}, and *NFKBIE* and *TERT* promoter mutations shows a nodular tumor that extends to the deep dermis. **(e)** The lesion is predominantly composed of large epithelioid melanocytes with focal areas of small more nevoid cells, predominantly near the dermal-epidermal junction. **(f)** Many dermal mitoses are present (arrows).

histopathologic features associated with Spitz nevi with these mutations. Two of the Spitz melanomas had genetic features that indicate malignancy in low- and high-CSD melanoma (Cases 12 and 23). Two additional Spitz melanomas had bi-allelic inactivation of *CDKN2A*. Case 3 with an *NTRK1* fusion, but without other definitely pathogenic mutations demonstrated copy number loss upstream of *TERT*, suggesting a structural rearrangement. Similar rearrangements reactivate *TERT* in neuroblastoma and have been reported in lethal Spitz melanoma in the absence of a *TERT* promoter mutation [13]. By design, our targeted assay cannot detect structural rearrangements far upstream

of *TERT* and therefore we may have missed similar alterations in other cases.

Most of our spitzoid melanomas were not classifiable as Spitz melanomas based on their genetic alterations. We further subdivided these “non-Spitz” spitzoid melanomas into those without any identifiable MAP-kinase pathway mutations and those with MAPK pathway mutation(s) not associated with Spitz nevi such as in *NRAS*, *BRAF*, *KIT*, *NF1*, and/or *MAP2K1/2*.

The melanomas in the latter of these two groups occurred in older patients, with more solar elastosis in the adjacent dermis and had a higher number of pathogenic mutations

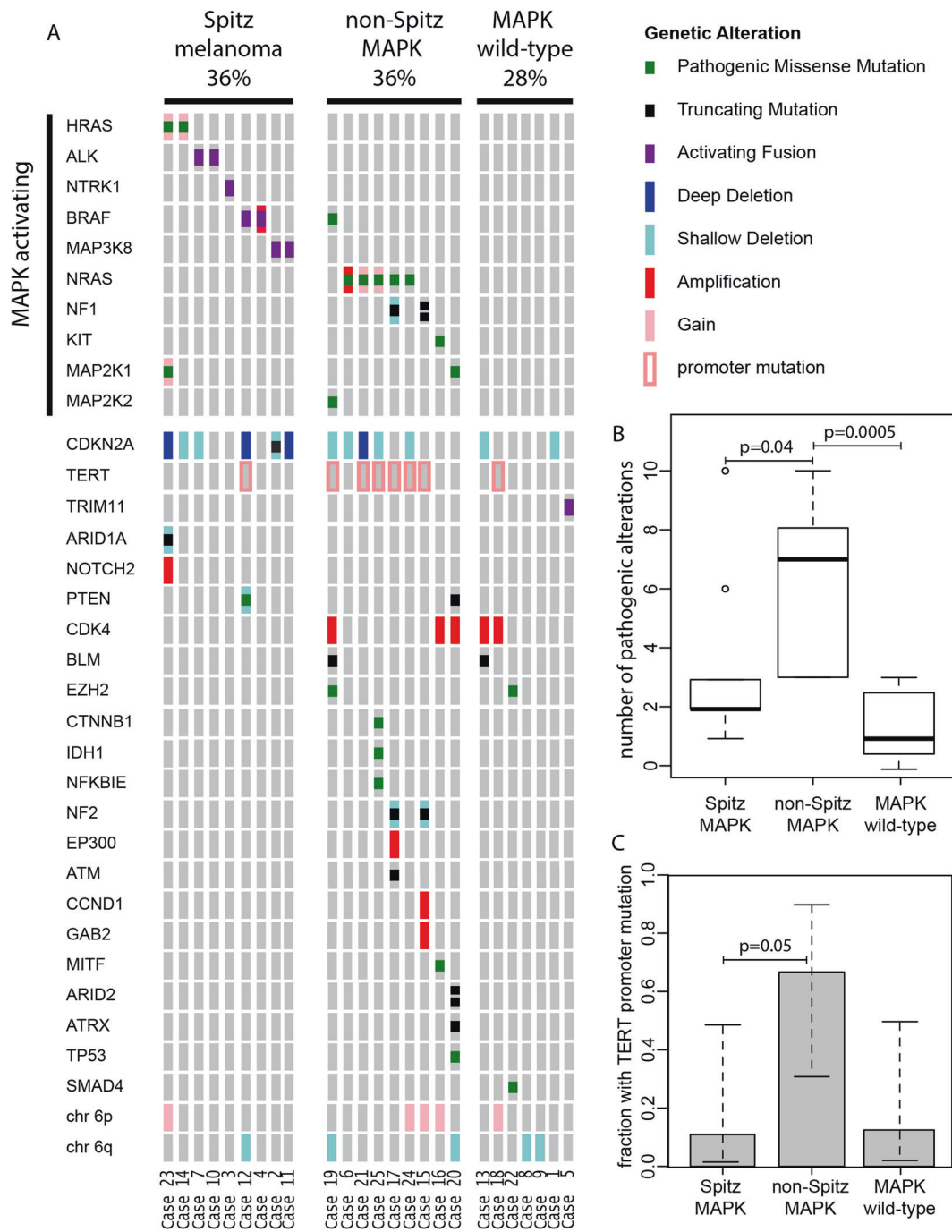


Fig. 7 Genetic alterations in spitzoid melanomas. **a** Tiling plot highlighting the genetic landscape of spitzoid melanomas in our cohort, stratified by those with classical Spitz drivers, non-Spitz MAPK drivers, and those without an identifiable MAPK mutation.

b Number of pathogenic mutations in each subset. **c** Proportion of cases with *TERT* promoter mutations. Note that those tumors with non-Spitz MAPK drivers demonstrated an increase in both the number of pathogenic mutations as well as *TERT* promoter mutations.

than the rest of our spitzoid melanomas, suggesting that they have a higher somatic mutation burden due to UV exposure. One notable exception was a spitzoid melanoma with focused high-level amplification of mutant *NRAS* that occurred on the ear of a teenager and demonstrated minimal solar elastosis and no additional pathogenic alterations. Focused high-level amplification of mutant *NRAS* has been

described in spitzoid tumors on the ear of children [71]. Perhaps secondary amplification of mutant *NRAS* leads to spitzoid cytomorphologic features by a mechanism similar to *HRAS* mutation.

The different histopathologic features of conventional and Spitz nevi likely reflect differences in the oncogenic signaling outputs of their initiating mutations. Melanocytes

of a conventional nevus may acquire secondary genetic alterations that lead to additional proliferation and a change in the cytomorphology of the neoplastic melanocytes. For example, when a melanocyte of a conventional nevus, which was initiated by a *BRAF* (or rarely *RAS*) mutation, acquires an activating *CTNNB1* mutation in addition to the mutation which initiated the nevus, a secondary clonal expansion of melanocytes characterized by increased cell size and pigmentation results in a component of deep penetrating nevus (melanocytoma) [51]. Bi-allelic loss of *BAP1* in a melanocyte of a conventional nevus results in a secondary proliferation of epithelioid melanocytes with distinctive spitzoid cytomorphology [72–75]. Similarly, bi-allelic loss of *PRKARIA* leads to development of a pigmented epithelioid melanocytoma within a conventional nevus [76]. While we identified a *CTNNB1* mutation in one spitzoid melanoma, we did not identify any pathogenic *BAP1* mutations in our series. This may indicate that cutaneous melanomas with *BAP1* loss are rare and represent only a small fraction of spitzoid melanoma, or that the spitzoid cytomorphologic features of melanocytic tumors with *BAP1* loss are not retained during malignant progression. Intriguingly, two spitzoid melanomas harbored activating *EZH2* mutations. Loss of *BAP1* has been shown to lead to increased *EZH2* activity and *EZH2*-dependent transformation [77]. Perhaps mutational activation of *EZH2* is functionally similar to loss of *BAP1* and also results in a spitzoid phenotype.

Most spitzoid melanomas without identified MAP-kinase pathway mutations occurred in young patients with little solar elastosis, similar to Spitz melanomas. Their genomes did not contain many deletions and amplifications suggesting they are distinct from “triple wild-type” melanomas in the TCGA that characteristically contain many copy number aberrations and structural rearrangements [45]. In one case we identified a *CRTC1-TRIM11* fusion which is not predicted to affect MAPK pathway signaling and has been recently described in dermal melanocytic tumors composed of epithelioid and spindled cells with minimal pigmentation that resemble clear cell sarcoma [67]. This case lacked melanin (pigmentation score of 0) and was characterized by epithelioid to spindled melanocytes with vesicular chromatin and a single prominent nucleolus, findings very characteristic of clear cell sarcoma. Future studies employing comprehensive DNA and RNA sequencing or interrogation of DNA methylation status may identify additional alterations that lead to spitzoid cytomorphology, particularly in cases without an identified MAP-kinase pathway mutation.

Interestingly, our findings differ from those of the study performed by Lazova et al., in which the authors found a high genetic similarity between spitzoid and conventional melanomas with ~40% of the spitzoid melanomas in their series

having a *BRAF* V600 mutation [24]. Perhaps the younger age of patients in our study accounts in part for the differences as the average age in our study was 45 years, whereas the average age in the study by Lazova et al. was 64 years. More likely, the disparate results may reflect different histopathologic criteria for spitzoid melanoma across institutions.

While our study was limited by a lack of clinical follow-up and somatic mutation burden was not quantified as we did not sequence matched normal DNA, our findings suggest that multiple evolutionary pathways can lead to spitzoid melanoma, and that it is not a homogeneous category. Given the genomic diversity of Spitz melanoma, and indeed the likelihood that spitzoid melanomas without Spitz initiating oncogenes are also genomically diverse, the failure of histopathologic criteria to distinguish all Spitz nevi from all spitzoid melanomas is not surprising. Diagnostic criteria likely need to be developed for each molecular sublineage (i.e. *ALK*-fused Spitz tumors, *HRAS* mutant Spitz tumors) and may need to include molecular as well as histopathologic findings. Future studies will aim to determine differences in clinical behavior for Spitz melanoma as compared with low- or high-CSD melanoma and the impact of specific initiating mutation, additional pathogenic mutations, and mutation burden on clinical outcomes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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