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Massively Parallel Sequencing Analysis of Benign Melanocytic Nevi

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Abstract

Aims: Melanocytic nevi are benign lesions of the skin or mucosa, which may constitute non-obligate precursors of malignant melanoma, particularly when displaying lentiginous and dysplastic features. Here we sought to interrogate the repertoire of somatic genetic alterations in melanocytic nevi.

Methods and Results: DNA extracted from 12 melanocytic nevi and matching normal tissue were separately microdissected and subjected to targeted massively parallel sequencing targeting 300 cancer genes. A median of 5.5 (range 1–12) nonsynonymous somatic mutations was detected, with 10 cases harboring mutually exclusive *BRAF*V600E (6/12) or *NRAS* (4/12) clonal hotspot mutations. One of the two cases lacking *BRAF* and *NRAS* mutations was a dysplastic nevus harboring a *HRAS* Q61L hotspot mutation. Analysis of the laser-capture microdissected components of a nevus synchronously diagnosed with *in situ* and invasive malignant melanoma revealed a truncal, clonal *BRAF*V600E mutation, and the acquisition of a *CDKN2A* homozygous deletion in the invasive component in conjunction with additional clonal mutations affecting *NF2*, *FAT4*, and *KDR* in both *in situ* and invasive malignant components.

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AUTHORS' CONTRIBUTIONS

T.H. conceived the study. K.J.B., A.C.H., and T.J.H. provided tissue samples and clinical data. K.J.B. and T.J.H. performed pathology review. T.H. performed experiments. T.M. provided laser capture resources. P.S. and D.B. performed bioinformatics analyses. J.R.L., F.C.G., B.A., M.F.B., B.W., J.S.R.-F. and T.H. analyzed and interpreted the data. J.R.L., F.C.G., J.S.R.-F., and T.J.H. wrote the first manuscript, which was reviewed by all co-authors.

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CONFLICTS OF INTEREST

All other authors declare no conflicts of interest relevant to this study.

Conclusion: Melanocytic nevi harbor recurrent *BRAF*V600E or *NRAS* hotspot mutations with low mutational burdens. Our findings also further validate that progression from nevi to malignant melanoma may be driven by the acquisition of additional genetic alterations, including *CDKN2A* homozygous deletions.

INTRODUCTION

Melanocytic nevi represent a heterogeneous group of visible, circumscribed lesions of the skin or mucosa.^{1, 2} The term “melanocytic nevus” refers to a broad spectrum of neoplastic lesions including both congenital and acquired and hereditary and non-hereditary skin lesions.¹ Whilst the majority of nevi display benign behavior, malignant transformation into melanoma has been documented.^{3–5} In fact, melanocytic nevi displaying lentiginous growth patterns and dysplasia have been described as potential non-obligate precursors to malignant melanoma.^{6, 7}

Previous studies have elucidated the repertoire of recurrent genetic alterations underpinning the pathogenesis of melanoma.^{8, 9} Approximately 50% of melanomas have been shown to harbor mutations affecting the *BRAF*V600 hotspot mutations and approximately 20% are reported to display hotspot mutations affecting *NRAS*.^{3, 8–11} Studies have established the role of UV exposure in melanomagenesis, with UV mutagenesis contributing to a higher mutational load and an increased abundance of cytidine to thymidine (C>T) transitions.^{3, 9, 12, 13} In comparison to the well-defined mutational landscape of melanomas, there are limited studies defining the mutational landscape of nevi outside of the primary genetic alterations affecting melanomas. Akin to melanomas, nevi have been found to harbor recurrent mutations affecting *BRAF* (~80%) and *NRAS* (~20%).^{14–18} Here, we employed targeted massively parallel sequencing to investigate the repertoire of somatic genetic alterations affecting cancer genes in melanocytic nevi, including nevi displaying lentiginous growth and/ or varying levels of atypia.

METHODS

Cases

Twelve cases of melanocytic nevi were retrieved from the pathology archives of the Department of Pathology of Memorial Sloan Kettering Cancer Center (MSKCC), and reviewed by dermatopathologists (T.H and K.B.). One case (SKIN23) was composed of a benign nevus associated with malignant melanoma *in situ* (MMIS) and invasive melanoma (IM). All samples were anonymized prior to the analysis. Approval by the Memorial Sloan Kettering Cancer Center (MSKCC). Institutional review board was obtained, and patients provided a written informed consent as specified in the approved protocol.

Microdissection and DNA extraction

Representative histologic sections from formalin-fixed paraffin-embedded blocks of melanocytic nevi were stained with Nuclear Fast Red and subjected to microdissection with a sterile needle under a stereomicroscope (Olympus) to ensure >80% tumor content, as previously described.^{19–21} In SKIN23, the three components (nevus, MMIS and IM) were separately laser-capture microdissected, using a Leica LMD 6500 as previously described.²²

Microdissection was also performed for matched normal tissue to ensure that normal samples were devoid of neoplastic or atypical cells. DNA was extracted from microdissected tissue using the DNeasy Blood and Tissue Kit (Qiagen), according to the manufacturer's protocol, and quantified using the Qubit Fluorometer assay (Life Technologies).

Targeted massively parallel sequencing

DNA samples from tumor and matched normal tissue were subjected to targeted massively parallel sequencing at the MSKCC Integrated Genomics Operations using the Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) assay, which targets all exons of 300 cancer genes and non-coding regions of selected genes.^{19, 20} Extracted DNA (250 ng) was used to prepare barcoded sequence libraries (New England Biolabs, KapaBiosystems), as previously described.^{19, 20} Massively parallel sequencing was performed on an Illumina HiSeq2500 following validated protocols.

MSK-IMPACT data analysis was performed as described previously.²² In brief, reads were aligned to the reference human genome GRCh37 using the Burrows-Wheeler Aligner (BWA, v0.7.15)²³. Local realignment, duplicate removal and base quality score recalibration was performed using the Genome Analysis Toolkit (GATK, v3.1.1)²⁴. Somatic single nucleotide variants (SNVs) were identified using MuTect (v1.0)²⁵, small insertions and deletions (indels) using Strelka (v2.0.15)²⁶, VarScan2 (v2.3.7)²⁷, Lancet (v1.0.0)²⁸ and Scalpel (v0.5.3)²⁹, and further curated by manual inspection. SNVs and indels outside of the target regions were filtered out. We further excluded SNVs and indels for which the tumor mutant allele fraction (MAF) was <5 times that of the paired normal MAF, and SNVs and indels found at >5% global minor allele frequency of dbSNP (build 137), as previously described^{30, 31}. FACETS³² was used to calculate Log₂ ratios and B allele frequencies for heterozygous single nucleotide polymorphisms (SNPs); the segmented profiles were analyzed to determine copy number alterations (CNAs) and whether genes harboring a somatic mutation were targeted by loss of heterozygosity (LOH), as previously described^{33, 34}. The segmentation of the copy number data and the solutions for ploidy and tumor cell content were curated by analysts with experience in the interpretation of the output files from FACETS. ABSOLUTE (v1.0.6)³⁵ was employed to determine the cancer cell fraction (CCF) of each mutation, as previously described^{31, 33}. A mutation was classified as clonal if its probability of being clonal was >50%³⁶ or if the lower bound of the 95% confidence interval of its CCF was >90%^{31, 33}. Mutations affecting hotspot codons were annotated, as previously described^{31, 33, 37}. A combination of Mutation Taster,³⁸ CHASM (melanoma)³⁹ and FATHMM⁴⁰ was used to define the potential functional effect of each non-synonymous SNV, as previously described^{20, 22}.

RESULTS

Pathologic characteristics of melanocytic nevi

Twelve nevi displaying lentiginous, dysplastic, and/or compound features were included in this study (Figure 1). Their clinicopathologic features are summarized in Table 1. Notably, one case (SKIN11) presented as an atypical intraepidermal melanocytic proliferation arising in nevus in the context of vemurafenib therapy for metastatic melanoma. Another case

(SKIN23) displayed malignant transformation into malignant melanoma *in situ* and invasive melanoma. All but two cases were associated with ultra-violet exposure, whilst the other two cases were congenital. Central histologic review of the cases classified them into varying degrees of atypia: none (n=5), mild (n=2), and moderate (n=5) and scored them for UV damage based on solar elastosis: none (n=10), mild (n=1; SKIN11), and moderate (n=1; SKIN09).

Melanocytic nevi display low mutation burden and harbor recurrent somatic mutations affecting *BRAF* and *NRAS*

To characterize the repertoire of somatic genetic alterations affecting *bona fide* cancer genes in melanocytic nevi, MSK-IMPACT assay was employed. Sequencing was performed to median depths of 386x (range 129–556x) and 264x (range 89–430x) for tumor and matched germline DNA samples, respectively (Supplementary Table S1). Analysis revealed medians of 5.5 (range 1–12) and 2.5 (range 0–7) non-synonymous somatic and synonymous somatic mutations, respectively (Figure 2; Supplementary Table S2). Ten of the twelve melanocytic nevi (83%) harbored a hotspot mutation affecting either *BRAF* (50%, 6/12) or *NRAS* (33%, 4/12), which displayed a mutually exclusive pattern. All *BRAF* and all *NRAS* mutations were predicted to be clonal (Supplementary Figure S1, Supplementary Table S2), consistent with their likely role as founder genetic events.

All *BRAF* mutations affected the V600 hotspot (6 V600E), whereas all but one *NRAS* mutations affected the Q61 hotspot (two Q61K, one Q61R and one G13R; Figure 2; Supplementary Table S2). Other recurrently mutated genes affected by non-synonymous mutations included *EPHA6* (25%, 3/12), *NOTCH2* (17%, 2/12), *EGFR* (17%, 2/12) and *FAT4* (17%, 2/12). Notably, two cases harbored double nucleotide substitutions, typical of UV-induced DNA damage,^{3, 9, 12} affecting *PMS2* (SKIN07) and *ERBB2* (SKIN08), whilst another case displayed multiple mutations affecting *NOTCH2* (SKIN03; P250L and E2155G).

Interestingly, one of the *BRAF*^{WT}/*NRAS*^{WT} cases harbored a clonal *HRAS* Q61L hotspot mutation (SKIN03), whilst the other *BRAF*^{WT}/*NRAS*^{WT} case harbored a mutation affecting *GRM3* (SKIN14). As *HRAS* hotspot mutations have been well documented in Spitz nevi, we conducted a morphological re-examination of this case. We did not find any associated Spitzoid features, thereby making this, to the best of our knowledge, the first report of an *HRAS* mutation in a dysplastic nevus.

Copy number analysis revealed profiles with few copy number alterations (Supplementary Figure S2), consistent with previous reports.^{3, 41} Of note, however, the few cases displaying frequent gains and losses occurred in congenital nevi or in a melanoma-associated nevus.

Given that the nevi included in this study displayed a variety of histologic features, we performed an exploratory, hypothesis generating analysis of the repertoire of somatic genetic alterations and the histologic features of the nevi analyzed. No significant differences in the repertoire of genetic alterations were observed according to the level of atypia, and the presence of a lentiginous growth, compound features, and/or histologic scores for UV damage and solar elastosis ($P > 0.05$, Fisher's exact test).

Comparison to previous sequencing studies of nevi reveals heterogeneous genetic profiles

To ascertain whether the melanocytic nevi reported here harbored similar repertoires of somatic genetic alterations to that of previously reported nevi, we conducted an exploratory analysis comparing the somatic mutation landscapes and the frequencies of CNAs to that of previously reported sequencing studies^{17, 18}. Analysis of somatic mutation landscapes (Figure 3A) revealed similar mutation frequencies in *BRAF* and *NRAS* between our melanocytic nevi and in the dysplastic nevi reported by Melamed et al¹⁸ of 50% (6/12) and 33% (4/12) vs 60% (9/15) and 27% (4/15), respectively. In contrast the globular nevi and reticular nevi reported by Stark et al¹⁷ reported *BRAF* mutation frequencies of 75% (9/12) and 39% (7/18), respectively. Moreover, globular nevi lacked *NRAS* mutations, whereas only 6% (1/18) of reticular nevi harbored an *NRAS* mutation. There was little overlap in mutations, apart from *BRAF* and *NRAS*, between studies. Recurrently mutated genes across studies included *NOTCH2* (4/57), *PTPRD* (3/57), *PIK3C2G* (3/57), *SETD2* (3/57), and *ERBB4* (3/57). Overall, the nevi harbored heterogeneous mutational profiles across the studies.

Comparison of CNA frequencies was performed between this study and that of Stark et al¹⁷ (Figure 3B). Global comparison to their combined set of nevi revealed different copy number profiles. Whilst we observed few gains and losses in our cohort, Stark et al reported little to no gains and extensive, genome-wide losses in their cohort. Upon comparison to each subtype, the genome-wide losses were found to be almost exclusively in the reticular nevi, with the globular nevi displaying flat, simple genomes. In our study, all melanocytic nevi lacked high-level amplifications and deletions.

Molecular aberrations underpinning the transformation of nevus to malignant melanoma

Case SKIN23 originated from a 77-year-old female patient diagnosed with an invasive melanoma (IM) occurring in the forearm. Upon histological analysis, the IM displayed progression from precursor malignant melanoma *in situ* (MMIS) and an adjacent intradermal nevus (Figure 4A). The three components were separately laser-capture microdissected and extracted DNA samples were subjected to MSK-IMPACT assay. Somatic mutation analysis revealed three identical non-synonymous somatic mutations shared by the three components (Figure 4B), including a *BRAF*V600E mutation that was clonal in all three samples (i.e. early truncal mutation), consistent with the notion that *BRAF*V600E mutations are founder genetic events in the development of nevi and melanomas.⁴² Additional clonal likely-pathogenic mutations affecting *NF2*, *FAT4* and *KDR* were found only the MMIS and IM components, whereas mutations private to each on the components were also found. Potential pathogenic subclonal mutations affecting *GATA3*, *SYK*, and *PARK2* were restricted to the nevus, whereas the IM harbored two distinct subclonal mutations in *PTPRD*, which were both coupled with LOH and predicted to be likely-pathogenic. Subclonal mutations affecting *JAK2* and *NFKB2* were detected in and found to be private to the MMIS (Supplementary Table S2). However, we cannot rule out the hypothesis that these mutations may not stem from tumor cells, but rather from tumor-infiltrating lymphocytes and/ or epidermal cells contaminating the sample. Nonetheless, our somatic mutations analysis

suggests clonal relatedness between and intra-lesion genetic heterogeneity among the three components.

Given that clonal relatedness based on the presence of highly frequent shared mutations, such as the *BRAF*V600E mutation, may be ambiguous, we sought to define whether the proposed clonal relatedness between the nevus, MMIS, and IM would be supported by their pattern of copy number alterations. Copy number analysis revealed a similar pattern of copy number alterations in the MMIS and IM, including gains affecting *BRAF*, *NOTCH1*, and *TERT* and losses affecting *CDK2NA*, *CDK2NB*, *MAP2K1*, and *PTEN*, whilst the nevus component displayed a flat copy number profile (Figure 4C). Notably, a homozygous deletion of *CDKN2A/B* was found solely in the IM component.

Taken together, our data support the notion that the nevus likely constituted the substrate for the development of the malignant melanoma components. Whilst the *BRAF*V600E was a founder genetic event and *CDKN2A* homozygous deletions potentially driving progression to the invasive malignant phenotype (Figure 4D). Nonetheless, intra-lesion heterogeneity and private mutations were found in each component, indicative of branched evolution.

DISCUSSION

Here, we investigated the repertoire of somatic genetic alterations affecting 300 key cancer genes in melanocytic nevi and confirmed the high prevalence of recurrent hotspot mutations affecting *BRAF* and *NRAS*. Moreover, we identified a clonal *HRAS* hotspot mutation in a *BRAF*^{WT}/*NRAS*^{WT} nevus, which to the best of our knowledge, is the first report of an *HRAS* mutation in a dysplastic nevus. We also compared the somatic mutation landscape and CNA frequencies of our study to previous sequencing studies of nevi, revealing heterogeneous repertoire of somatic genetic alterations, apart from *BRAF* and *NRAS* mutations. Furthermore, we genetically characterized the progression of a benign melanocytic nevus to an invasive melanoma, which was associated with parallel evolution and likely driven by loss of *CDKN2A*.

Previous studies have reported on the landscape of somatic mutations in melanomas and nevi. {Hodis, 2012 #9; Poynter, 2006 #16; Tschandl, 2013 #10; Kumar, 2004 #15; Pollock, 2003 #14; Poynter, 2006 #16; Dhomen, 2009 #37; Curtin, 2005 #11; Shain, 2015 #3; Young, 2014 #39; Melamed, 2017 #43; Stark, 2018 #44} Compared to the high frequency of *BRAF* mutations observed in benign nevi samples previously described (80%^{14–16}), the melanocytic nevi reported here displayed lower frequencies of mutations affecting the *BRAF*V600 hotspot (50%). However, when we compare the melanocytic nevi reported here to that of melanomas associated with nevi, we observe comparable frequencies of mutations affecting the *BRAF*V600 hotspot (50% vs 50%^{10, 16}). These similar mutation frequencies affecting *BRAF* indicate that melanocytic nevi may serve as potential precursor lesions to melanomas.

Our exploratory, hypothesis generating analysis revealed that within this group of benign nevi harboring predominantly *BRAF* and *NRAS* mutations, our targeted exome sequencing of 300 genes with associated copy number analysis does not provide causation for the

morphologic variation in atypia. Furthermore, within our series, these morphologic variations were found not to be associated with “UV signature” or solar elastosis. A larger sample size and more comprehensive analyses by whole-exome or, ideally, whole-genome sequencing and a detailed epigenetic analysis may be helpful to uncover additional mutations.

Our genetic characterization of the progression case (SKIN23) supports the notion that melanomas harboring *BRAF*V600 mutations can arise from benign nevi,^{3, 14} and that hotspot *BRAF*V600E mutations are early events in the development of melanocytic lesions. The presence of additional, clonal, and likely-pathogenic mutations in the MMIS and IM demonstrate, however, that *BRAF* mutations alone are not sufficient for the malignant progression of benign nevi to melanoma. In this case, we identified a homozygous deletion of *CDKN2A* as a potential driver event in the progression from MMIS to malignant invasive melanoma, respectively, consistent with previous findings.^{3, 43, 44}

This study has several limitations. First, the number of cases analyzed here is relatively small. Despite the small sample size, we were able to identify recurrent mutations affecting melanocytic nevi and provide molecular evidence for the progression of a nevus to MMIS to IM. Second, the cohort of cases is heterogeneous, hence, alongside the small sample size, the findings of our study are limited to being hypothesis generating. Third, the results of this study are limited to the 300 key cancer genes included in the MSK-IMPACT sequencing assay, hence we cannot exclude the possibility of differences in genetic alterations between nevi with different histologic characteristics nor the possibility of mutations affecting genes outside of this panel driving the progression of a nevus to invasive melanoma. Lastly, within this small cohort, severely atypical nevi were not represented as we deliberately focused on “benign” nevi.

Despite these limitations, our study characterizes the genetic alterations affecting cancer genes in melanocytic nevi, confirming that melanocytic nevi are underpinned by hotspot *BRAF*V600E and hotspot *NRAS* mutations. We also report a clonal, hotspot *HRAS* Q61L mutation in an atypical dysplastic nevus. Moreover, we provide a detailed characterization of the molecular progression of a benign nevus with a *BRAF*V600E mutation to MMIS to IM. Further sequencing analyses are warranted to define the mutational landscape of benign nevi and determine the spectrum of genetic alterations that can drive malignant transformation towards melanoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Happle R What is a nevus? A proposed definition of a common medical term. *Dermatology* 1995;191;1–5.
2. Lentigenes Weedon D., nevi and melanomas. *Skin Pathology*. Edinburgh: Churchill Livingstone, 2002;803–858.
3. Shain AH, Yeh I, Kovalyshyn I et al. The Genetic Evolution of Melanoma from Precursor Lesions. *New England Journal of Medicine* 2015;373;1926–1936. [PubMed: 26559571]
4. Smolle J, Kaddu S, Kerl H. Non-random spatial association of melanoma and naevi - a morphometric analysis. *Melanoma Research* 1999;9;407–412. [PubMed: 10504061]
5. Elder DE. Dysplastic naevi: an update. *Histopathology* 2010;56;112–120. [PubMed: 20055909]
6. Elder DE. Precursors to melanoma and their mimics: nevi of special sites. *Mod Pathol* 2006;19;S4–S20. [PubMed: 16446715]
7. King R, Page RN, Gooe PB, Mihm MC Jr. Lentiginous melanoma: a histologic pattern of melanoma to be distinguished from lentiginous nevus. *Mod Pathol* 2005;18;1397–1401. [PubMed: 15976811]
8. Akbani R, Akdemir Kadir C, Aksoy BA et al. Genomic Classification of Cutaneous Melanoma. *Cell* 2015;161;1681–1696. [PubMed: 26091043]
9. Hodis E, Watson IR, Kryukov GV et al. A Landscape of Driver Mutations in Melanoma. *Cell* 2012;150;251–263. [PubMed: 22817889]
10. Tschandl P, Berghoff AS, Preusser M et al. NRAS and BRAF Mutations in Melanoma-Associated Nevi and Uninvolved Nevi. *PLOS ONE* 2013;8;e69639.
11. Curtin JA, Fridlyand J, Kageshita T et al. Distinct Sets of Genetic Alterations in Melanoma. *New England Journal of Medicine* 2005;353;2135–2147. [PubMed: 16291983]
12. Brash DE. UV Signature Mutations (). *Photochemistry and photobiology* 2015;91;15–26. [PubMed: 25354245]
13. Anna B, Blazej Z, Jacqueline G, Andrew CJ, Jeffrey R, Andrzej S. Mechanism of UV-related carcinogenesis and its contribution to nevi/melanoma. *Expert review of dermatology* 2007;2;451–469. [PubMed: 18846265]
14. Pollock PM, Harper UL, Hansen KS et al. High frequency of BRAF mutations in nevi. *Nature Genetics* 2003;33;19+.
15. Kumar R, Angelini S, Snellman E, Hemminki K. BRAF mutations are common somatic events in melanocytic nevi. *The Journal of investigative dermatology* 2004;122;342–348. [PubMed: 15009715]
16. Poynter JN, Elder JT, Fullen DR et al. BRAF and NRAS mutations in melanoma and melanocytic nevi. *Melanoma Res* 2006;16;267–273. [PubMed: 16845322]
17. Stark MS, Tan J-M, Tom L et al. Whole-Exome Sequencing of Acquired Nevi Identifies Mechanisms for Development and Maintenance of Benign Neoplasms. *Journal of Investigative Dermatology* 2018;138;1636–1644. [PubMed: 29476775]
18. Melamed RD, Aydin IT, Rajan GS et al. Genomic Characterization of Dysplastic Nevi Unveils Implications for Diagnosis of Melanoma. *Journal of Investigative Dermatology* 2017;137;905–909. [PubMed: 27890785]
19. Piscuoglio S, Ng CK, Murray M et al. Massively parallel sequencing of phyllodes tumours of the breast reveals actionable mutations, and TERT promoter hotspot mutations and TERT gene amplification as likely drivers of progression. *The Journal of pathology* 2016;238;508–518. [PubMed: 26832993]
20. Piscuoglio S, Geyer FC, Burke KA et al. Massively parallel sequencing analysis of synchronous fibroepithelial lesions supports the concept of progression from fibroadenoma to phyllodes tumor. *NPJ Breast Cancer* 2016;2;16035. [PubMed: 28721388]
21. Piscuoglio S, Burke KA, Ng CK et al. Uterine adenosarcomas are mesenchymal neoplasms. *The Journal of pathology* 2016;238;381–388. [PubMed: 26592504]

22. Lozada JR, Burke KA, Maguire A et al. Myxoid fibroadenomas differ from conventional fibroadenomas: a hypothesis-generating study. *Histopathology* 2017;71;626–634. [PubMed: 28513873]
23. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 2009;25;1754–1760. [PubMed: 19451168]
24. McKenna A, Hanna M, Banks E et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res* 2010;20;1297–1303. [PubMed: 20644199]
25. Cibulskis K, Lawrence MS, Carter SL et al. Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. *Nat Biotechnol* 2013;31;213–219. [PubMed: 23396013]
26. Saunders CT, Wong WS, Swamy S, Becq J, Murray LJ, Cheetham RK. Strelka: accurate somatic small-variant calling from sequenced tumor-normal sample pairs. *Bioinformatics* 2012;28;1811–1817. [PubMed: 22581179]
27. Koboldt DC, Zhang Q, Larson DE et al. VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. *Genome Res* 2012;22;568–576. [PubMed: 22300766]
28. Narzisi G, Corvelo A, Arora K et al. Genome-wide somatic variant calling using localized colored de Bruijn graphs. *Comms Bio* 2018;1;1–9.
29. Narzisi G, O’Rawe JA, Iossifov I et al. Accurate de novo and transmitted indel detection in exome-capture data using microassembly. *Nature methods* 2014;11;1033–1036. [PubMed: 25128977]
30. Ng CKY, Piscuoglio S, Geyer FC et al. The Landscape of Somatic Genetic Alterations in Metaplastic Breast Carcinomas. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2017;23;3859–3870. [PubMed: 28153863]
31. Weigelt B, Bi R, Kumar R et al. The Landscape of Somatic Genetic Alterations in Breast Cancers From ATM Germline Mutation Carriers. *Journal of the National Cancer Institute* 2018.
32. Shen R, Seshan VE. FACETS: allele-specific copy number and clonal heterogeneity analysis tool for high-throughput DNA sequencing. *Nucleic acids research* 2016;44;e131.
33. Geyer FC, Li A, Papanastasiou AD et al. Recurrent hotspot mutations in HRAS Q61 and PI3K-AKT pathway genes as drivers of breast adenomyoepitheliomas. *Nat Commun* 2018;9;1816. [PubMed: 29739933]
34. Weigelt B, Bi R, Kumar R et al. The Landscape of Somatic Genetic Alterations in Breast Cancers From ATM Germline Mutation Carriers. *J Natl Cancer Inst* 2018;110;1030–1034. [PubMed: 29506079]
35. Carter SL, Cibulskis K, Helman E et al. Absolute quantification of somatic DNA alterations in human cancer. *Nat Biotechnol* 2012;30;413–421. [PubMed: 22544022]
36. Landau DA, Carter SL, Stojanov P et al. Evolution and impact of subclonal mutations in chronic lymphocytic leukemia. *Cell* 2013;152;714–726. [PubMed: 23415222]
37. Chang MT, Asthana S, Gao SP et al. Identifying recurrent mutations in cancer reveals widespread lineage diversity and mutational specificity. *Nat Biotechnol* 2016;34;155–163. [PubMed: 26619011]
38. Schwarz JM, Rodelsperger C, Schuelke M, Seelow D. MutationTaster evaluates disease-causing potential of sequence alterations. *Nature methods* 2010;7;575–576. [PubMed: 20676075]
39. Carter H, Chen S, Isik L et al. Cancer-specific high-throughput annotation of somatic mutations: computational prediction of driver missense mutations. *Cancer research* 2009;69;6660–6667. [PubMed: 19654296]
40. Shihab HA, Gough J, Cooper DN et al. Predicting the functional, molecular, and phenotypic consequences of amino acid substitutions using hidden Markov models. *Hum Mutat* 2013;34;57–65. [PubMed: 23033316]
41. Bastian BC, Olshen AB, LeBoit PE, Pinkel D. Classifying melanocytic tumors based on DNA copy number changes. *The American journal of pathology* 2003;163;1765–1770. [PubMed: 14578177]
42. Dhomen N, Reis-Filho JS, da Rocha Dias S et al. Oncogenic Braf induces melanocyte senescence and melanoma in mice. *Cancer cell* 2009;15;294–303. [PubMed: 19345328]

43. Young RJ, Waldeck K, Martin C et al. Loss of CDKN2A expression is a frequent event in primary invasive melanoma and correlates with sensitivity to the CDK4/6 inhibitor PD0332991 in melanoma cell lines. *Pigment Cell & Melanoma Research* 2014;27;590–600. [PubMed: 24495407]
44. Mozraitien J, Bielskien K, Atko ius V, Labeikyt D. Molecular alterations in signal pathways of melanoma and new personalized treatment strategies: Targeting of Notch. *Medicina* 2015;51;133–145. [PubMed: 28705475]

Figure 1: Morphologic features of melanocytic nevi.

Representative micrographs of melanocytic nevi included in this study. A) SKIN3T. B) SKIN5T. C) SKIN6T. D) SKIN7T. E) SKIN8T. F) SKIN9T. G) SKIN11T. H) SKIN13T. I) SKIN14T. J) SKIN15T. K) SKIN16T.

Figure 2: Repertoire of non-synonymous somatic mutations detected by targeted capture massively parallel sequencing in melanocytic nevi.

Heatmap indicating the non-synonymous somatic mutations identified in the SKINs analyzed. Each column represents a sample; mutated genes are reported in rows. The types of somatic mutations identified are color-coded according to the legend. Loss of heterozygosity (LOH) is identified by a diagonal bar. Genes significantly mutated in melanomas are highlighted with an asterisk on the right side of the heatmap.⁸

Figure 3: Comparison of somatic genetic alterations in melanocytic nevi to that of previously reported sequencing studies in nevi.

A) Heatmaps displaying recurrent and/or shared non-synonymous somatic mutations between the nevi reported in this study ($n=12$; left) to that of dysplastic nevi reported by Melamed et al¹⁸ ($n=15$; middle) and globular and reticular nevi reported by Stark et al¹⁷ ($n=12$ and $n=18$, respectively; right). Each column represents a sample; mutated genes are reported in rows. The types of somatic mutations identified are color-coded according to the legend. B) Frequency plots and Fisher's exact test corrected for multiple testing comparing copy number gains and losses between the melanocytic nevi from this study ($n=12$) and the combined cohort of nevi ($n=30$; left), globular nevi ($n=12$; middle), or reticular nevi ($n=18$; right) from Stark et al¹⁷. The frequency of gains (green bars) or losses (purple bars) for each gene is plotted on the y-axis according to genomic location (x-axis). Inverse Log_{10} values of the Fisher's exact test p -values are plotted according to genomic location. All statistical tests were two-sided.

Figure 4: Malignant transformation of a nevus to malignant melanoma *in situ* to invasive melanoma.

A) Representative micrographs of SKIN23T representing a benign nevus (* in top panel) and invasive melanoma (** in top panel) and malignant melanoma *in situ* (bottom panel). B) The results of the targeted capture MPS analysis showing cancer cell fractions (CCFs) which were based on an integrative analysis of mutant allele fractions, tumor cell content, ploidy, and local copy number using ABSOLUTE and reported for the non-synonymous and promoter somatic mutations identified in a given lesion. CCFs are color-coded according to the legend. Clonal mutations are highlighted in orange boxes. Genes significantly mutated in

melanomas are highlighted with an asterisk on the right side of the heatmap.⁸ C) Heatmap illustrating the repertoire of copy number alterations as defined by targeted capture MPS. Samples are represented as columns and chromosomes are represented along the y axis. Absolute copy number was defined by ABSOLUTE and are color-coded according to the legend to the right of the heatmap. D) Phylogenetic tree depicting the evolution of the progression from benign nevus to invasive melanoma. The colored branches represent each of the subclones identified, and selected somatic genetic alterations that define a given clone are illustrated along the branches.

Table 1:

Summary of Clinicopathologic and Genomic Findings

Case ID	Age	Gender	Diagnosis	Level of Atypia	Solar Elastosis	Tissue Site	# Non-Synonymous Mutations	BRAF	NRAS	HRAS
SKIN03	63	M	LCDN	Moderate	None	Flank: lower	11	WT	WT	Q61L
SKIN05	73	M	CN	None	None	Back: lower	4	V600E	WT	WT
SKIN06	44	M	LCDN	Moderate	None	Abdomen	2	WT	Q61K	WT
SKIN07	43	F	LCDN	Mild	None	Back: middle	3	V600E	WT	WT
SKIN08	55	M	LCDN	Moderate	None	Back: upper	6	V600E	WT	WT
SKIN09	68	F	CN	Mild	Moderate	Arm: left	6	WT	Q61R	WT
SKIN11	62	M	AIMP Arising in Nevus	None	Mild	Back: middle	10	WT	G13R	WT
SKIN13	18	M	CN (congenital)	None	None	Hip: left	1	V600E	WT	WT
SKIN14	60	F	LCN and blue nevus	None	None	Scapula: right	5	WT	WT	WT
SKIN15	72	M	LCN (congenital)	Moderate	None	Back: R upper	1	WT	Q61K	WT
SKIN16	45	M	LCDN	Moderate	None	Back: right	12	V600E	WT	WT
SKIN23	77	F	Nevus	None	None	Forearm	9	V600E	WT	WT

LCDN: lentiginous dysplastic compound nevus, CN: compound nevus, AIMP: atypical intraepidermal melanocytic proliferation, LCN: lentiginous compound nevus, MMIS: malignant melanoma in situ, IM: invasive melanoma