Title page:

Title: BRAF/NRAS wild-type melanomas have a high mutation load correlating with histological and molecular signatures of UV damage

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Mutation load in BRAF/NRAS wild-type melanomas

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Abstract

Purpose: The mutation load in melanoma is generally high compared to other tumor types due to extensive UV damage. Translation of exome sequencing data into clinically relevant information is therefore challenging. This study sought to characterize mutations identified in primary cutaneous melanomas and correlate these with clinico-pathologic features.

Experimental design: DNA was extracted from 34 fresh frozen primary cutaneous melanomas and matched peripheral blood. Tumor histopathology was reviewed by two dermatopathologists. Exome sequencing was performed and mutation rates were correlated with age, sex and tumor site and histopathologic variables. Differences in mutations between categories of solar elastosis, pigmentation and BRAF/NRAS mutational status were investigated.

Results: The average mutation rate was 12 per megabase, similar to published results in metastases. The average mutation rate in severely sun damaged (SSD) skin was 21 per Mb compared to 3.8 per Mb in non-SSD skin (p=0.001). BRAF/NRAS wild-type tumors had a higher average mutation rate compared to BRAF/NRAS mutant tumors (27 versus 5.6 mutations per Mb; p=0.0001). Tandem CC>TT/GG>AA mutations comprised 70% of all dinucleotide substitutions and were more common in tumors arising in SSD skin (p=0.0008) and in BRAF/NRAS wild-type tumors (p=0.0007). Targetable and potentially targetable mutations in wild-type tumors, including NF1, KIT and NOTCH1, were spread over various signaling pathways.
Conclusion: Melanomas arising in SSD skin have higher mutation loads and contain a spectrum of molecular subtypes compared to *BRAF*-mutant and *NRAS*-mutant tumors indicating multi-gene screening approaches and combination therapies may be required for management of these patients.
**Translational Relevance**

Targeted therapy directed at the oncogene, *BRAF*, has improved melanoma patient outcome in recent years. Targeted strategies for *NRAS*-mutant melanoma are currently under investigation. Here we show that *BRAF/NRAS* wild-type tumors are a complex group, more commonly arising in sun-exposed sites associated with severe solar elastosis. Consistent with this, they have a high mutation load with a large proportion of C>T transitions as well as dinucleotide CC>TT transitions specific for UV-induced damage. It is likely that a number of genomic insults are required cumulatively for melanoma progression in this group. Potentially targetable mutations in *BRAF/NRAS* wild-type melanomas are spread over several different signaling pathways and this may in the future have implications for therapeutic approaches to patients harboring such tumors. Classification of melanoma into *BRAF* mutant, *NRAS* mutant and high mutation load groups may assist in identification of patients more likely to respond to particular combined drug therapies.

**Introduction**

The development of *BRAF* inhibitors, vemurafenib and dabrafenib, marked a turning point in the treatment and prognosis of patients with advanced stage metastatic melanoma. The *BRAF* oncogene is mutated in up to 50% of cutaneous melanomas and its clinical and histological associations have been well characterized\(^1\)\(^2\). *BRAF* V600E mutations occur more commonly in patients under the age of 50 years with higher nevus counts, and are more common in melanomas arising on the trunk in intermittently sun-
exposed skin\textsuperscript{1, 3}. They are also more prevalent in superficial spreading melanomas compared to other melanoma subtypes and have characteristic histopathologic features\textsuperscript{1, 4, 5}. In contrast, \textit{BRAF} V600K mutations are more common with increasing age and in melanomas on the head and neck\textsuperscript{6}. \textit{BRAF} mutant melanomas are more likely to metastasize to regional lymph nodes, compared to \textit{BRAF} wild-type tumors, which more commonly metastasize to non-nodal sites\textsuperscript{4}. Mutually exclusive of \textit{BRAF}, mutations in \textit{NRAS} are found in approximately 15% of cutaneous melanomas, which tend to be thicker, with a higher mitotic rate\textsuperscript{7}. Therapeutic targeting of \textit{NRAS} has to date been less successful than targeting of \textit{BRAF}\textsuperscript{2, 8}, though inhibitors targeting downstream of \textit{NRAS} show promise and are currently undergoing clinical trials\textsuperscript{8}. Tumors that are wild-type for \textit{BRAF} and \textit{NRAS} are less well characterized and are likely to be more heterogeneous\textsuperscript{4}.

In recent years, exome sequencing of melanoma has provided valuable insights into the complexity of this tumor. The mutation load in melanoma is far greater than other tumor types, mostly due to UV damage\textsuperscript{9}. Consequently, uncovering new therapeutic targets by sifting out driver mutations responsible for tumor development and progression from inconsequential passenger mutations is a major challenge. The majority of exome studies to date have focused on sequencing data from cell lines derived from metastatic melanomas\textsuperscript{10-16}. Few studies have included samples from primary melanomas\textsuperscript{14, 15, 17} and none have correlated exome data with clinical and histological variables of the primary tumor.
Integration of molecular changes with clinical and pathological characteristics of the primary tumor may assist the translation of exome data to a clinically relevant understanding of tumor biology. The aim of this study was to identify mutations associated with primary cutaneous melanomas and correlate these with clinico-pathologic features.

**Materials and Methods**

**Selection of samples for discovery phase.** Fresh frozen tissue from primary cutaneous melanomas was collected prospectively from melanoma clinics at the Victorian Melanoma Service, Alfred Hospital and Peter MacCallum Cancer Centre (n=5) as well as retrospectively from the Victorian Cancer Biobank (n=8) and Melanoma Institute Australia Biospecimen bank (n=21). Matched patient blood was also collected to distinguish somatic mutations from germ line mutations. In one case, matched blood was not available, therefore normal DNA was extracted from dissected adjacent normal tissue (case NM002). All patients gave informed consent and ethics approval was obtained from the Peter MacCallum Cancer Centre Ethics Committee (11/25) for all human tissues and clinicopathological data used in this study. The histopathology of all cases was reviewed by two dermatopathologists (CM and RS) and various histopathologic features were scored (detailed below). Thirty-two tumors were verified on haematoxylin and eosin stain as containing >80% tumor. The remaining two samples (NM002 and NM019) contained 50-60% tumor and were included as the sensitivity of mutation detection by next generation sequencing remains high even for impure samples\(^{18}\). Tumor and matched normal DNA was extracted using the Qiagen Gentra Puregene kit (Qiagen, Hilden,
Germany) according to the manufacturer’s protocol. Extracted DNA was quantified using a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA) and a Quant-iT dsDNA HS kit (Invitrogen).

**Assessment of clinical and histological variables.** Patient information including date of birth, date of surgery, gender and site of tumor was collected. Histological variables Breslow thickness (mm), ulceration, mitotic rate (no./mm²), melanoma subtype, presence of regression, lymphovascular invasion, neurotropism and microsatellite lesions were assessed. Melanoma subtype was classified according to WHO criteria into nodular melanoma (NM), superficial spreading melanoma (SSM) and lentigo maligna melanoma (LMM)¹⁹. Solar elastosis was graded (0, none; 1, mild; 2, moderate; 3, severe) according to the amount of elastotic fibres in normal skin adjacent to the melanoma in the excision specimen³. Histological evidence of solar damage was also dichotomized; tumors arising on non-severely sun damaged (non-SSD) (solar elastosis scores of 0 or 1) and tumors arising on SSD skin (solar elastosis scores of 2 or 3). Pigmentation was graded according to the amount of pigment within constituent melanocytes³. Tumor lymphocytic infiltration was graded 0-3 according to the density and distribution of the lymphocytic infiltrate within the dermal component of the tumor²⁰. Features of nesting, scatter, epidermal contour and circumscription were also assessed³.

**Exome sequencing.** A schematic summary of how samples were sequenced and analyzed is shown in Supplementary Figure 1. One µg of DNA was sheared to approximately 200 bp by sonication (Covaris). Exome enrichment was performed using the NimbleGen EZ
Exome Library v2.0 kit (n=25) or the Agilent SureSelect Human All Exon version 2 kit (n=9) according to recommended protocols. Sequencing was performed on an Illumina HiSeq2000 instrument. Samples were loaded in an indexed pool of 3 samples per lane, and an average coverage of 141× was achieved across all samples. We did not observe any significant differences in performance of the different exome capture platforms. Library preparation and sequencing information for each sample is provided in Supplementary Table 1.

**Sequencing alignment and variant calling.** Sequence reads were aligned to the human genome (hg19 assembly) using the Burrows–Wheeler Aligner (BWA) program\(^{21}\). Local realignment around indels and base quality score recalibration were performed using the Genome Analysis Tool Kit (GATK)\(^{22}\) software, and duplicate reads removed using Picard\(^{23}\). Single nucleotide variants (SNVs) and indels were identified using the GATK Unified Genotyper, Somatic Indel Detector\(^{24}\) and MuTect (Broad Institute)\(^{25}\). Variants were annotated with information from Ensembl\(^{26}\) Release 64 using the Ensembl Perl Application Program Interface including SNP Effect Predictor. Data with SNVs identified in this cohort is provided in Supplementary Table 2.

**Candidate variant identification.** Variants were first filtered for confident calls using a quality score cutoff of ≥30 and a read depth of ≥20. Next, variants were filtered to include only somatic mutations, located in canonical transcripts (the most prevalent transcript as detailed by the UniProt Knowledgebase\(^{27}\)), with bidirectional read support, and mutations predicted to be potentially deleterious (mutations which potentially change the coding of a protein i.e. non-synonymous, splice site, indels, stop codon lost and stop
codon gain mutations). A list of potentially ‘actionable’ mutations, predicted to have diagnostic, prognostic or therapeutic implications, was generated by comparing our list of filtered mutations to potential targets listed by Catalogue of Somatic Mutations in Cancer (COSMIC)\textsuperscript{28} and The Genomics of Drug Sensitivity in Cancer Project (GDSC), Sanger Institute UK\textsuperscript{29}. 

In order to assess the frequency of commonly mutated genes in this cohort, a literature search was performed to identify mutations validated as occurring in >10% of melanomas.

**Pathway analysis**

For each tumor, the number of mutations in 8 pathways, well described in melanoma\textsuperscript{30}, was assessed. Gene(s) listed in each pathway are described in Supplementary Table 3.

**Validation of mutations**

The \textit{BRAF} and \textit{NRAS} mutation status of each tumor was determined using high resolution melting analysis (HRM) and Sanger sequencing as previously described\textsuperscript{31}.

Actionable mutations shown in Supplementary Table 4 were validated using Sanger sequencing. PCR amplification was performed on a Rotor-Gene Q (Qiagen, Hilden, Germany) or a LightCycler 480 (Roche Diagnostics, Penzberg, Germany). Primers sequences and specific PCR reaction conditions are available upon request. Following PCR amplification, a 1/10 dilution PCR product was sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA).
sequencing products were purified with Agencourt CleanSEQ beads (Beckman Coulter, Pasadena, CA), followed by capillary electrophoresis on an ABI 3730 DNA Sequencing instrument (Applied Biosystems Foster City, CA). Data analysis was performed with Sequencher software, version 4.6 (Gene Codes, Ann Arbor, MI).

**Validation of mutation load in **BRAF/NRAS** wild-type tumors**

To validate our concept of a high mutation load group of melanomas, we retrospectively examined exome data from Hodis et al.\textsuperscript{14} and Krauthammer et al.\textsuperscript{15}, which sequenced independent cohorts with a total of 198 cutaneous melanomas and described the mutation rate together with **BRAF** and **NRAS** status. Acral, mucosal and uveal melanomas were excluded.

**Statistical analysis**

Non-parametric Spearman correlation was used to investigate associations between mutation rate and other continuous variables (thickness and mitotic rate) to avoid any undue influence of outliers. The Mann-Whitney U and Kruskal-Wallis tests were used to assess associations between mutation rate and respectively dichotomous variables and variables with 3 or more categories. Ordered logistic regression was used to assess associations between solar elastosis scores and continuous variables. Multinomial logistic regression was used to assess for associations between **BRAF** V600E, **BRAF** V600K, **NRAS** and WT tumors. All analyses were performed using Stata12 statistical software (Stata Corporation, College Station, TX).
Results

A total of 34 primary cutaneous melanomas were sequenced from 34 patients (59% female) with a median age at diagnosis of 67 years. Melanomas were from the lower limb (47%), head and neck (26%), trunk (18%) and upper limb (9%). Fifty-three percent of cases arose in non-SSD skin. Notably, none of the cases were from acral or mucosal sites. Seventeen cases were NM, 16 SSM and 1 LMM. Of the 16 SSM, 5 had a prominent dermal nodule. Thirty percent of melanomas were amelanotic. The median thickness was 6.2mm and median mitotic rate 10 per mm² (Table 1).

Landscape of mutations in primary melanoma samples.

A total of 139,962 somatic mutations located in canonical transcripts were identified (see Methods), with an average of 4,117 (range 105-28,507) mutations per tumor (or on average 33 mutations per Mb). Of these, 37,981 mutations (12 mutations per Mb) were predicted to be deleterious (Figure 1), consistent with rates of non-synonymous changes reported in other studies\textsuperscript{14,15}

Signatures of UV-induced damage.

The ratio of non-synonymous to synonymous changes was 1.7, consistent with other melanoma exome studies\textsuperscript{11,32}. A breakdown of mutational consequences based on the deleterious filter is shown in Figure 2A. Eighty-five percent of single nucleotide variants were composed of C>T or G>A transitions consistent with UV-induced DNA damage (Figure 2B). Ninety-nine percent of all single nucleotide variants were at a dipyrimidine site and of those 86% were C>T transitions, similar to results published elsewhere\textsuperscript{10,15}. 


The UV signature of DNA damage was also confirmed by investigating dinucleotide
CC>TT/GG>AA transitions, which is a more specific signature of UV damage since the
likelihood of obtaining two tandem transitions is very low compared to single nucleotide
variant C>T changes which may not be due to UV damage. All but 6 tumors showed
tandem CC>TT changes, which made up 73% of all dinucleotide substitutions (Figure
2C). Tumors arising in SSD skin had an average of 26 CC>TT transitions, compared to
an average of 4 in tumors arising in non-SSD skin (p=0.0008). The number of CC>TT
transitions as a percentage of total dinucleotide transitions in tumors with no solar
elastosis was 36% compared to 74% in tumors with severe solar elastosis (p=0.04)
(Figure 2C). BRAF/NRAS wild-type tumors had an average of 34 CC>TT transitions
compared to an average of 6 in both NRAS and BRAF mutant tumors (p=0.0007).

Tumors which showed no CC>TT/GG>AA changes (n=6) were from relatively sun-
protected sites (such as the lower limb and trunk) with low solar elastosis scores (Figure
2C) and were either BRAF or NRAS mutant. Only one of these tumors arose in SSD skin
(solar elastosis score of 2) and was BRAF V600K mutant.

**Correlation of mutation rate with clinical and histopathologic variables.**

Melanoma arising on sun-exposed anatomic sites (head, neck and upper limb) had higher
mutation rates compared to those arising on the lower limb and trunk as would be
expected, although this difference did not reach statistical significance (p=0.07).
Mutation rates were over 5-fold greater in tumors arising in skin with severe solar elastosis compared to tumors arising in skin with no solar elastosis ($p=0.001$) (Figure 3).

Interestingly there was an inverse correlation between thickness and mutation rate ($r=-0.4$, $p=0.02$) (Figure 4). There was no statistically significant association between mutation rate and other clinico-pathologic features such as age, sex, mitotic rate, tumor subtype, pigmentation, features of regression or tumor-infiltrating lymphocytes.

**Mutation rate in tumors with classical melanoma mutations.**

Thirty-five percent of tumors in the discovery cohort had a canonical $BRAF$ V600E or V600K mutation and 35% were $NRAS$ mutant in exon 3 (Figure 1). Interestingly, the proportion of $BRAF$ V600K (7/34) mutations was greater than $BRAF$ V600E mutations (5/34). All $BRAF$ and $NRAS$ mutations were independently confirmed by Sanger sequencing. Consistent with previous reports$^6$, most $BRAF$ V600K mutations were in tumors arising in SSD skin, whereas $BRAF$ V600E mutations were more common in tumors from sun-protected sites with low solar elastosis scores ($p=0.02$).

The presence of a $BRAF$ or $NRAS$ mutation was associated with a lower mutation load within the tumor compared to WT tumors ($p<0.0001$). This association was strongest for $BRAF$ V600E mutant tumors (Relative Risk Ratio=0.38 per mutation 95%CI 0.16-0.91), and less strong for $BRAF$ V600K and $NRAS$ mutant tumors (RRR=0.91 per mutation 95%CI 0.82,1.02 and RRR=0.82 per mutation 95%CI 0.69,0.97 respectively).
This cohort of melanomas exhibited a range of non-synonymous mutations in genes classically associated with melanoma including c-KIT (n=3), RAC1 (n=2) and CDKN2A (n=3). Other melanoma-associated mutations validated in the literature as occurring in >10% of cases are shown in Figure 1. The majority of these mutations clustered in tumors with high mutation loads.

**BRAF/NRAS wild-type tumors**

Ten patients in this study had tumors lacking canonical BRAF and NRAS mutations. The median age was 78 years compared to 66 years in the BRAF/NRAS mutant group, although this difference was not statistically significant. BRAF/NRAS wild-type tumors, compared to tumors carrying NRAS or BRAF mutations, were more common on the head and neck (p=0.04) and from SSD skin (p=0.01). This is in keeping with previous observations in BRAF mutant tumors. BRAF/NRAS wild-type tumors had a higher average mutation rate (27 versus 5.6 mutations per Mb; p=0.0001).

Previous studies have shown that BRAF mutant melanomas are commonly of SSM subtype and typically display characteristic histopathologic features including epidermal thickening and peripheral circumscription. In our study, 6 BRAF/NRAS wild-type tumors were SSM, 3 NM and 1 LMM subtype. The contour of the epidermis within the radial growth phase of BRAF/NRAS wild-type tumors was thinner compared to the adjacent epidermis, with a discontinuous transition from tumor to normal skin compared to a more abrupt cut-off in mutant tumors (p=0.02). There was no statistically significant association between BRAF/NRAS wild-type tumors and other histopathologic features.
(tumor thickness, mitotic rate, ulceration, regression, pigmentation, tumor-infiltrating lymphocytes, cell size or predominant cell type).

Most of the **BRAF/NRAS** wild-type tumors harbored a number of recently validated melanoma gene mutations (Figure 1). From our filtered list of mutations, those occurring in genes reported in the COSMIC database were identified. The average number of COSMIC mutations was 48 in the **BRAF/NRAS** wild-type group compared to 12 in the **BRAF** and **NRAS** groups (p=0.0004) (Figure 5A). While COSMIC details mutations which may or may not be actionable, the Genomics of Drug Sensitivity in Cancer (GDSC) database contains an enriched set of 70 genes which have drug sensitivity information linked to mutational profiles of cancer cells. The average number of potentially actionable mutations in genes reported in the GDSC database was 11 in the **BRAF/NRAS** wild-type group compared to 3 in **BRAF** and 4 in **NRAS** groups (p=0.0003) (Figure 5A).

Examination of somatic mutations in these tumors indicated that a majority had actionable mutations in known cancer genes as shown in Supplementary Table 4. All potentially actionable mutations were independently confirmed by Sanger sequencing. Five tumors had mutations in the tumor suppressor gene **NF1**. **KIT** mutations were present in 3 tumors, all with SSD skin (p=0.05) and a higher than average mutation rate of 30 per Mb (p=0.02). Actionable hotspot mutations in **RAC1** (P29S)1415, **PDGFRA** (E996K)34 and **HRAS** (G13D)35 were also identified in individual tumors. Additionally,
the patient with a somatic HRAS mutation also had a point mutation in the KIT gene together with a truncating mutation in CDKN2A (Supplementary Table 4).

Three tumors in the BRAF/NRAS wild-type group had fewer than 10 mutations per Mb. Interestingly, 2 of these tumors had mutations in NOTCH1, one of which had a concurrent TP53 mutation. Other potentially actionable mutations in this group are shown in Supplementary Table 4.

**Validation of high mutation load in BRAF/NRAS wild-type tumors**

Of 198 cutaneous melanomas (42 primary and 156 metastatic samples, excluding acral, uveal, mucosal and unknown primary melanomas) sequenced by Hodis et al.\textsuperscript{14} and Krauthammer et al.\textsuperscript{15}, 46\% were BRAF V600E mutant, 9\% were BRAF V600K, 23\% were NRAS mutant and 21\% were BRAF/NRAS wild-type. The mean number of somatic substitutions was 397, 577, 495 and 940 for BRAF V600E, V600K, NRAS and WT tumors, respectively (p=0.006).

**Pathway analysis**

An analysis of the number of mutations across 8 well-described melanoma pathways in BRAF and NRAS mutant tumors compared to BRAF/NRAS wild-type tumors is shown in Figures 5B and 5C. Whilst the MAPK pathway contains the majority of mutations for all groups, BRAF/NRAS wild-type tumors have multiple mutations spread across 7 of these pathways, in particular the PI3K-Akt and p53 pathways. NRAS mutants on the other hand,
had a slight preponderance to other pathways but \textit{NRAS} remains the dominantly mutated gene.
Discussion

This study shows that *BRAF* and *NRAS* wild-type melanomas are a complex group with a high mutation load due to extensive UV damage. This finding was validated in a larger cohort of 198 primary and metastatic melanomas. *BRAF*/*NRAS* wild-type melanomas are strongly associated with UV damage as evidenced clinically by the higher degree of solar elastosis, and on a molecular level, with a high proportion of C>T transitions at a dipyrimidine, and more specifically, more frequent tandem CC>TT transitions. It is likely that different treatment strategies will be required when treating patients with high mutation load melanomas, which harbor an array of potentially targetable mutations. Classification of melanoma into *BRAF* mutant, *NRAS* mutant and high mutation load groups may be helpful for identification of patients suitable for particular combined drug therapies.

To our knowledge, this is the first study to sequence primary cutaneous melanomas and correlate molecular data with clinical characteristics. Whilst previous studies have shown an association between molecular signatures of UV damage and cutaneous melanomas\(^1^4\)\(^1^5\), none have quantified the degree of solar elastosis adjacent to the primary tumor. In fact, melanomas arising in non-glaborous, non-mucosal sites are often universally classified as ‘sun-exposed’. Krauthammer *et al* also found that *BRAF*/*NRAS* wild-type cutaneous melanomas had a high mutation burden, but reported *BRAF*/*NRAS* mutant melanomas to have mutation loads in the mid range. That cohort was skewed by uveal, acral and mucosal tumors with low mutation burdens. Though acral melanomas can have *BRAF* or *NRAS* mutations and perhaps be associated with UV damage\(^3^6\), mucosal (which
may be NRAS or BRAF mutant\textsuperscript{14, 15, 37} and uveal melanomas are not UV related. With histological assessment of solar damage, we have been able to show distinct molecular signatures amongst common histological subtypes of cutaneous melanoma, which may have important implications for treatment.

The inverse correlation between mutation load and primary tumor thickness shown in this study was surprising. The clonal evolution model rests on the notion that cancer progresses from a low metastatic potential (thin early stage melanomas) to a strong metastatic state (thicker more locally advanced melanomas) through the accumulation of molecular alterations such as mutations which increase the invasive and proliferative potential of cancer cells as tumor burden increases\textsuperscript{38}. Our data suggest that in melanomas there is not a simple relationship between tumor burden and mutation load. Rather, it suggests that melanomas do not necessarily accumulate mutations as they get thicker. In support of this, a similar range of mutation rates published for metastatic disease and cell lines\textsuperscript{15} suggests that the majority of damage is done early in tumor development. BRAF mutations are known to occur in melanocytic nevi\textsuperscript{39} and \textit{in situ} melanomas. Figure 4 highlights that, if a key mutation is present (\textit{BRAF} V600E in particular), melanomas can become very thick with few additional mutations. Conversely, \textit{BRAF}/NRAS wild-type tumors contain a variety of low frequency driver mutations consistent with the requirement for multiple ‘hits’ in the genome to progress.

The median thickness of primary melanomas in this cohort (6.2mm) is considerably thicker than the median thickness of melanomas at the time of diagnosis for the general
population. In Victoria, Australia, the median thicknesses of superficial spreading and nodular melanomas at diagnosis are 0.6mm and 2.6mm, respectively. Females were over-represented in this cohort compared to the general melanoma population. This ascertainment bias relative to the general melanoma population is due to the difficulties associated with acquiring fresh tissue samples from primary melanomas. Tumors need to be thick in order to take a fresh sample for tumor banking whilst ensuring adequate material is available for routine pathologic assessment and sufficient tumor material remains for DNA extraction and sequencing. Whilst thick melanomas (>2mm) represent only approximately 20% of cases, they contribute over 60% of melanoma deaths and are therefore an important group to study. This cohort was intentionally enriched for NM due to their significant contribution to mortality that is disproportionate to their prevalence and as a result similar in total mortality burden to the more common superficial spreading melanomas.

Given all primary tumors were thick (>2mm) and numbers in this cohort were small, it is not possible to conclude that BRAF mutant tumors are thicker in general. In fact, larger studies have shown that the frequency of BRAF V600E mutations decreases with tumor thickness. Our findings are in agreement with other studies, which show that BRAF V600E mutations are more prevalent in non-SSD skin, and V600K and KIT mutations are more prevalent in SSD skin. Furthermore, our findings that BRAF and NRAS wild-type tumors tend to have a thinner epidermis in contrast to adjacent normal skin and a tendency for the transition from tumor to normal skin to be discontinuous are also supported by larger studies.
The correlation between \textit{BRAF} status and mutation rate suggests that where a predominant driver mutation is present, the mutation rate will often remain low. Whilst \textit{KIT} mutations occurred in 3 \textit{BRAF/NRAS} wild-type tumors, these all had a high mutation burden. There were 3 tumors in the \textit{BRAF/NRAS} wild-type group with mutation rates less than 10 per Mb. Two of these tumors had a mutation in \textit{NOTCH1}, which has recently been implicated in growth and invasion of uveal melanoma\textsuperscript{45} and is potentially targetable\textsuperscript{46,47}.

Inactivating mutations in the tumor suppressor gene \textit{NF1} were present in 50\% of wild-type tumors compared to just 4\% of \textit{BRAF/NRAS} mutant tumors. Hodis \textit{et al.} reported a similarly high frequency of \textit{NF1} mutations amongst \textit{BRAF/NRAS} wild-type compared to mutant tumors (25\% vs 2\%)\textsuperscript{14}. Loss of \textit{NF1} tumor suppression can lead to constitutive \textit{MAPK} pathway activation through Ras\textsuperscript{48} (Figure 5B). This highlights the potential for \textit{NF1} to become an important therapeutic target in wild-type tumors.

It is important to note that, given the current knowledge on the sensitivity and resistance of therapeutics to specific mutations, classifying mutations into driver and passenger mutations as well as actionable and/or druggable is difficult. An ‘actionable mutation’ as defined above, is a genetic alteration which may have significant diagnostic, prognostic, or therapeutic implications for a patient. A subset of these may be ‘drugable’, that is, predict for sensitivity or resistance to a specific drug\textsuperscript{49}. Therapeutic targeting of mutations in tumour suppressor genes is particularly challenging, as research into
restoring normal gene function in patients (i.e gene therapy) is currently ineffective for most tumour suppressor genes.

Whilst systematic identification of actionable mutations in this cohort is particularly challenging due to the large number of mutations present, we have attempted to identify actionable mutations by comparing variants to a number of cancer mutation databases. BRAF/NRAS wild-type tumors contain a number of low frequency driver mutations and therefore require a larger number of UV-induced insults to the genome to progress. Importantly, they do contain a number of potentially targetable mutations, though these are spread over different pathways. Like BRAF mutant tumors, the MAPK pathway is most frequently involved in BRAF/NRAS wild-type tumors, however this is accompanied by greater burden of mutations in this group overall. Whilst there is significant cross-talk between pathways, results from this study suggest that therapeutic targeting of multiple pathways may be necessary rather than focused targeting of a single pathway in BRAF/NRAS wild-type tumors with a high mutation burden.

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References


**Figure Legends:**

**Figure 1. Genetic landscape of primary melanomas.** Numbers of somatic non-synonymous mutations across 34 matched melanoma samples per Mb are shown in blue bars from highest to smallest. The mutational status of tumors is indicated for classical melanoma genes including *BRAF*, *NRAS*, *KIT* and *TP53*. Only mutations in exon 3 of *NRAS*, exon 15 of *BRAF* and the *RAC1* P29S are shown. For *BRAF*, V600E, V600K and L597R mutations are represented by an ‘E’, ‘K’ or ‘R’ in the square, respectively. Below classical genes are the presence of other melanoma-associated mutations validated in the literature as occurring in >10% of cases. For all genes, the number and percentage of cases with at least one mutation in the gene are indicated (grey bars).

**Figure 2. Molecular signature of UV-induced DNA damage.** Samples have been grouped according to degree of solar elastosis (dashed lines). (A) Breakdown of mutational consequences based on the deleterious filter. The percentage of non-synonymous, splice site, Indels, stop codon lost and stop codon gain mutations are shown for each sample. (B) Breakdown of mutations based on the type of nucleotide substitution. The percentage of A>C/T>G, A>T/T>A, C>A/G>T, C>G/G>C, C>T/G>A and T>C/A>G mutations are shown for each sample. (C) The percentage of dinucleotide CC>TT/GG>AA over total number of dinucleotide changes for each sample. Three samples as indicated by the red lines have no CC>TT/GG>AA dinucleotide changes and these belong to the none (NM016 and SSM006) and mild (SSM009) solar elastosis groups. Samples SSM001 and NM001 in the mild solar elastosis group and NM004 in the
moderate solar elastosis group have 1 or 2 dinucleotide changes but no CC>TT/GG>AA changes.

Figure 3. High mutation load tumors are associated with more severe solar elastosis of the surrounding skin. Box-and-whisker plot of mutation rate (per Mb) vs. the degree of solar elastosis ranking from mild (score=0, n=5), mild (score=1, n=13), moderate (score=2, n=6) and severe (score=3, n=10). There was a significant association between higher solar elastosis scores and increasing mutation rate (p=0.001).

Figure 4. \textit{BRAF} mutant melanomas have lower mutations rates compared to wild-type tumors. Mutation rate (per Mb) vs. Breslow thickness (mm) of each tumor. The mutational status of each tumor is described with \textit{BRAF} V600E (Green triangle), \textit{BRAF} V600K (Orange square), \textit{NRAS} exon 3 positive (Red diamond) and non \textit{BRAF}/\textit{NRAS} positive or WT (Blue cross) marked for each tumor on the graph. There was an inverse correlation between thickness and mutation rate (Spearman correlation, r=-0.4, p=0.02)

Figure 5. \textit{BRAF}/\textit{NRAS} wild-type tumors display a range of actionable mutations in various melanoma signaling pathways. (A) Identification of deleterious mutations in genes listed in the COSMIC and GDSC datasets across \textit{BRAF}, \textit{NRAS} and WT tumors. \textit{BRAF}/\textit{NRAS} WT tumors showed more hits in genes listed on both the COMSIC and GDSC datasets compared to \textit{BRAF} and \textit{NRAS} mutant tumors (p=0.0004 and p=0.0003, respectively for the two datasets). (B) Schematic diagram of the molecular subtypes/pathways in melanoma (adapted from Vidwans et al., \textit{PLoS One}...
2011;6(3):e18257). The pathways are colour coded for simplicity: GNAQ/GNA11 (Gold), MAPK (Red), CDK (Blue), P53/BCL (Purple), MITF (Orange), NRAS (Yellow), c-KIT (Pink) and AKT/PI3K (Green). (C) Pie charts illustrating the proportion of mutations in the 8 pathways of melanoma based on NRAS and BRAF status. Segments of the pie chart are colour coded according to their signaling pathway with adjacent values representing average number of mutations in pathway per sample.
Table 1. Clinical and Histopathologic characteristics of patient cohort

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HN head and neck, UL upper limb, T trunk, LL lower limb, SSM superficial spreading melanoma, SSM+N superficial spreading melanoma with a prominent dermal nodule, NM nodular melanoma, LMM lentigo maligna melanoma.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
BRAF/NRAS wild-type melanomas have a high mutation load correlating with histological and molecular signatures of UV damage

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