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Molecular characterisation of cutaneous melanoma: creating a framework for targeted and immune therapies

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Large-scale genomic analyses of cutaneous melanoma have revealed insights into the aetiology and heterogeneity of this disease, as well as opportunities to further personalise treatment for patients with targeted and immune therapies. Herein, we review the proposed genomic classification of cutaneous melanoma from large-scale next-generation sequencing studies, including the largest integrative analysis of melanoma from The Cancer Genome Atlas (TCGA) Network. We examine studies that have identified molecular features of melanomas linked to immune checkpoint inhibitor response. In addition, we draw attention to low-frequency actionable mutations and highlight frequent non-coding mutations in melanoma where little is known about their biological function that may provide novel avenues for the development of treatment strategies for melanoma patients.

Cutaneous melanoma is the deadliest form of skin cancer that originates in melanocytes. Early-stage melanoma is generally curable with early detection and surgery. Patient prognosis significantly decreases for patients with late-stage metastatic disease (Balch *et al*, 2009). Historically, metastatic melanoma has been considered an untreatable disease, where previous standard of care treatment regimens of either dacarbazine, interferon- α -2b (in the adjuvant setting) and high-dose IL-2 (HD IL-2) for advanced melanoma produce variable benefits to patients, with few patients exhibiting durable responses (reviewed in Girotti *et al*, 2014). Following the discovery of the *BRAF* V600 mutation in melanoma and with the advances in immune checkpoint inhibitors, melanoma has emerged as the poster child for both targeted and immune therapy.

Hotspot mutations in the V600 codon of *BRAF* in ~50% of melanomas (Davies *et al*, 2002) and the G12, G13 and Q61 codons of *NRAS* in ~25% of cases (Sekiya *et al*, 1984; Albino *et al*, 1989; van't Veer *et al*, 1989) were identified before the era of next-generation sequencing. These findings led to the development of *BRAF* inhibitors (e.g., vemurafenib and dabrafenib) and MEK kinase inhibitors (e.g., trametinib). Unfortunately, although the antitumour responses to MAPK inhibitors have been marked, resistance remains a clinical challenge. Conversely, response to immune checkpoint antibodies, such as anti-CTLA4 (e.g., ipilimumab) and anti-PD-1 (e.g., nivolumab and pembrolizumab), have produced more durable responses in the treatment of

metastatic melanoma patients; however, not all patients respond. As a result, understanding the molecular mechanisms that modulate the response to immunotherapies are critical.

Herein, we review the latest large-scale genomic studies of cutaneous melanoma, including the largest integrative analysis from The Cancer Genome Atlas (TCGA) Network (Cancer Genome Atlas Network, 2015), and present how such information has been proposed to inform clinical decision-making (Table 1). We briefly provide clinical updates on MAPK inhibitors and immunotherapies and highlight findings from biomarker analyses from immune checkpoint inhibitor studies in melanoma. In addition, we draw attention to low-frequency actionable mutations that could inform on potential rare combination strategies to further personalise treatment options for patients. Finally, we examine the discoveries of non-coding mutations that occur in a large fraction of patients where little is known about their biology that provide new avenues of investigation in melanoma.

THE CHALLENGE OF IDENTIFYING SIGNIFICANTLY MUTATED GENES IN MELANOMA

Cutaneous melanoma has one of the highest mutation burdens of any cancer sequenced to date (Hodis *et al*, 2012; Krauthammer *et al*, 2012), with the latest estimate of ~17 mutations per Mb

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Table 1. Implications for clinical management of melanoma patients modified from TCGA comprehensive analysis

	BRAF	RAS	NF1	Triple wild-type
Genomic Subtypes				
• MAPK pathway	• BRAF V600 E/K/R, K601E CDKN2A mut/del/hyper-meth (~60%)	• (N/K) RAS G12, G13, Q61 CDKN2A mut/del/hyper-meth (~70%)	• NF1 LoF mut **[BRAF non-V600 mut]	• KIT COSMIC mut/amp, PDGFRA amp, KDR (VEGFR2) amp • rare COSMIC GNA11 mut, GNAQ mut CDKN2A mut/del/hyper-meth (~40%)
• Cell cycle pathway	CCND1 amp (~3%) CDK4 amp (3%) / # (rare CDK4 COSMIC mutations)	CCND1 amp (8%) # CDK4 amp (7%) / # (rare CDK4 COSMIC mutations)	# CCND4 amp (4%)	CCND1 amp (1%) # CDK4 amp (~15%)
*DNA Damage Response & Cell death pathways	RBI1 mut (2%) TP53 mut (~10%) Note: ~90% wild-type	RBI1 mut (8%) TP53 mut (20%)	RBI1 mut (11%) TP53 mut (~30%)	TP53 mut (7%) *BCL2 upregulation
• PI3K/AKT pathway	*MDM2 amplification (1%) †PTEN mut/del (17%) †AKT3 overexpression (<10%) †(rare AKT1/3 and PIK3CA COSMIC mut)	*MDM2 amplification (3%) †PTEN mut/del (8%) †AKT3 overexpression (~40%) †(rare AKT1/3 and PIK3CA COSMIC mut)	*MDM2 amplification (4%) †PTEN mut/del (7%) †AKT3 overexpression (~30%)	*MDM2 amplification (~15%) †PTEN mut/del (7%) †AKT3 overexpression (~20%)
• Epigenetics	#IDH1 mut (rare EZH2 COSMIC mut) #ARID2 mut (~15%)	#IDH1 mut (rare EZH2 COSMIC mut) #ARID2 mut (~15%)	#IDH1 mut (rare EZH2 COSMIC mut)	#IDH1 mut (rare EZH2 COSMIC mut)
Telomerase pathway	Promoter mut (~75%)	Promoter mut (~70%)	Promoter mut (~85%)	Promoter mut (<10%) TERT amp (~15%)
Highlighted additional pathways	PD-L1 amp, MITE amp, PPP6C mut (7%) CTNNB1 T41, S45 mut (~0.5%)	PPP6C mut (~14%) CTNNB1 G34 mut (~0.5%)	CTNNB1 D32,G34 mut (~0.5%)	CTNNB1 T41, S45 mut (~1%)
Beta-catenin	~30%	~25%	~25%	~40%
High immune infiltration (pathology)	~90%	~95%	~95%	~30%
Mutational burden	12 mut/mb	17 mut/mb	39 mut/mb	9.2 mut/mb
Class 1: Clinically Actionable	• BRAE inhibitors (e.g. dabrafenib, vemurafenib)	• MEK inhibitors (e.g. trametinib, cobimetinib)	• MEK inhibitors (e.g. trametinib, cobimetinib)	• C-KIT inhibitors (e.g. imatinib, dasatinib, nilotinib, sunitinib) • VEGF inhibitors (AEB071)
Class 2: Transitionally Actionable	Immuno-therapies (e.g. mAb against immune checkpoint proteins (ipilimumab, nivolumab, pembrolizumab), high dose bolus IL-2, Interferon-α2b)	• BRAE inhibitors (e.g. dabrafenib, vemurafenib)	• MEK inhibitors (e.g. trametinib, cobimetinib)	• MDM2/p53 interaction inhibitors (e.g. AMG322)
	• MDM2/p53 interaction inhibitors (e.g. AMG322)	• ERK inhibitors	• CDK inhibitors (e.g. palbocicli)	
		pan-RAF inhibitor (e.g. LY3009120)	#IDH1 inhibitors (e.g. AG-120, IDH305)	
			#EZH2 inhibitors (e.g. GSK126, EPZ005678, E11)	
			†PI3K/mTOR inhibitors (e.g. MLN117, OSI4027)	
			†Akt inhibitor (e.g. GSK2141795)	
Class 3: Pre-clinical	• PPP6C/Aurora kinase inhibitors (e.g. MLN8237)	• ARID2 Chromatin remodelers (synthetic lethality)		• BCL2/BH3 mimetics
	Mutant RAF dimer inhibitors (e.g. BGB6659)			

Abbreviations: LoF = loss-of-function; GoF = gain-of-function; Mb = Megabases; mut = mutation; TCGA = The Cancer Genome Atlas. Canonical pathways and frequency of significant genetic alterations in BRAF, RAS (N-, H- and K-), NF1 and Triple wild-type melanomas are given. LoF and GoF mutations are indicated in blue and red panels, respectively. Potential predictive biomarkers are represented with symbols (•, #, *, †, ‡, §). Class 1: clinically actionable alterations that impact point-of-care decisions; Class 2: translationally actionable alterations that are deemed likely to be clinically relevant but more evidence is required to support use in point-of-care decision making; and Class 3: alterations that have biological importance based on preclinical studies, but clinical relevance is unclear. Modified from latest cutaneous melanoma TCGA study.

calculated by TCGA from whole-exome sequencing (WES) of 318 primary and metastatic melanomas originating from non-glabrous (hair-bearing) skin (Cancer Genome Atlas Network, 2015). Over 75% of the cutaneous melanoma samples sequenced in the TCGA cohort possessed a UV signature, defined by having C>T transitions at dipyrimidine sites accounting for >60%, or CC>TT mutations in >5%, of the total mutation burden (Brash, 2015). Ultraviolet A and UVB are the two wavelengths humans are exposed to from solar radiation. Ultraviolet B-induced DNA damage results in the formation of cyclobutane pyrimidine dimers (CPDs). The most widely accepted mutational mechanism leading to the generation of the UV signature involves the deamination of cytosines or 5-methyl-cytosines in the CPDs to uracils or thymidines, which are then replicated in an error-free manner to produce C>T transitions at dipyrimidines (Taylor, 2015). Experimental systems have shown G>T substitutions as being the major mutations resulting from UVA-mediated by oxidative damage (Brash, 2015). However, recent studies have shown that UVA can induce C>T signature mutations through a reactive oxygen species (ROS)-dependent mechanism involving both the photoprotective black eumelanin and the phototoxic red pheomelanin (Noonan *et al*, 2012; Premi *et al*, 2015). Furthermore, pheomelanin is thought to be phototoxic itself through the generation of ROS, which has shown to increase incidence of melanoma in mice even in the absence of UV exposure (Mitra *et al*, 2012). As a result, identifying driver mutations in melanoma is challenging given the high mutation burden, especially in the case of tumour suppressors and infrequently mutated oncogenes.

This problem was first raised in the analyses of over 100 melanoma exomes in 2012 (Hodis *et al*, 2012; Krauthammer *et al*, 2012). For example, more than 500 genes were mutated in at least 10% of the 121 melanomas sequenced – many of which were not expressed in melanocytes and melanoma cell lines. To address this problem, sophisticated statistical tools were developed, including InVEx, which uses sequencing data from intronic and untranslated regions (UTRs) to infer gene-specific mutation burdens (Hodis *et al*, 2012). Using this tool, genes that were found to be significantly mutated included well-established melanoma oncogenes and tumour suppressors, *BRAF*, *NRAS*, *MAP2K1*, *PTEN* and *CDKN2A*, as well as novel significantly mutated genes (SMGs) containing hotspot and loss-of-function mutations that were expressed in melanoma cell lines. These novel SMGs that possessed hotspot mutations caused by C>T transitions were the Rho GTPase, *RAC1* (p. P29S), the catalytic subunit of the heterotrimeric PP6 protein phosphatase complex, *PPP6C* (p. R301C), and the serine threonine kinase, *STK19* (p. D89N). Novel SMGs possessing loss-of-function mutations included *ARID2*, which encodes a component of the SWI/SNF chromatin-remodeling complex. These novel SMGs were concurrently identified in an exome study of 147 melanomas using an alternative approach to define SMGs, which included consideration of gene expression (Krauthammer *et al*, 2012). This represented an advance to the field as hotspot mutations caused by C>T transitions in oncogenes were not well known.

Algorithms developed before InVEx, such as MutSig, have evolved over the years to take into consideration factors shown to affect regional mutation rates (e.g., gene size, replication time and expression). A pancancer study in which MutSigCV was used to identify SMGs across many cancers estimated that to identify genes possessing driver mutations in $\geq 2\%$ of melanoma patients, up to 5300 samples would need to be sequenced given the high mutation burden of this cancer (Lawrence *et al*, 2014). Ensuing studies have sequenced a fraction of this sample size (TCGA consortium, $n = 318$; Yale cohort, $n = 213$), revealing additional SMGs possessing coding mutations in melanoma, which include *NF1*, *RBI* and *IDH1* (Cancer Genome Atlas Network, 2015; Krauthammer *et al*,

2015). These genes were previously implicated in melanoma through focused sequencing or functional studies, but were found for the first time to be significantly mutated. Almost all *IDH1* mutations encoded the p.R132C amino-acid substitution with an incidence of $\sim 5\%$ and $\sim 2\%$ in samples of the TCGA and Yale cohorts, respectively. Furthermore, *DDX3X*, a novel SMG was identified by InVEx in $\sim 6\%$ of samples (Cancer Genome Atlas Network, 2015).

GENOMIC CLASSIFICATION OF MELANOMA: *BRAF*, *RAS*, *NF1* AND TRIPLE WILD-TYPE

Following *BRAF* and *NRAS*, the next most frequently SMG possessing coding mutations was *NF1*, found in $\sim 15\%$ of patients (Figure 1). Germline mutations in *NF1* are known to cause an inherited multisystem genetic disorder, neurofibromatosis type 1, which is characterised by changes in skin colouring pigmentation (e.g., café-au-lait spots) and the growth of both benign and malignant tumours (Andersen *et al*, 1993). *NF1* encodes for neurofibromin, a RAS-GTPase-activating protein, which negatively regulates RAS signalling by facilitating hydrolysis of RAS-GTP to the RAS-GDP-inactive form. Both the Cancer Genome Atlas Network (2015) and Krauthammer *et al* (2015) observed that close to 40–50% of melanomas that lacked a hotspot mutation in *BRAF* (p.V600 or K601E) or *NRAS* (p. G12, G13 or Q61) possessed loss-of-function mutations in *NF1*. Previous studies have clearly demonstrated the mutual exclusivity of hotspot *BRAF* and *NRAS* mutations in melanoma; however, *NF1* mutations were also significantly anticorrelated with hotspot *BRAF*, but not *NRAS*, mutations. Altogether, these results supported the categorisation of cutaneous melanoma into four genetic subgroups by MAPK driver mutations: *BRAF*, *RAS* (N-H-K), *NF1* (lacking a *BRAF* p.V600 or *RAS* p.G12, G13 and Q61 hotspot mutation) and *Triple wild-type* (WT) melanomas (Table 1). *NF1* subtype patients were older, had a higher mutation burden (Cancer Genome Atlas Network, 2015) and were reported to possess significant co-occurring mutations in additional RASopathy genes (e.g., *RASA1*, *RASA2*, *PTPN11* and *SOS1*) (Arafah *et al*, 2015; Krauthammer *et al*, 2015). Furthermore, WES analysis of desmoplastic melanoma, a rare form of melanoma with sarcomatous histology that occur in chronically sun-exposed skin, revealed significant loss-of-function mutations in *NF1* in over 50% of samples (Shain *et al*, 2015a). Notably, *BRAF* p.V600E and *NRAS* p.Q61L/R mutations were not found, providing strong genetic evidence of a role for *NF1* in melanomagenesis.

A number of molecular and clinical features were associated with the genomic subtypes. For example, only $\sim 30\%$ of *Triple WT* samples harboured a UV signature compared with over 90% for the other three subtypes (Cancer Genome Atlas Network, 2015). Somatic copy number analysis revealed *Triple WT* melanomas had a higher fraction of samples possessing significant copy number amplifications consistent with other studies (e.g., Curtin *et al*, 2005). Amplicons included the 4q12 minimal common region containing *KIT*, *PDGFRA* and *KDR* (also known as *VEGFR2*), as well as amplifications in loci encompassing *TERT*, *CDK4* and *CCND1*. Fusion analysis from RNA sequencing (seq) and whole-genome sequencing (WGS) data revealed that *Triple WT* melanomas had more complex structural rearrangements and candidate fusion drivers (although few recurrent fusions were identified). Furthermore, *Triple WT* melanomas had more frequent recurrent *KIT* mutations (Cancer Genome Atlas Network, 2015). We suspect that the established genomic framework based on somatic alterations including MAPK mutations can aid in personalised therapeutic decision-making in melanoma (Table 1).

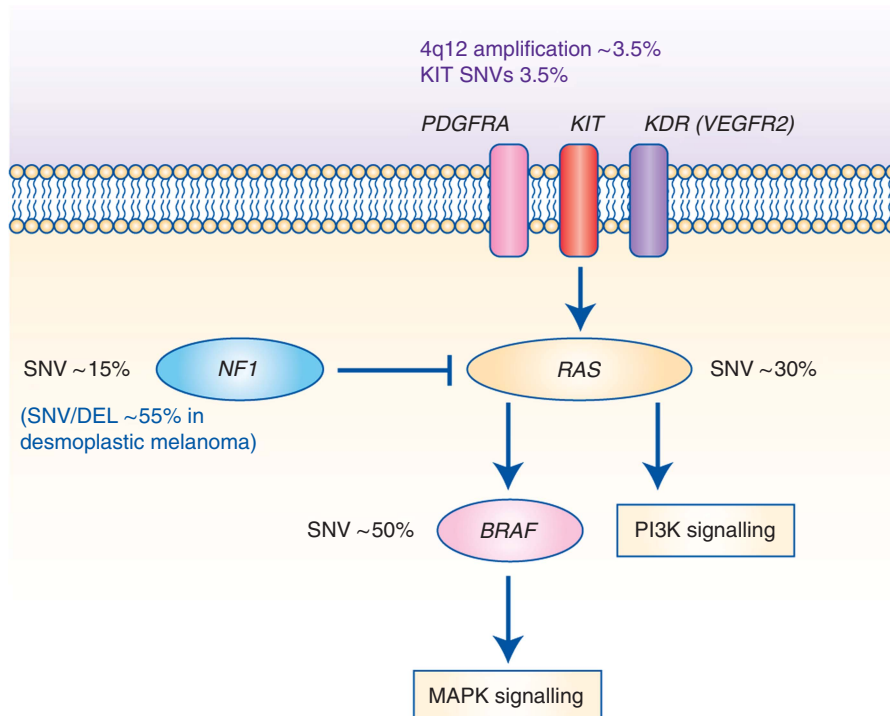


Figure 1. MAPK pathway genetic alterations that constitute the genomic subtypes. Approximate frequency of single-nucleotide variants (SNVs) of SMGs in *BRAF*, *RAS* and *NF1* are shown. In the latest TCGA study, the estimate of *NRAS* SNVs in melanomas is ~30%, with the majority encoding amino-acid Q61 changes, although low-frequency SNVs encoding for alterations in amino acids G12, G13 and Q61 in *HRAS* and *KRAS* were also found in ~2% of samples. Significant amplification of the 4q12 amplicon as well as recurrent *KIT* mutations were found more frequently in melanomas lacking MAPK mutations (*Triple WT*). Of note, *NF1* is either mutated or deleted (DEL) in ~55% of desmoplastic melanomas, where no hotspot *BRAF* and few hotspot *RAS* mutations have been reported.

MELANOMA HETEROGENEITY

A key question in melanoma pathogenesis concerns the temporal acquisition of driving genetic alterations. To address this problem, a unique study sequenced over 290 cancer-associated genes in 150 areas of 37 primary melanomas alongside neighbouring precursor lesions. The various pathogenic stages of melanoma (benign, intermediate, intraepidermal and invasive) were identified through independent histopathological scoring from eight dermatopathologists (Shain *et al*, 2015b). This group found benign lesions possessed *BRAF* (p.V600E) mutations exclusively. Intermediate lesions harboured *BRAF* (p.V600K/K601E) and *NRAS* mutations, as well as additional driver events that included *TERT* promoter mutations, which were commonly found in both intermediate lesions and melanomas *in situ*. Biallelic inactivation of *CDKN2A* and mutations in chromatin remodellers (e.g., *ARID2*) were found in invasive melanomas. In addition, *PTEN* and *TP53* mutations were found in advanced tumours.

Exome sequencing of matched primary and metastatic samples from the same patients have revealed important insights into the processes involved in metastatic spread. Specifically, a recent study provided evidence that primary tumours metastasise in parallel to different anatomical sites, often from a common parental sub-population, rather than sequentially from one site to the next (Sanborn *et al*, 2015). Conversely, individual metastases were sometimes founded by more than one cell population in the primary tumour, which could explain why specific resistance variants are found in different sites after initial response to therapy. Single-cell RNA sequencing analysis of bulk melanomas provided further evidence of tumour heterogeneity at the transcriptional level, revealing that different malignant cells within the same tumour can possess distinct transcriptional states that are

associated with either sensitivity or resistance to MAPK targeted therapies (Tirosh *et al*, 2016).

MAPK INHIBITORS IN BRAF MUTANT MELANOMAS

In the United States between 2011 and 2013, the FDA approved *BRAF* inhibitors, vemurafenib (Chapman *et al*, 2011; McArthur *et al*, 2014) and dabrafenib (Hauschild *et al*, 2012), as well as the MEK inhibitor, trametinib (Flaherty *et al*, 2012), for the treatment of unresectable or metastatic melanoma after the demonstration of improved PFS (progression-free survival) and/or OS (overall survival) in comparison with dacarbazine in *BRAF* mutant melanoma patients. *BRAF* inhibitor treatment causes a number of side effects, which include photosensitivity, pyrexia and secondary cutaneous squamous cell carcinomas (SCCs), as well as keratoacanthomas (reviewed in Carlino *et al*, 2015). *BRAF* inhibitor-induced SCCs and keratoacanthomas are thought to be induced by the well-characterised paradoxical MAPK activation, which is caused by *BRAF* inhibitor-mediated homo- and heterodimerisation of non-mutant RAF isoforms leading to the activation of MAPK pathway in *BRAF* wild-type cells (reviewed in Girotti *et al*, 2014). Furthermore, the development of resistance emerges in ~50% of patients within 7 months of treatment (Chapman *et al*, 2011; Hauschild *et al*, 2012).

Subsequent combined *BRAF* and MEK inhibitor phase III clinical trials for patients with a *BRAF* (p.V600E or p.V600K) mutation were shown to increase PFS and/or OS compared with *BRAF* inhibitor monotherapy (Larkin *et al*, 2014; Long *et al*, 2014; Robert *et al*, 2015). Inhibition of the MAPK pathway targeting two nodes resulted in a reduction in *BRAF* inhibitor resistance in ~25% of patients and a decrease in secondary SCCs and

keratoacanthomas, presumably by preventing paradoxical MAPK activation. However, resistance is still a problem, and combination immune and targeted therapy strategies are next steps in treating *BRAF* mutant melanoma patients (reviewed in Prieto *et al*, 2016). Clinical trials to date of ipilimumab in combination with *BRAF* and MEK inhibitors have shown an increase in severe adverse effects, such as hepatotoxicity and bowel perforation. Nonetheless, different combinations of *BRAF* inhibitors and ipilimumab are well tolerated (e.g., dabrafenib and ipilimumab) (Ribas *et al*, 2013; Puzanov *et al*, 2014). Furthermore, combination therapies with anti-PD-1 and anti-PD-L1 appear to be more promising given their reduced toxicity. Studies have demonstrated that *BRAF* inhibitor treatment can produce a more favourable microenvironment leading to increased CD8+ T-cell infiltration and a decrease in immunosuppressive cytokines (Boni *et al*, 2010). Some caution has been suggested regarding use of MEK inhibitors with immune therapy because of their effects on T-cell function; however, studies using mouse models have demonstrated the feasibility of combination *BRAF* and MEK inhibitors with anti-PD-1 therapeutics (Hu-Lieskovan *et al*, 2015). Interestingly, transcriptomic analyses of melanomas acquiring MAPK inhibitor resistance demonstrated in half of disease progressing melanomas potential cross-resistance mechanisms to salvage anti-PD-1/PD-L1 immunotherapy through T-cell exhaustion and loss of antigen presentation (Hugo *et al*, 2015). As a result, identifying the most optimal treatment regimens for combination targeted and immune therapy remains an important avenue of investigation (Grob *et al*, 2015).

A number of preclinical studies and characterisation of patient samples treated with *BRAF* inhibitors have identified numerous mechanisms that lead to resistance, which will not be covered here and have been reviewed elsewhere (e.g., Carlino *et al*, 2015). However, the majority of these mechanisms involve reactivation of the MAPK pathway, including mutations of *NRAS*, promotion of the splice variant, p61 *BRAF* p.V600, and *BRAF* p.V600E amplification, which all lead to increased *BRAF* dimerisation and constitutive signalling (Carlino *et al*, 2015). Recent studies of *RAF* inhibitors able to inhibit both *BRAF* V600E and *BRAF* mutant dimers may have more clinical promise and may reduce incidence of resistance (Girotti *et al*, 2015; Yao *et al*, 2015; Zhang *et al*, 2015).

GENOMIC CLASSIFICATION: IMPLICATIONS FOR CLINICAL MANAGEMENT OF PATIENTS WITH TARGETED AND IMMUNE THERAPIES

Given the clinical utility of MAPK targeted therapy and the anticorrelative relationship of hotspot *BRAF*, *RAS* and loss-of-function *NFI* mutations, we suspect that the framework generated by the TCGA study will be useful to guide personalised therapeutic decisions for cutaneous melanoma (Table 1). Currently, ongoing clinical trials of MEK and CDK4 inhibitors have shown clinical promise in treating patients with *NRAS* mutant melanomas (Sosman *et al*, 2014), which were based on preclinical studies that demonstrated MEK inhibitors induce low-level apoptosis in melanoma, but not cell cycle arrest in a *NRAS*-driven mouse model (Kwong *et al*, 2012). The discovery of the *NFI* subtype in cutaneous and desmoplastic melanomas provides a potential new subclass of melanomas to be targeted with MAPK inhibitors (Cancer Genome Atlas Network, 2015; Krauthammer *et al*, 2015; Shain *et al*, 2015a). Early, preclinical studies have demonstrated that *NFI*-null melanomas activate *RAS* leading to MEK kinase dependence as well as sensitivity to MAPK inhibitor treatment (Maertens *et al*, 2013; Nissan *et al*, 2014). Other studies have shown not all *NFI* mutant melanomas respond to MEK inhibitors (Krauthammer *et al*, 2015; Ranzani *et al*, 2015). Further investigation is required to determine the mechanisms that mediate MEK inhibitor response in *NFI* mutant melanomas,

which may aid in further stratifying patients who could benefit from such therapies in combination with immune checkpoint inhibitors given the co-occurring high mutation burden of this subtype (see below and Table 1). In addition, other studies have suggested that pan-*RAF* and MEK inhibitor combinations may be a useful strategy to target *NFI* mutant cancers (Whittaker *et al*, 2013; Whittaker *et al*, 2015). Combination therapy targeting a third node of the MAPK pathway may also be useful for all three subtypes in the form of ERK inhibitors (Carlino *et al*, 2015). Finally, *Triple WT* melanomas were found to possess more frequent *KIT* recurrent mutations as well as amplification of the 4q12 amplicon containing *KIT*, *PDGFRA* and *KDR* (Cancer Genome Atlas Network, 2015). Clinical trials for patients harbouring *KIT* aberrations with tyrosine kinase inhibitors, imatinib and dasatinib, have produced responses in a subset of patients (Hodi *et al*, 2008; Woodman *et al*, 2009). The observation of coamplification of *PDGFRA* and *KDR* support consideration of combination therapies with receptor tyrosine kinase inhibitors, sorafenib, crenolanib, regunafenib and pasopanib, in *KIT*-amplified melanomas.

Integrative analysis of the genomic subtypes in combination with clustering data from additional data platforms including mRNA, reverse phase protein array (RPPA) and histopathological analysis revealed a subset of samples with evidence of lymphocytic infiltration associated with improved patient survival, which were equally distributed across the genomic subtypes (Cancer Genome Atlas Network, 2015) (Table 1). Clustering mRNA-seq data revealed three subgroups of melanoma enriched for genes involved in immune function ('immune' subgroup), skin and neuronal development ('keratin' subgroup) and low expression of the melanocytic lineage-specific transcription factor, MITF ('MITF-low' subgroup). The immune subgroup also correlated with lymphocytic infiltration by pathology, and high LCK protein expression from RPPA data. Approximately 50% of samples within each subtype possessed an immune signature, which included PD-L1. Presumably, these are potential subset of patients who will respond to immune therapies.

MOLECULAR FEATURES LINKED TO IMMUNE THERAPY RESPONSE IN MELANOMA

The importance of immunotherapy in melanoma has long been recognised, which is demonstrated, in part, by the fact that HD IL-2 treatment can produce long-term remissions in ~6–8% of patients (Girotti *et al*, 2014). Immune therapies to treat melanoma can be broadly characterised into categories that include cytokines/chemokines (e.g., HD IL-2), cancer vaccine therapies, oncolytic virus therapies (e.g., T-VEC), immune checkpoint inhibitors (e.g., anti-CTLA-4 and anti-PD-1) and adoptive cell transfer (ACT) (Sharma and Allison, 2015).

The biggest clinical success in melanoma has come from the immune checkpoint antibodies targeting PD-1 (the programmed cell death-1) and CTLA-4 (cytotoxic T lymphocyte antigen-4). Programmed cell death-1 and CTLA-4 are coinhibitory T-cell receptors that drive a negative signal to diminish T-cell activation and induce immune tolerance. Programmed cell death-1 has two ligands, PD-L1 that is expressed in many cell types including tumours cells, and PD-L2, which is predominately expressed in antigen-presenting cells (Sharma and Allison, 2015). Anti-PD-1 (e.g., nivolumab and pembrolizumab), anti-PD-L1 and anti-CTLA-4 (e.g., ipilimumab) antibodies can reverse immune suppression and activate T cells, triggering an antitumour response. Ipilimumab was the first agent to improve OS in advanced melanoma patients (Hodi *et al*, 2010), and in combination with dacarbazine improved OS to 11.2 months compared with 9.1 months with higher survival rates at 3 years to 20.8% vs 12.2% (hazard ratio for death, 0.72; $P < 0.001$) (Robert *et al*, 2011) compared with dacarbazine and placebo. Longer-term follow-up studies demonstrated a

'plateauing' effect of the OS curve with 20% survival beginning at 3 years (Schadendorf *et al*, 2015).

Early-phase anti-PD-1 clinical trials reported higher response rates and fewer immune-related toxicities produced compared with anti-CTLA-4 (reviewed in Girotti *et al*, 2014; Prieto *et al*, 2016). Combination anti-PD-1 and anti-CTLA-4 studies revealed further impressive results (Wolchok *et al*, 2013; Larkin *et al*, 2015; Postow *et al*, 2015). However, more severe adverse effects (grade 3 or 4) were observed. In the phase III Checkmate 067 study that evaluated monotherapies of either nivolumab or ipilimumab compared with combination therapy (nivolumab and ipilimumab), stratifying patients by *BRAF* mutation, disease stage and PD-L1 expression (PD-L1 positivity cutoff $\geq 5\%$ on tumour cells with any staining of any intensity on the cell surface) (Larkin *et al*, 2015), revealed an overall response rate (ORR) of 57.6% for the combination group vs 43.7% and 19% for monotherapies nivolumab and ipilimumab, respectively. Notably, the PD-L1-positive cohort reported median PFS of 14 months (combination or nivolumab therapy) vs 3.9 months (ipilimumab monotherapy), whereas the PD-L1-negative cohort displayed increased success with combination therapies with a PFS of 11.2 months over both nivolumab (5.2 months) and ipilimumab (2.8 months) monotherapies (Larkin *et al*, 2015). One could interpret these results that given the increased toxicity of dual checkpoint inhibitor treatment, combination anti-PD-1 and anti-CTLA-4 could be reserved for PD-L1-negative patients who have shown improved ORR and PFS compared with monotherapy (Larkin *et al*, 2015). Whereas PD-L1-positive patients would receive anti-PD-1 monotherapy as a result of having similar benefit compared with combination treatment. The major caveat for this proposition being that overall survival results have not been reported yet, which is the best indicator for immune therapy response (Ascierto *et al*, 2015). In contrast, it should be noted that the Checkmate 069 study demonstrated the superiority of combination therapy (ipilimumab and nivolumab) over ipilimumab monotherapy with an ORR of 61% compared with 11% in *BRAF* wild-type patients (similar results found in *BRAF* mutant patients) (Postow *et al*, 2015), and also that the ORR was independent of PD-L1 status with similar response rates in PD-L1-positive and -negative tumours (Postow *et al*, 2015). Quantitative immune profiling studies that examined not only immune cell presence but also density and PD-1/PD-L1 proximity within melanoma samples revealed that pre-existing CD8-positive T cells negatively regulated by the PD-1/PD-L1 axis are predictive of tumour regression to anti-PD1 therapy (Tumeh *et al*, 2014). PD-L1 as a biomarker for response is challenged by the fact that PD-L1 expression is dynamic and heterogeneous, both within different portions of the tumour and immune cells. Furthermore, detection methods, time of measurement (e.g., pre-treatment vs on treatment) and determining appropriate cutoffs for positive staining complicates standardisation between studies (Schalper *et al*, 2016).

To date, a number of studies have revealed molecular features of melanoma that modulate response to immune therapy, which include: mutational load, intrinsic transcriptomic states and intestinal microbiome, as well as the composition of peripheral immune cells in patients and melanoma immune infiltrates. For example, WES data has been used to identify neoantigens with implications for immunotherapy in a number of cancers (reviewed in Schumacher and Schreiber, 2015), including melanoma (van Rooij *et al*, 2013; Snyder *et al*, 2014; Van Allen *et al*, 2015). Neoantigens can be defined as 'non-self' peptides produced from somatic mutations that are presented on major histocompatibility complex class I molecule that are recognised by the adaptive immune system. The earliest multipatient melanoma exome sequencing study with anti-CTLA-4 blockade response data revealed that a high mutational burden was associated with increased survival (Snyder *et al*, 2014). The authors also observed a number of four amino-acid (tetrapeptide) motifs within predicted neoantigens that were shared by patients with long-term clinical

benefit, but absent in patients that had minimal or no clinical benefit (Snyder *et al*, 2014). Other studies have not observe this tetrapeptide signature in larger patient cohorts (Schumacher *et al*, 2015; Van Allen *et al*, 2015). A study of 110 patients with complete WES and a subset of RNA-seq data determined mutational and neoantigen load, as well as expression of cytolytic markers in the microenvironment were associated with clinical benefit to anti-CTLA4 treatment (Van Allen *et al*, 2015). Specifically, *granzyme A* (*GZMA*), *perforin* (*PRF1*), *PD-L2* and *CTLA-4* were all significantly upregulated in the cohort that showed clinical benefit. In addition, studies performing integrative analysis from publicly available TCGA data revealed a WNT/ β -catenin signature associated with an absence of a T-cell expression signature (Spranger *et al*, 2015). Follow-up functional studies in preclinical melanoma mouse models demonstrated that activation of the WNT/ β -catenin pathway produces resistance to anti-PD1 therapy. Furthermore, transcriptomic analysis of pre-treatment melanoma biopsies with anti-PD-1 checkpoint inhibitor response data identified increased expression of genes involved in mesenchymal transition, cell adhesion, extracellular matrix remodeling, angiogenesis and wound healing as barriers to anti-PD-1 response (Hugo *et al*, 2016). Expression markers of the CD8 T-cell cytolytic score, *CTLA-4*, and *PD-L2* that correlated with response to anti-CTLA4 (Van Allen *et al*, 2015) were not upregulated in anti-PD-1-responsive melanomas, suggesting differential response mechanisms between the two checkpoint inhibitors (although sample data sets are still relatively small). Although few specific gene mutations have been found to modulate response to immune therapy, mutations in *BRCA2* have been linked to improved response (Hugo *et al*, 2016) and *PTEN* mutations/deletions in resistance to anti-PD-1 checkpoint inhibitors (Peng *et al*, 2016). A number of environmental factors have been linked to response to immune therapy including the presence of cytokines, chemokines and ratios of peripheral and intratumoral immune cells. For example, clinical studies have reported a high peripheral neutrophil-to-lymphocyte ratio measured before ipilimumab treatment to be correlated with reduced OS (Ferrucci *et al*, 2015; Zaragoza *et al*, 2016). An increased eosinophil count detected early in ipilimumab-treated patients was associated with improved OS, whereas elevated levels of monocytic myeloid-derived suppressor cells were found in non-responders (Delyon *et al*, 2013; Gebhardt *et al*, 2015). Work from mouse models have shown activation of intratumoral antigen-presenting CD103+ dendritic cells that increase CD8+ T-cell priming improve response in mice to *BRAF* and PD-L1 blockade (Salmon *et al*, 2016). Interestingly, two preclinical studies recently demonstrated intestinal microbiota of mice influence the response to anti-CTLA-4 and anti-PD-L1 checkpoint inhibitors, suggesting that manipulating the microbiota in human patients could be used as a strategy to improve response to immunotherapy (Sivan *et al*, 2015; Vetizou *et al*, 2015).

One of the best examples of personalised medicine in the melanoma arena is ACT therapy whereby tumour-infiltrating T-lymphocytes are isolated from a patient's tumour, expanded and activated *ex vivo* and reinfused back into the patient (Rosenberg and Restifo, 2015). This strategy has been shown to lead to complete tumour regressions in metastatic melanoma patients. Combining this strategy with WES analysis, neoantigen prediction, followed by expansion of T cells obtained from the tumour or peripheral blood that react to predicted neoantigens, has produced impressive improvements to personalise ACT response (Cohen *et al*, 2015). One of the benefits of this strategy being that this approach leads to the targeting of neoantigens solely in the tumour-limiting immune-related side effects. Combining ACT strategies with dendritic cell immunisation (Carreno *et al*, 2015) and immune checkpoint inhibitor treatment, as well as targeted therapy, may provide opportunities to increase long-term durable responses in metastatic melanoma patients.

POTENTIAL LOW-FREQUENCY ACTIONABLE MUTATIONS

In an attempt to identify low-frequency actionable and driver mutations without sequencing the estimated 5300 samples required to identify driver mutations in ≥2% of melanoma patients (Lawrence *et al*, 2014), studies have cross-referenced the COSMIC mutation database that compiles mutation data from all next-generation sequencing (NGS) cancer studies. Such analyses has revealed low frequency actionable mutations in *EZH2*, *AKT3* and *PIK3CA*, as well as driver mutations frequently found in other types of melanoma, such as *GNAQ* and *GNAI1* (Hodis *et al*, 2012; Cancer Genome Atlas Network, 2015). Therapeutic strategies to target these frequently mutated G proteins in uveal melanomas include combination MAPK- (e.g., PD0325901 or MEK162) and PKC- (e.g., AEB071) targeted therapies (Chen *et al*, 2014). In addition, hotspot mutations in epigenetic regulators, such as *IDH1* (p. R132), and less the frequently mutated *EZH2* (p.Y641), indicate another potential mode of action for targeted therapy in melanoma. IDH1 inhibitors (e.g., AG-120) are currently in phase 1/2 clinical studies for patients with glioma and acute myeloid leukaemia (Popovici-Muller *et al*, 2012), and recent preclinical studies have demonstrated the clinical utility of EZH2 inhibitors in melanoma (Tiffen *et al*, 2015). Furthermore, *AKT1/3* and *PIK3CA* mutations observed in *BRAF* and *RAS* mutant melanomas may be useful biomarkers for possible combination therapy utilising MAPK and PI(3)K/AKT/mTOR inhibitors (Cancer Genome Atlas Network, 2015). Understanding the relationships of these low-frequency actionable mutations within the different genomic subtypes may inform clinicians on rare therapeutic combinations that may potentially lead to durable response in combination with MAPK and immune targeted therapies (Table 1). In contrast, a number of studies have identified frequent non-coding mutations in genes where the biological significance is still unclear, but affect druggable pathways that may lead to new therapeutic approaches.

DARK MATTER: NON-CODING MUTATIONS IN MELANOMA

In the era of next-generation sequencing, some of the most novel genomic insights in cutaneous melanoma aetiology have come from the analyses of non-genic portions of the genome. For example, telomerase activity is upregulated in almost all cancers,

including melanoma. However, the mechanisms that mediate these processes were not entirely clear. In 2013, two concurrent studies identified two mutually exclusive recurrent *TERT* promoter mutations found in ~70% of cutaneous melanoma (Horn *et al*, 2013; Huang *et al*, 2013). These hotspot mutations were discovered through interrogation of familial melanomas (Horn *et al*, 2013) and the analysis of WGS data (Huang *et al*, 2013). In the familial study, linkage analysis and high-throughput sequencing of a melanoma prone family revealed a disease-segregating germline mutation in the *TERT* promoter (Horn *et al*, 2013). Subsequent sequencing of sporadic melanomas did not identify the same *TERT* promoter mutation, but instead identified 74% of metastatic melanoma cell lines, 85% of metastatic tissue and 33% of primary melanomas that possessed *TERT* promoter mutations predominantly at two positions, chr 5: 1 295 228 C>T (C228T) and chr 5: 1 295 250 C>T (C250T), in a mutually exclusive manner (Table 2). These mutants produce a new ETS/TCF binding motif that led to increased transcriptional activity and *TERT* expression (Horn *et al*, 2013; Huang *et al*, 2013). A number of telomerase inhibitor strategies have been developed, and are currently in clinical trials for the treatment of polycythemia vera and multiple myeloma treatment (Ruden and Puri, 2013). In addition to *TERT*, six genes harbouring recurrent non-coding mutations at frequencies between 5 and 10% in cutaneous melanoma have been discovered (Table 2).

Sequencing analyses of 20 desmoplastic melanomas identified hotspot promoter mutations in *NFKBIE* in ~15% of cases (Table 2), altering the binding motifs for several transcription factors (e.g., ELF1; Shain *et al*, 2015a). The protein, IκBε encoded by *NFKBIE*, inhibits the NF-κB signalling pathway via the cytoplasmic retention of transcription factors. Remarkably, these mutations were concurrently identified in six non-desmoplastic melanomas. In addition, a number of frequent non-coding mutations causing C>T transitions have been found in cutaneous melanoma, including the mutation in the 5'-UTR of *RPS27* identified in ~10% of samples by combining data sets generated from four NGS studies with unpublished data (Dutton-Regester *et al*, 2014) (Table 2). *RPS27* is a component of the 40S subunit of ribosomes whose aberrant expression has been linked to a number of cancers, including melanoma (Santa Cruz *et al*, 1997). The *RPS27* mutation is thought to increase its expression by expanding the 5'TOP element (Dutton-Regester *et al*, 2014), a motif known to control mRNA translation regulated through the PI(3)K/AKT and mTOR pathways. Additional genes whose expression is positively

Table 2. Recurrent non-coding mutations in melanoma

Gene name	Gene symbol	Reported genomic coordinates for predominant SNV	Frequency (cutaneous melanoma) (%)	mRNA expression
Telomerase reverse transcriptase	<i>TERT</i>	Chr5: 1 295 228 C>T Chr5: 1 295 250 C>T	~ 70	Increase
Ribosomal protein S27	<i>RPS27</i>	Chr1: 153 963 239 C>T	~ 10	Increase
Succinate dehydrogenase complex, subunit D, integral membrane protein	<i>SDHD</i>	Chr11: 111 957 523 C>T Chr11: 111 957 541 C>T	~ 5–10	Decrease
Mitochondrial ribosomal protein S31	<i>MRPS31</i>	Chr13: 41 345 346 C>T	~ 5	Unknown
NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 9	<i>NDUFB9</i>	Chr8: 125 551 344 C>T	~ 5	Decrease
Diphthamide biosynthesis 3	<i>DPH3</i>	Chr3: 16 306 504 C>T Chr3: 16 306 505 C>T/A	~ 10	Increase
Oxidoreductase NAD-binding domain containing 1	<i>OXNAD1</i>	Chr3: 16 306 504 C>T Chr3: 16 306 505 C>T/A	~ 10	Increase
Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, epsilon	<i>NFKBIE</i>	Chr6: 44 233 400 C>T (clustered C>T from chr6: 44 233 379 to -44 233 439)	~ 15	Unknown (proposed GoF)

Abbreviations: SNV = single-nucleotide variant; 5'-UTR = untranslated region. Genomic coordinates (hg19) of predominant single-nucleotide variant (SNV), frequency and effect on mRNA expression are indicated for recently discovered promoter, 5'-UTR and non-coding mutations in cutaneous and desmoplastic melanomas.

regulated by non-coding mutations include a bi-directional promoter mutation in ~10% of melanomas in two genes, *diphthamide biosynthesis 3 (DPH3)* (Fredriksson *et al*, 2014) and *oxidoreductase NAD-binding domain containing 1 (OXNAD1)* genes (Denisova *et al*, 2015) (Table 2). *DPH3* is required for the generation of a unique post-translational modification in the elongation factor-2, which is essential for protein synthesis. Although reporter assays have suggested that the recurrent mutations in *DPH3* and *OXNAD1* results in a significant increase in promoter activity, increased mRNA expression of these genes in tumours was not observed (Denisova *et al*, 2015).

In contrast, WGS studies revealed promoter mutations that reduce expression in two genes, which included the *succinate dehydrogenase complex, subunit D (SDHD)* (Weinhold *et al*, 2014) and the *NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 9 (NDUFB9)* (Poulos *et al*, 2015) (Table 2). A pancancer WGS analysis first revealed the *SDHD* promoter mutations, which disrupt ETS binding sites, resulting in decreased expression (Weinhold *et al*, 2014). *SDHD* is one of the four subunits of the succinate dehydrogenase (SDH) enzyme whose function is to anchor SDH to the matrix of the mitochondrial inner membrane and has an important function as it is involved in both the citric acid cycle and oxidative phosphorylation energy conversion pathways. Decreased *SDHD* expression caused by the promoter mutations is consistent with its tumour suppressor function (Weinhold *et al*, 2014). The recurrent *NDUFB9* promoter mutation was discovered from the first sequencing of a melanoma cell line, COLO-829, and was subsequently validated with TCGA data and functional studies, which determined that ~5% of melanomas possess this mutation. Luciferase assays demonstrated modest effects on the promoter activity, and the expression of the corresponding gene in tumour data is not significant between mutant and wild-type samples. This may be due to issues related to tumour purity, but still requires further investigation. In the TCGA study, MutSig CV determined *RPS27*, *NDUFB9* and the *mitochondrial ribosomal protein S31 (MRPS31)* possessing a novel 5'-UTR mutation in ~6% of samples to be significantly mutated (Cancer Genome Atlas Network, 2015). However, the biological function of the majority of these newly discovered genes in melanoma with non-coding mutations is unclear.

FUTURE STUDIES

We still have much to learn about the biology and accompanying biomarkers that influence response to immune therapy in melanoma. With the long-term benefits and durable responses produced by immune checkpoint inhibitors, combination targeted and immune therapy treatment strategies are evident next steps in the evolution of melanoma therapies. Identifying biomarkers that predict response and the mechanisms of resistance to combination targeted and immune therapy will be vital. Although there is a growing list of molecular determinants of immune therapy response in melanoma, analysis using additional data platforms and comprehensive examination of multiple patient biopsies during the course of treatment in large cohorts will likely reveal new factors that influence immune checkpoint inhibitors response. Furthermore, genomic studies to date have revealed actionable, low-frequency driver mutations in melanoma. This raises the possibility of the use of additional combination strategies with MAPK inhibitors and immune therapies with drugs targeting low-frequency driver events, to further personalise treatment options for melanoma patients. It is clear that preclinical studies are needed to determine efficacy of such treatment strategies to target less well-understood mutations in melanoma, including *NF1* mutant melanomas. Finally, understanding the biological

significance of novel recurrent non-coding mutations in genes with poorly understood function that occur in a relatively large fraction of patients may provide new therapeutic strategies to treat melanoma.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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