See related commentary on pg 335

Beyond BRAF^{V600}: Clinical Mutation Panel Testing by Next-Generation Sequencing in Advanced Melanoma

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The management of melanoma has evolved owing to improved understanding of its molecular drivers. To augment the current understanding of the prevalence, patterns, and associations of mutations in this disease, the results of clinical testing of 699 advanced melanoma patients using a pan-cancer next-generation sequencing (NGS) panel of hotspot regions in 46 genes were reviewed. Mutations were identified in 43 of the 46 genes on the panel. The most common mutations were *BRAF*^{V600} (36%), *NRAS* (21%), *TP53* (16%), *BRAF*$^{Non\text{-}V600}$ (6%), and *KIT* (4%). Approximately one-third of melanomas had >1 mutation detected, and the number of mutations per tumor was associated with melanoma subtype. Concurrent *TP53* mutations were the most frequent events in tumors with *BRAF*^{V600}and *NRAS* mutations. Melanomas with *BRAF*$^{Non\text{-}V600}$mutations frequently harbored concurrent *NRAS* mutations (18%), which were rare in tumors with *BRAF*^{V600} mutations (1.6%). The prevalence of *BRAF*^{V600} and *KIT* mutations were significantly associated with melanoma subtypes, and *BRAF*^{V600} and *TP53* mutations were significantly associated with cutaneous primary tumor location. Multiple potential therapeutic targets were identified in metastatic unknown primary and cutaneous melanomas that lacked *BRAF*^{V600}and *NRAS* mutations. These results enrich our understanding of the patterns and clinical associations of oncogenic mutations in melanoma.

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Abbreviations: NGS, next-generation sequencing; TCGA, the Cancer Genome Atlas; WES, whole-exome sequencing

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INTRODUCTION

According to the American Cancer Society estimates, 76,100 patients are expected to be diagnosed with melanoma and 9,710 patients are predicted to die from the disease in 2014 (Siegel *et al.*, 2014). Melanoma is a complex, heterogeneous disease with multiple signaling pathways implicated in its molecular pathogenesis. A key advance in the understanding and treatment of this disease was the discovery of frequent recurrent somatic mutations that result in substitutions of the valine at position 600 in the gene encoding the BRAF serine–threonine kinase (*BRAF*^{V600} mutations) in the RAS-RAF-MEK-ERK signaling pathway (Davies *et al.*, 2002). Large single-center studies, meta-analyses, and whole-exome sequencing efforts have subsequently confirmed that *BRAF*^{V600} mutations are the most common activating genetic events detected in cutaneous melanomas (Hocker and Tsao, 2007; Hodis *et al.*, 2012; Jakob *et al.*, 2012; Krauthammer *et al.*, 2012). Studies of clinically annotated specimens have identified significant clinical associations with *BRAF*^{V600} mutations, including melanoma subtype, primary tumor location, and prognosis (Curtin *et al.*, 2005; Bauer *et al.*, 2011; Long *et al.*, 2011; Jakob *et al.*, 2012). More recent studies have also identified

significant differences in demographics, primary tumor features, and clinical outcomes between patients with the two most common *BRAF* substitutions observed in melanoma, $BRAF^{V600E}$ and $BRAF^{V600K}$ (Menzies *et al.*, 2012; Bucheit *et al.*, 2013). Significant clinical associations have also been identified with oncogenic *NRAS* and *KIT* mutations (Handolias *et al.*, 2010; Jakob *et al.*, 2012). These findings support the concept that studies of mutations may not only help identify therapeutic targets but also provide insights into the molecular pathogenesis and natural history of melanoma. There is also growing evidence that mutations correlate with differential clinical benefit with approved systemic therapies for this disease (Nathanson *et al.*, 2013; Trunzer *et al.*, 2013). Notably, recent whole-exome sequencing (WES) studies have demonstrated that melanoma has one of the highest rates of somatic mutations among all cancers, and have identified many additional genes that are mutated recurrently in this disease (Berger *et al.*, 2012; Hodis *et al.*, 2012; Krauthammer *et al.*, 2012).

Owing to the approval and clinical testing of treatments that target specific genetic mutations, molecular testing is now performed routinely for patients with advanced melanoma. The expansion of therapeutic options concurrent with technical advances has led to the development of a growing number of clinical molecular testing platforms. Increasingly, panel-based testing approaches are being used in order to maximize the efficient use of both patient materials and clinical testing infrastructure and resources. In addition to providing an opportunity to identify therapeutic options for patients, the data generated by such testing provide an opportunity to improve our understanding of the molecular heterogeneity of this disease. Such data can also be used to identify hypotheses for prospective studies that may improve patient testing and/or clinical management (Curtin *et al.*, 2005; Curtin *et al.*, 2006; Woodman *et al.*, 2012).

In this study, we reviewed our institution's results from clinical molecular testing by next-generation sequencing (NGS) of commonly mutated regions in 46 genes using a pan-cancer panel (AmpliSeq panel, Life Technologies, Carlsbad, CA; Supplementary Table S1 online) in 699 consecutive patients with advanced melanoma. These data have been analyzed for the prevalence and overlap of mutations, and their concordance in a subset of patients with testing on multiple samples. The molecular data have also been analyzed for associations with clinical subtypes (ie, cutaneous, acral, mucosal, uveal, and unknown primary melanoma) and primary tumor location. The results of this study reinforce the molecular complexity of this disease and identify clinical associations for *TP53* and $BRAF^{Non\text{-}V600}$ mutations.

RESULTS

Mutation prevalence

The cohort ($n=699$) included advanced melanoma patients with known cutaneous ($n=484$, 69%), acral ($n=54$, 8%), mucosal ($n=43$, 6%), and uveal ($n=13$, 2%) primary melanomas. A subset of patients had metastatic disease without a known primary tumor (referred to as ''unknown primary'', $n=104$, 15%). NGS of regions affected recurrently by mutations in cancer identified at least one mutation in 43 of the 46 tested genes in the full cohort of patients (Supplementary Figures S1, S2 and Table S2 online). The most prevalent mutations in the entire cohort were $BRAF^{V600}$ ($n=251$; 36% of all patients), *NRAS* ($n=150$; 21%), *TP53* ($n=110$; 16%), $BRAF^{Non\text{-}V600}$ ($n=39$; 6%), and *KIT* ($n=27$; 4%) substitutions.

The most common mutations in cutaneous melanomas were $BRAF^{V600}$ (41%), *NRAS* (22%), *TP53* (17%), and $BRAF^{Non\text{-}V600}$ (7%; Figure 1a). Metastatic melanomas without a known primary tumor demonstrated a very similar mutation spectrum (39% $BRAF^{V600}$, 22% *NRAS,* 19% *TP53*, 4% $BRAF^{Non\text{-}V600}$; Figure 1b). The most common mutations in acral melanomas were *NRAS* (24%), $BRAF^{V600}$ (19%), *KIT* (11%), and *TP53* (6%; Figure 1c). The most common mutations in mucosal melanomas were *NRAS* (21%), *KIT* (16%), *TP53* (9%), and $BRAF^{V600}$ (7%; Figure 1d). The majority (92%) of the small cohort of uveal melanomas had no mutations detected in the 46 gene panel, which notably did not include *BAP1, GNAQ*, or *GNA11*, which are mutated frequently in this melanoma subtype.

Characteristics and overlap of detected mutations

There is evidence that different substitutions in individual oncogenes correlate with distinct molecular and clinical characteristics, including *BRAF* (Wan *et al.*, 2004; Garnett *et al.*, 2005; Menzies *et al.*, 2012; Bucheit *et al.*, 2013). In this cohort, the most common $BRAF^{V600}$ substitutions were V600E (76% of all V600 mutations; 28% of all patients), V600K (17%; 6%), and V600R (2.4%; 0.8%; Supplementary Figure S3a online). Mutations resulting in 20 different substitutions at sites other than V600 in BRAF were also detected. The most frequent $BRAF^{Non\text{-}V600}$ substitutions were G469E (18% of $BRAF^{Non\text{-}V600}$mutations; 1% of all patients), G469R (13%; 0.7%), and K601E (11%; 0.5%; Supplementary Figure S3b online). For *NRAS*, mutations affecting Q61 were the most prevalent (77% of all *NRAS* mutations), followed by G12/13 (20%; Supplementary Figure S3c online). The most common *KIT* mutations were L576P (27% of KIT mutations, 1% of all patients), K642E (20%; 0.8%), and N822Y (10%; 0.4%; Supplementary Figure S3d online). Possible UVR–related mutations (C>T or G>A transitions; Berger *et al.*, 2012) were detected overall in 39 of the 43 mutated genes (Supplementary Figure S4 online). Among the genes mutated in >5% of the samples, *TP53* displayed the highest frequency of UVR signature mutations (66%). Likely UVR-associated substitutions represented only 11% of detected *NRAS* mutations, 8% of *BRAF* mutations, and none of the *KIT* mutations. CC>TT substitutions, which also provide strong evidence of UVR-induced DNA damage, were detected in 15 tumors, with *TP53* ($n=5$) being the most frequently affected gene (Supplementary Table S2 online).

Approximately one-third ($n=213$) of the melanomas had ≥2 mutations. Concomitant mutations were present in 35% of tumors with $BRAF^{V600}$mutations, 55% of tumors with $BRAF^{Non\text{-}V600}$ mutations, and 50% of tumors with *NRAS* mutations (Figure 2a–c). *TP53* mutations were the most common overlapping mutations in tumors with *NRAS* (17%) and

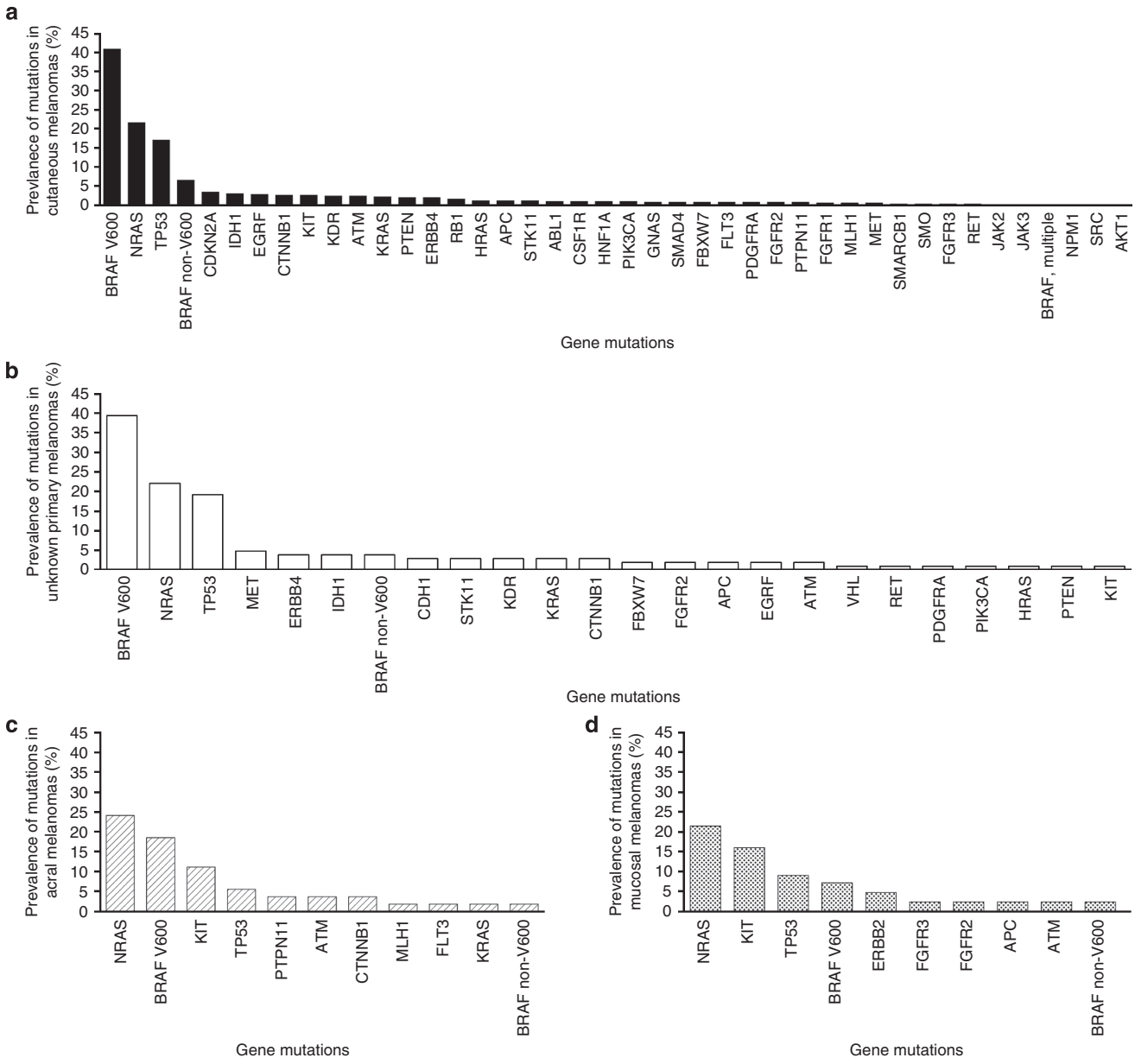

Figure 1. Prevalence of detected gene mutations by melanoma subtype. Panels show the rate of gene mutations observed in (**a**) cutaneous melanomas ($n=484$); (**b**) unknown primary melanomas ($n=104$); (**c**) acral melanomas ($n=54$); and (**d**) mucosal melanoma ($n=43$).

BRAF^{V600} (12%) mutations (12% of V600E; 7% of V600K; Supplementary Figure S5 online). The most frequent overlapping mutation in melanomas with *BRAF*$^{Non\text{-}V600}$ mutations was *NRAS,* which was mutated in 18% of these tumors. In contrast, concurrent *NRAS* mutations were detected in only 1.6% of tumors with *BRAF*^{V600}mutations. Mutations in *TP53* (13%) and *KRAS* (10%) were also relatively common in tumors with *BRAF*$^{Non\text{-}V600}$ mutations. *ATM* (11%), *NRAS* (7%), and *CTNNB1* (7%) were the most prevalent concomitant mutations in tumors with *KIT* mutations (Figure 2d) The rate of co-occurring *BRAF* and *NRAS* mutations in tumors with rare but potentially targetable mutations (ie, PI3K-AKT pathway, EGFR, MET) are presented in Supplementary Table S3 online.

Associations with melanoma subtype and primary tumor location

The overall rate of mutations varied significantly by melanoma subtype. Mucosal (44%) and acral (33%) melanomas were more likely to have no mutations detected than cutaneous (15%) and unknown primary (20%) melanomas ($P<0.0001$) (Supplementary Figure S6 online). As mentioned previously, no mutations were detected in the majority (92%) of the small cohort of uveal melanomas.

BRAF^{V600}, *NRAS, TP53, BRAF*$^{Non\text{-}V600}$, and *KIT* mutations, which were the most frequent events overall in the cohort, were assessed for associations with clinically defined melanoma subtypes (Figure 3a). This analysis identified significant associations for *BRAF*^{V600} ($P<0.001$) and *KIT* ($P<0.001$)

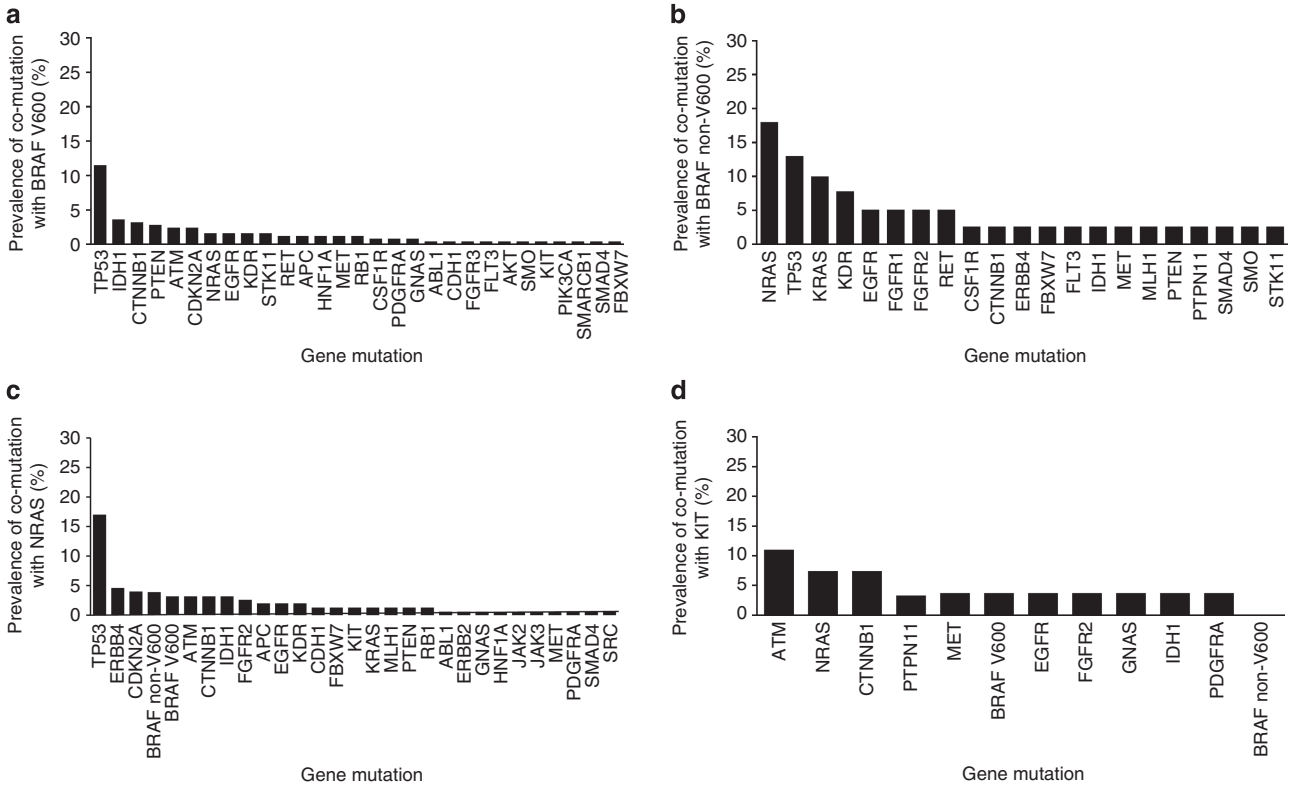

Figure 2. Prevalence of concurrent mutations among melanomas with common gene mutations. Panels show the rate of concurrent mutations present in melanomas with (**a**) *BRAF*^{V600}; (**b**) *BRAF*$^{Non\text{-}V600}$; (**c**) *NRAS*; and (**d**) *KIT*.

mutations. *BRAF*^{V600} mutations were more frequent in cutaneous and unknown primary melanomas, whereas *KIT* mutations were more prevalent in acral and mucosal melanomas. *TP53* mutations were also more common in cutaneous and unknown primary melanomas, but this differential distribution did not reach statistical significance ($P=0.059$). The prevalence of *NRAS* mutations varied very little by subtype.

Among nonacral cutaneous melanomas, the prevalence of *BRAF*^{V600} ($P=0.001$) and *TP53* ($P=0.0002$) mutations were significantly associated with primary tumor location (Figure 3b). The rate of *BRAF*^{V600}mutations was higher in the primary tumors of the trunk (49%) compared with the head/neck (30%, $P=0.0004$). *TP53* mutations were more frequent in primary tumors of the head/neck (26%) compared with the trunk (16%, $P=0.03$) or extremities (8%, $P<0.0001$). *BRAF*$^{Non\text{-}V600}$ mutations trended toward an association with primary tumor location ($P=0.055$), and were more common in primary tumors of the head/neck (10%) compared with the extremities (4%) on pairwise comparison ($P=0.025$). *NRAS* mutations were not significantly associated with the primary tumor site.

Among the cutaneous and unknown primary melanomas, 113 tumors (19%) were wild type for both *BRAF*^{V600} and *NRAS* mutations. The most common mutations in this cohort were *TP53* ($n=51$; 45%), *BRAF*$^{Non\text{-}V600}$ ($n=24$; 19%), and *KIT* ($n=13$; 10%; Figure 4). Other potentially targetable genes in which mutations were detected in this cohort included *EGFR* ($n=7$; 6%), *ERBB4* ($n=7$; 6%), *CDKN2A* ($n=5$; 4%), *PIK3CA* ($n=4$; 3%), *PDGFRA* ($n=3$; 2%), and *PTEN* ($n=2$; 2%). Mutations in *KRAS* ($n=7$; 7%) and *HRAS* ($n=5$; 4%) were also identified in the cohort.

Concordance of mutations

Thirty-seven patients had NGS data available for more than one tumor, including 23 patients with paired primary tumors and metastases. Highly concordant results were observed for *BRAF* (100%) and *NRAS* (97%) among the matched primary melanomas and metastases (Supplementary Figure S7 online). One patient with two primary melanomas had discordant *BRAF* testing results. However, this was not unexpected, as the patient had a primary mucosal melanoma (*BRAF* wild type) and a primary cutaneous melanoma (*BRAF*^{V600}). Three patients (8%) had discordant results for *TP53*. Two patients had a *TP53* mutation in the primary lesion that was not detected in the metastasis (H179Y→WT; L194F→WT), whereas the third patient had a wild-type *TP53* in the primary tumor and mutation in the metastasis (WT→R306*). One patient each demonstrated discordance in *PTEN*, *KRAS*, *CTNNB1*, *APC*, and *FBXW7*.

Mutation prevalence between primary ($n=248$) and metastatic ($n=486$) samples overall demonstrated similar distribution. For both primary and metastatic samples, *BRAF*^{V600} (32% primary; 37% metastatic), *NRAS* (18%; 23%), and *TP53* (17%; 15%) were the most common gene mutations in descending order. *KIT* (6%) was the next most common mutation in primary tumors followed by *BRAF*$^{Non\text{-}V600}$ (5%), whereas the

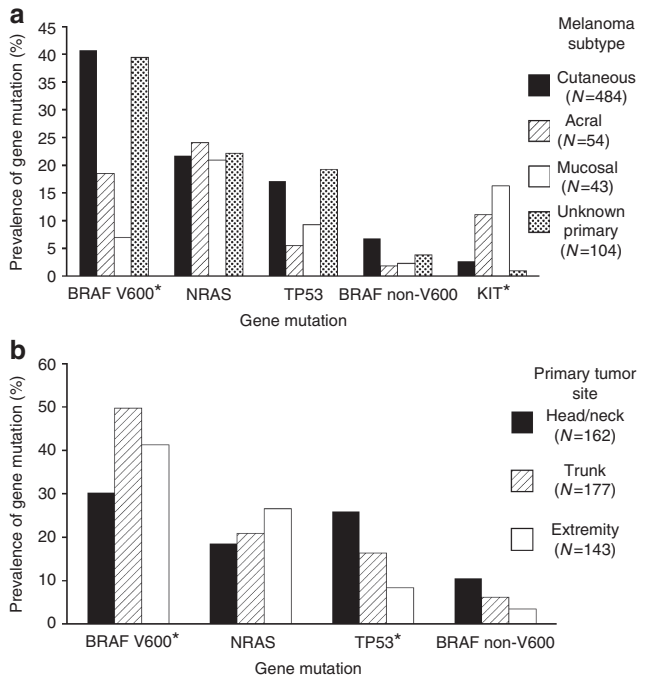

Figure 3. Associations of mutations with clinical subtypes and primary tumor location. (**a**) Gene mutation rates in different melanoma subtypes (black, cutaneous; striped, acral; white, mucosal; spotted, unknown primary). *, $BRAF^{V600}$ mutations ($P<0.001$) were significantly associated with cutaneous and unknown primary melanomas, whereas *KIT* mutations ($P<0.001$) were significantly associated with acral and mucosal melanomas. (**b**) Primary tumor location of prevalent gene mutations in cutaneous melanomas (black, head/neck; striped, trunk; white, extremity). *, $BRAF^{V600}$ mutations were significantly associated with the trunk compared with the head/neck ($P=0.0004$), whereas *TP53* mutations were significantly associated with the head/neck compared with the trunk ($P=0.03$) or extremities ($P<0.0001$).

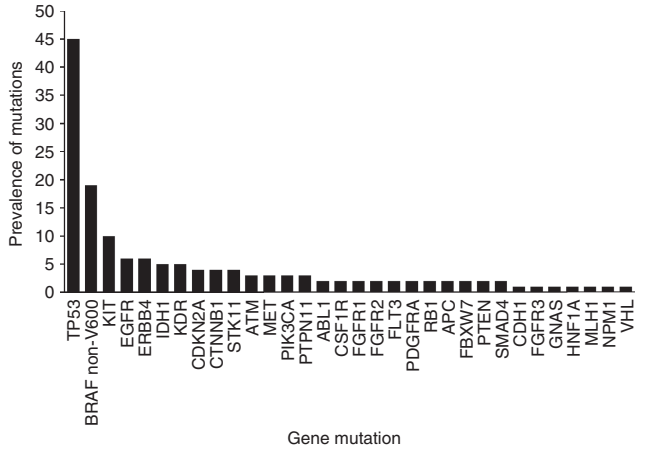

Figure 4. Mutations detected in cutaneous and unknown primary melanomas without a $BRAF^{V600}$ or *NRAS* mutation ($n=113$).

reverse was true for metastatic tumors ($BRAF^{Non\text{-}V600}$ 6%; *KIT* 4%). Only *STK11* showed a significant difference in prevalence between metastatic samples (2%) and primary tumors (0%, $P=0.033$). A total of 68 patients (10%) received chemotherapy before the removal of the lesion that was used for sequencing. Only 2 genes showed significant increases in mutation rates in the post-chemotherapy tumors compared with the chemotherapy-naïve tumors (*MLH1,* 3% vs. 0.3%, $P=0.05$; *RB1,* 4% vs. 1%, $P=0.03$).

DISCUSSION

Molecular testing is now performed routinely for patients with advanced melanoma. In addition to guiding therapeutic decision-making, the information from such testing can also provide insights into the molecular basis and heterogeneity of this disease. In this study, we have reviewed the results of clinical NGS of regions of 46 genes in a pan-cancer panel in a cohort of 699 melanoma patients. The results represent the largest cohort of melanomas to date analyzed by multiplexed NGS and add insights to some of the discoveries from recent WES efforts, including, to our knowledge previously unreported, molecular and clinical associations for *TP53* and $BRAF^{Non\text{-}V600}$mutations (Hodis *et al.*, 2012; Krauthammer *et al.*, 2012). Although this pan-cancer panel does not examine certain melanoma-specific genes of interest, it does provide an opportunity to assess other genes that are not commonly tested by focused, single-gene approaches in this tumor type.

Mutations affecting the V600 site of *BRAF* and hotspots in *NRAS* were the most frequent mutations observed in our cohort of 699 patients who underwent NGS for regions of 46 genes. Despite the possible bias that could have occurred owing to patients being selected for molecular testing in the clinical setting, the mutation rates for both of these genes, particularly in the cutaneous melanomas, are similar to other large series and meta-analyses of melanoma patients tested for these mutations (Hocker and Tsao, 2007; Jakob *et al.*, 2012). Hotspot mutations in $BRAF^{V600}$ and *NRAS* were also the most common mutations identified in two recent WES studies of cohorts of 121 and 147 melanomas (Hodis *et al.*, 2012; Krauthammer *et al.*, 2012), and in the preliminary publicly available results reported for the melanoma component of the Cancer Genome Atlas (TCGA) effort (TCGA; Research Network; https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm; sample batches 180, 198, 206, and 240; accessed on 04/01/2014). Although the rates of $BRAF^{V600}$ mutations in the cutaneous melanomas in the published WES studies were slightly higher than those observed here, one of those studies included cell lines, and both studies were limited by the requirement for frozen tumors with sufficient DNA for WES. In addition, patients with known $BRAF^{V600E}$ mutations detected by outside testing before patients were seen at our institution may not have undergone CMS46 analysis, thus potentially contributing to the lower percentage of $BRAF^{V600E}$ mutations in this cohort.

Consistent with previous studies by ourselves and others, $BRAF^{V600}$mutations were more frequent in cutaneous melanomas than in acral and mucosal melanomas (Hocker and Tsao, 2007; Jakob *et al.*, 2012). The rates of $BRAF^{V600}$, $BRAF^{Non\text{-}V600}$, *NRAS*, and *TP53* mutations in melanomas with an unknown primary tumor were nearly identical to the rates observed in cutaneous melanomas. This result is also consistent with similar rates observed between unknown primary melanomas and melanomas with a known cutaneous

primary in our previous analysis of patients at our center who underwent DNA pyrosequencing for $BRAF^{V600}$mutations and *NRAS* hotspot mutations only, and also consistent with a more recent study that analyzed the mutational status of *BRAF, NRAS,* and *KIT* in 44 patients with unknown primary melanoma (Jakob *et al.*, 2012; Egberts *et al.*, 2014). The additional data for the similar rates of $BRAF^{Non\text{-}V600}$ and *TP53* mutations provide further support for the hypothesis that the majority of melanomas with an unknown primary tumor likely had an occult cutaneous primary tumor.

In addition to position 600, our pan-cancer panel included sequencing of multiple other residues in exons 11 and 15 of *BRAF,* where most mutations in this gene have been identified (Davies *et al.*, 2002). Mutations that affected sites in the BRAF protein other than V600 ($BRAF^{Non\text{-}V600}$) were detected overall in 6% of the patients in this study. $BRAF^{Non\text{-}V600}$ mutations were detected in 7% of cutaneous, 4% of unknown primary, 2% of acral, and 2% of mucosal melanomas. Among the cutaneous and unknown primary tumors that did not have $BRAF^{V600}$ or *NRAS* mutations ($n=113$), $BRAF^{Non\text{-}V600}$ mutations (19%) were the second most common mutations detected. Dahlman *et al.* (2012) previously reported an 8% prevalence of $BRAF^{Non\text{-}V600}$ mutations in a smaller cohort ($n=49$) of patients without $BRAF^{V600}$or *NRAS* mutations. A review of publicly available preliminary data from the melanoma TCGA effort, which is restricted to melanomas with a primary tumor arising on nonglabrous skin but includes sequencing of all exons of *BRAF,* identified 21 $BRAF^{Non\text{-}V600}$ mutations in 266 melanomas (8%). Among the TCGA melanoma cases that were wild type for $BRAF^{V600}$ and *NRAS* mutations ($n=72$), the rate of $BRAF^{Non\text{-}V600}$ mutations was 21%. As 10 of the 21 $BRAF^{Non\text{-}V600}$ mutations detected in the TCGA were in exons other than 11 or 15, it is possible that the actual prevalence of $BRAF^{Non\text{-}V600}$ mutations in our cohort was slightly higher, although the functional significance of most of those mutations is unknown. A portion of these mutations also co-occurred with classic activating mutations in exon 15, and thus they are not favored to be oncogenic. Preclinical studies have demonstrated that $BRAF^{Non\text{-}V600}$ mutations do not respond to the Food and Drug Administration-approved BRAF inhibitors vemurafenib and dabrafenib, which were selected for development based on their specificity for BRAF proteins with V600E substitutions. However, other agents may be active in melanomas with $BRAF^{Non\text{-}V600}$ mutations, including MEK inhibitors and pan-RAF inhibitor sorafenib (Wan *et al.*, 2004; Garnett *et al.*, 2005; Smalley *et al.*, 2009; Dahlman *et al.*, 2012). Dramatic and durable clinical responses have been reported in isolated metastatic melanoma patients with $BRAF^{Non\text{-}V600}$ mutations affecting the L597 residue in early-phase clinical trials of the MEK inhibitors trametinib and TAK-733 (Dahlman *et al.*, 2012; Falchook *et al.*, 2012; Kim *et al.*, 2013). Clinical trials have been planned to systematically evaluate the activity of the MEK inhibitor trametinib in patients with $BRAF^{Non\text{-}V600}$ mutations.

$BRAF^{Non\text{-}V600}$ mutations showed significant clinical and molecular differences compared with $BRAF^{V600}$mutations. Although $BRAF^{V600}$mutations were detected at higher rates in melanoma patients with cutaneous and unknown primary tumors, the prevalence of $BRAF^{Non\text{-}V600}$ mutations was not significantly associated with melanoma subtype. However, the power to detect significant differences was limited by the comparatively lower prevalence of the $BRAF^{Non\text{-}V600}$ mutations. More strikingly, the pattern of co-mutations was distinct for $BRAF^{V600}$ and $BRAF^{Non\text{-}V600}$ mutations. In this large cohort, only 1.6% of the melanomas with detected $BRAF^{V600}$mutations had a concurrent *NRAS* mutation. This prevalence is similar to the rate observed in our previous pyrosequencing study (Jakob *et al.*, 2012). In contrast, concurrent *NRAS* (18%) and *KRAS* (11%) mutations were frequent events in melanomas with $BRAF^{Non\text{-}V600}$ mutations. Previous *in vitro* characterization of 15 $BRAF^{Non\text{-}V600}$ mutations demonstrated heterogeneous effects on the serine–threonine catalytic activity of the BRAF protein (Wan *et al.*, 2004; Garnett *et al.*, 2005). Although some mutations increase the activity of *BRAF* to a level comparable to that observed with V600 mutations, other mutations are only partially activating, and some cause the kinase activity to be decreased compared with the wild-type protein. Experimental data support that the nonactivating *BRAF* mutations still increase the activity of MAPK pathway signaling through increased formation of multiprotein complexes with CRAF and active RAS proteins (Wan *et al.*, 2004). The co-occurrence of $BRAF^{Non\text{-}V600}$ mutations with activating *RAS* mutations may therefore cooperate to activate the MAPK pathway to a greater degree than that achieved with either event alone.

One of the important insights from recent WES studies of melanoma was the identification of frequent *TP53* mutations. Although previous studies had suggested that *TP53* mutations were quite rare in melanoma (Castresana *et al.*, 1993; Albino *et al.*, 1994; Lubbe *et al.*, 1994), a recent WES study identified a rate of 19% (Hodis *et al.*, 2012). Although we covered most exons but did not fully sequence the *TP53* gene, and thus may underestimate their prevalence, we found a very similar rate of *TP53* mutations (16%) in this cohort. *TP53* mutations were the most common mutations identified after $BRAF^{V600}$ and *NRAS* mutations in both this study and the WES study, underscoring their frequency and potential significance. *TP53* mutations strongly trended ($P=0.06$) toward an association with melanoma subtype, with lower prevalence in acral and mucosal melanomas compared with cutaneous and unknown primary melanomas. *TP53* mutations were also associated with primary tumor location, with higher prevalence observed in melanomas with head/neck primary tumor location. This result could reflect an etiological role for UVR in *TP53* mutations. Consistent with this hypothesis, 66% of the observed *TP53* mutations were associated with typical UVR-induced changes. *TP53* mutations were frequent in melanomas with concurrent $BRAF^{V600}$, $BRAF^{Non\text{-}V600}$, and *NRAS* mutations. As *TP53* mutations have been significantly associated with clinical outcomes and therapeutic resistance in other cancers (Temam *et al.*, 2000; Poeta *et al.*, 2007; Hoffmann *et al.*, 2008; Lindenbergh-van der Plas *et al.*, 2011), future studies will test the predictive and prognostic significance of these events in melanoma. Notably, we did observe discordant results for *TP53* mutation status in three patients (13%) who had molecular

testing data for both primary tumors and metastases. This suggests that testing of archival material alone may not be adequate to accurately determine the significance of *TP53* mutations as a predictive marker of response to systemic therapies in patients with metastatic disease. However, the analysis of the paired specimens did overall demonstrate highly concordant results for this panel of genes mutated recurrently in cancer.

Although $BRAF^{V600}$and *NRAS* were the most frequent mutations observed in our study, 19% of patients with cutaneous and unknown primary melanomas had neither of these mutations. The identification of therapeutic targets in these patients is a key challenge and clinical need. The most common genes in this cohort in which mutations were detected by our panel were *TP53* (45%), $BRAF^{Non\text{-}V600}$ (19%), and *KIT* (10%). As described above, clinical responses have been observed in early-phase clinical trials of the MEK inhibitors trametinib and TAK-733 in metastatic melanoma patients with $BRAF^{Non\text{-}V600}$ mutations (Dahlman *et al.*, 2012; Falchook *et al.*, 2012; Kim *et al.*, 2013). A number of nonrandomized clinical trials with the KIT inhibitor imatinib have been conducted in advanced melanoma patients with *KIT* mutations. The clinical response rates in these trials have ranged from 16 to 29% (Carvajal *et al.*, 2011; Guo *et al.*, 2011; Hodi *et al.*, 2013). One imatinib study identified the presence of a concurrent *NRAS* mutation, which we observed in 7% of the *KIT*-mutant patients in this cohort, as a predictor of resistance. Other genes that are potentially actionable in which rare mutations were detected include *EGFR* (6%), *ERBB4* (6%), *PIK3CA* (3%), and PDGFRA (2%). However, the functional and clinical significance of the majority of the mutations detected in these genes is currently unknown.

As described above, the AmpliSeq 46-gene panel can provide important clinical information. Notably, the panel allows for the consolidated evaluation of multiple important cancer genes and oncogenic mutations in one assay, including a number of clinically actionable aberrations. However, we recognize that this pan-cancer panel has significant limitations for this study. The panel includes a number of genes that currently have unknown relevance to melanoma. The panel also fails to include certain mutations that have been detected in cutaneous melanomas in recent WES studies, such as TERT, NF1, and RAC1, as well as mutations detected in other melanoma subtypes (ie, BAP1, GNAQ, GNA11). The high frequency of acral, mucosal, and uveal melanomas with no mutations detected supports the need to consider other molecular testing panels for those subtypes, or the augmentation of the panel with genes relevant for those subtypes. In addition, the sequencing of only certain regions of many of the genes may not be adequate to annotate certain genes, particularly tumor suppressors that can be affected by frameshift mutations at many loci. For example, the observed mutation rates for *CDKN2A* (2.4%) and *PTEN* (1.6%) in our cohort are lower than those observed in recent WES studies. The inclusion of only 46 genes also makes it technically challenging to accurately quantify significant copy number variation, which could have affected these and other genes in the panel. This may further explain why tumor suppressors that show frequent losses through deletions that are not adequately detected by this platform, such as *CDKN2A* and *PTEN*, were less prevalent in our study than those that are more frequently affected by missense and frameshift mutations, such as *TP53*. Exploratory studies are underway to determine what copy number variations can be detected and validated reliably using the AmpliSeq platform, and will be reported in the future. Such copy number variation information may also be forthcoming from panels that include sequencing of all exons for genes of interest, and larger numbers of genes. Currently, our pan-cancer panel is expanding to include more comprehensive coverage of prevalent cancer genes, including those that may prove to be clinically relevant to melanoma. However, gene translocations, such as those recently reported for *BRAF* (Botton *et al.*, 2013; Hutchinson *et al.*, 2013), are not assessable using this panel. Ultimately, the integration of multiple types of molecular data that will be available in the near future from the melanoma TCGA effort will likely provide additional molecular insights into the mutations observed in this study. Subsequent studies in which molecular data can be integrated with clinical characteristics and outcomes will be critical to personalizing and optimizing patient management.

In summary, our study presents the results for the largest cohort to date of melanoma patients to be analyzed by clinical multiplexed NGS. Consistent with previous studies, we found that $BRAF^{V600}$and *NRAS* hotspot mutations were the most common molecular aberrations detected. We also observed frequent *TP53* mutations, consistent with recent data from WES studies, with information about their associations with melanoma subtypes and primary tumor location. In addition, our study adds to the growing understanding of the prevalence and molecular patterns of $BRAF^{Non\text{-}V600}$ mutations, which have emerged as a therapeutic target. These results provide a basis for future focused studies of these molecular events, and further supports the rationale for integrated analyses of clinical molecular testing data from other centers.

MATERIALS AND METHODS

Mutation testing

Molecular testing was performed on DNA extracted from formalin-fixed, paraffin-embedded tissues from melanoma primary tumors or metastases for which molecular testing was clinically indicated. DNA was extracted using standard methods, and was analyzed for mutations using the AmpliSeq sequencing panel (Life Technologies), as previously described (Singh *et al.*, 2013). Detailed methodology is provided in Supplementary Materials online. The regions analyzed for mutations in each of the 46 genes in the panel are listed in Supplementary Table S1 online.

Data analysis

This study was conducted according to the Declaration of Helsinki Principles, and all analyses were performed under a protocol approved by the Institutional Review Board. In accordance with this protocol, all samples used were obtained from patients who gave their written informed consent for the use of their archival tissue for research purposes or from deceased patients who have samples stored

in the Department of Pathology at MD Anderson Cancer Center. Clinical NGS data, patient demographics, and disease characteristics were obtained from institutional pathology and clinical databases. Review of the publicly available TCGA melanoma samples (batches 180, 198, 206, and 240) was performed through the online TCGA data matrix (https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm; accessed on 04/01/2014). Associations were evaluated using either Fisher's exact tests or Freeman–Halton tests using SAS v9.3 for Windows. *P*-values less than 0.05 were considered statistically significant.

CONFLICT OF INTEREST

JM serves on the Board of Merrimack Pharmaceuticals. The remaining authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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