Published in final edited form as:
Clin Cancer Res. 2013 September 1; 19(17): 4868–4878. doi:10.1158/1078-0432.CCR-13-0827.

Tumor genetic analyses of patients with metastatic melanoma treated with the BRAF inhibitor Dabrafenib (GSK2118436)

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Abstract

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Conflicts of interest: KLN, SO'D and H-TA have received research funding from GlaxoSmithKline; A-MM, JG, RG, BM and MC are employees of, and have stock ownership of, GlaxoSmithKline; BW, RL, KD'A and AP have no conflicts of interest to declare; JRI has acted as an uncompensated consultant or advisor for GlaxoSmithKline; GSF has received research funding and other remuneration from GlaxoSmithKline; MM has acted as a compensated consultant or advisor for GlaxoSmithKline; MPB has acted as a compensated consultant or advisor for, and has received research funding from, GlaxoSmithKline; MAD has served on advisory boards for GlaxoSmithKline, Genentech and Novartis, and has received research funding from GlaxoSmithKline, Genentech, AstraZeneca, Merck, Myriad and Oncothyreon; PFL was an employee of GlaxoSmithKline; RK has acted as a compensated consultant or advisor for GlaxoSmithKline; GVL has acted as a compensated consultant or advisor for GlaxoSmithKline, Roche, Novartis, Amgen and Bristol-Myers Squibb, and has received honoraria from Roche.

Purpose—Dabrafenib is a selective inhibitor of V600-mutant BRAF kinase, which recently demonstrated improved progression free survival (PFS) as compared with dacarbazine, in metastatic melanoma patients. The current study examined potential genetic markers associated with response and PFS in the phase I study of dabrafenib.

Experimental Design—Baseline (pre-treatment or archival) melanoma samples were evaluated in 41 patients using a custom genotyping melanoma-specific assay, sequencing of *PTEN*, and copy number analysis using multiplex ligation amplification and array based comparative genomic hybridization. Nine patients had on-treatment and/or progression samples available.

Results—All baseline patient samples had *BRAF*$^{V600E/K}$ confirmed. Baseline PTEN loss/mutation was not associated with best overall response (BOR) to dabrafenib, but it showed a trend for shorter median progression free survival (PFS) (18.3 [95% confidence interval (CI) 9.1–24.3] vs. 32.1 weeks [95% CI 24.1–33], p=0.059). Higher copy number of *CCND1* (p=0.009) and lower copy number of *CDKN2A* (p=0.012) at baseline were significantly associated with decreased PFS. Although no melanomas had high level amplification of BRAF, the two patients with progressive disease as their best response had *BRAF* copy gain in their tumors.

Conclusions—Copy number changes in *CDKN2A*, *CCND1*, and mutation/copy number changes in *PTEN* correlated with the duration of PFS in patients treated with dabrafenib. The results suggest that these markers should be considered in the design and interpretation of future trials with selective BRAF inhibitors in advanced melanoma patients.

Keywords

Melanoma; BRAF inhibitors; correlative studies; BRAF mutation; PTEN

Introduction

Melanoma is the most lethal form of skin cancer. In the United States, it is estimated that there will be 76,250 new cases and 9,180 deaths related to melanoma in 2012, and the incidence in increasing (1). Melanomas are characterized by a high rate of single base mutations, as compared to other solid cancer types, associated with a UV light induced signature, as well as a characteristic profile of genomic amplifications and deletions (2, 3). Mutations in *BRAF* are found in ~45% of melanomas (4, 5), the majority of which are at codon 600 and result in constitutive kinase activity of BRAF and subsequent downstream signaling through the MAP kinase pathway (6). Seventy to ninety percent of BRAF mutations are due to the substitution of glutamic acid for valine (V600E mutation; c. 1799T>A), and 10–30% are due to the substitution with lysine (V600K) (4, 5, 7). Common genomic changes in melanoma include deletion of *PTEN*, and *CDKN2A* and amplifications of *KIT*, *MITF*, *TERT*, *CCND1*, among others (3). *PTEN* deletions are most commonly observed in conjunction with *BRAF* mutations, in approximately 30%, whereas *CDKN2A* mutations are seen across all mutational (*BRAF*/*NRAS*/WT) sub-types of melanomas (8, 9). The frequency of these genomic changes allows their study in conjunction with outcome upon treatment of melanoma.

Until recently, most systemic therapy options available for patients with advanced stage melanoma were ineffective, and the five year survival rate was less than 15%. In addition to immunotherapeutic agents, therapies directed at the MAPK signaling pathway have been developed, in particular targeted inhibition of the mutant V600E BRAF protein. Dabrafenib is a reversible ATP-competitive inhibitor that selectively inhibits mutant BRAF^{V600E}, with an IC_{50} (concentration required for 50% inhibition) fivefold lower than for wildtype BRAF or CRAF (10). Treatment of BRAF-mutant metastatic melanoma with the RAF inhibitors dabrafenib or vemurafenib, results in response rates of approximately 50% and significantly

improved progression free survival (PFS) and overall survival (OS) as compared with dacarbazine chemotherapy (11–14). The emergence of acquired resistance in the majority of patients, for which multiple mechanisms have been described, involving both reactivation of the MAPK signaling pathway and by-pass mechanisms, remains the greatest barrier to better clinical outcomes (15–22).

Pre-clinical studies have also identified several mechanisms of *de novo* or intrinsic resistance to BRAF inhibitors, including PTEN loss (alone or in conjunction with Rb1 loss), MET and SRC activation (associated with amplification of *MET*, *CTNNB1* and *CCND1*), activation of P70S6K and S6, and HGF mediated MET expression (21–26). In order to improve our understanding of the clinical significance of these DNA-based aberrations implicated in intrinsic resistance, and other candidate genes previously identified as aberrant in melanoma, we report here the molecular analysis of a large collection of human melanoma tumor samples from patients treated on the phase I clinical trial of the BRAF inhibitor dabrafenib (11).

Patients and Methods

Patients and clinical outcome

Melanoma tumor samples were collected from patients enrolled on the first-time-in-human BRF112680 phase I trial of dabrafenib (GSK2118436; clinical trial number NCT00880321) between May 27, 2009 and March 20, 2012, at eight study centers in Australia and the United States (11). Samples were collected either at baseline (‘pre-treatment’ and/or archival samples), ‘on-treatment’, and when possible, at time of progression. Informed consent was obtained from all patients before the start of treatment and collection of tumor samples; the study complied with all local guidelines. Patients commenced on variable doses of dabrafenib (35mg – 300mg) (Table 1); all were escalated to a total daily dose of ≥300mg/day. The clinical outcome measures used in this study included the objective response and progression-free survival using computed tomography (CT) and RECIST 1.0 as previously reported (11).

Tumor QC, DNA extraction

All samples were formalin-fixed and paraffin embedded (FFPE) and processed for hemotoxylin and eosin (H&E) staining and pathologist review of tumor content at the ACC Histology Core Facility by QCY. Samples with over 70% tumor content were processed for DNA extraction directly, while those with less than 70% tumor underwent H&E-guided macrodissection prior to DNA extraction. DNA was extracted using standard methods.

iPlex genotyping and PTEN sequencing

Genotyping performed using a custom iPlex (Sequenom, Inc.) single nucleotide extension panel Genotyping was done at the Perelman School of Medicine Molecular Profiling Facility. Analysis of data was done using the iSeq™ software; we assessed the ratio of wildtype:mutant (T:A) nucleotide in *BRAF*^{V600E} using the peak height chromatograms. Sanger sequencing of *PTEN* exons 1 through 9 was performed using standard methods and published primers (27). Detailed methods are included in Supplementary Methods.

Copy number analysis

Multiplex ligation dependent probe amplification (MLPA) was used to detect copy number aberrations of genes located on chromosome 10q23 (MLPA kit P225-B2 PTEN, MRC-Holland). MLPA was performed according to the manufacturer’s instructions and analyzed with their software - MRC-Coffalyser Stand Alone Alpha Version 1.0.0.43 software. The

fragments were analyzed on ABI 3130xl capillary sequencer using Genemapper software (Applied Biosystems, Inc.). Variation in peak height was evaluated by comparing each test sample to three normal controls present in the same experiment. Normalization was done intra-sample by dividing the peak area of each probe's amplification product by the total area of only the reference probes in this probe mix. Single regression for control and tumor data slope correction was performed. Normal ratio limits were set at −0.70 and 1.2. This program identifies a peak as deleted when showing a ratio < | 0.7 | and amplified when showing a ratio ≥ 1.2. The copy number of *PTEN* was measured in 17 samples using both MLPA and aCGH for cross-validation; all samples had the same copy number profiles.

Array based comparative genomic hybridization was done using the Agilent SurePrint G3 Human CGH 1x1M microarrays following manufacturer's instructions. Arrays were scanned using Agilent's High-Resolution C Scanner. Extracted data was analyzed using BioDiscovery's Nexus 6 copy-number software (Nexus Genomics Inc., Mountain View, CA, USA). Copy number variation was assessed using the CBS-like Rank Segmentation algorithm provided with Nexus 6; genes mapping was done to hg19, Feb 2009 build. Copy number gain was defined as log2 scale value ≥ 0.3 and loss as log2 scale value ≤ −0.3, with at least three contiguous SNPs needed. Segments were particularly examined for the presence of high copy gains (log2 scale value ≥ 1.14) and homozygous loss (log2 scale value ≤ −1.1). Additionally, only segments derived from > 16 probes were included in subsequent analysis.

Chromosomal instability analysis

Characterizing cytogenetic instability was used as an alternative to analyzing specific, recurring copy number changes across the tumor set. To this end, copy number alterations were first identified in the segmented data for each tumor and mapped to a specific chromosomal arm. Considering gains and losses separately, the total accumulation of copy number altered regions was calculated for each arm (i.e. total bases altered) and the fraction of the arm altered (total bases gained or lost/total size of arm).

Statistical Analysis

Progress free survival (PFS) was compared in patients whose tumors had wild-type or non-deleted/mutant *PTEN* status versus those with deleted or mutant *PTEN* using the log-rank test and Kaplan-Meier analysis. Correlation between copy number values among 36 genes was assessed using Spearman's correlation analysis. Association between copy number values and PFS was evaluated using proportional hazards regression. In this analysis, $p<0.05$ was considered statistically significant. Cytogenetic instability was analyzed between patients with pre or early dose tissue. Comparisons were made using the median PFS of 24 weeks, with 12 patients having a PFS over 24 weeks and 11 with a PFS less than 24 weeks, using a t-test.

Results

Description of patient population

A total of 91 samples were available from 77 patients. Eleven patients had multiple samples available. Twenty-seven samples were determined to have no tumor or in an amount too small to allow for adequate DNA extraction. We obtained *BRAF* mutational data on 57 melanoma tumor samples from 45 patients. Of these samples, two were on-treatment and eleven were progression samples, with the remaining 44 baseline (pre-treatment or archival) samples. Patient characteristics for the 41 patients with baseline samples available are in Table 1.

Mutational information

In the 41 patients with baseline samples available, six had *BRAF*^{V600K}, 35 *BRAF*^{V600E} mutations (Table 2). Two melanoma samples carrying the *BRAF*^{V600E} mutation exhibited a T(wildtype):A(mutant) ratio favoring the mutant allele, one suggestive of amplification of that allele (patient 32, 1:4). Of the seven *BRAF*^{V600K} mutations observed, two samples showed T:A ratio favoring the mutant allele, suggestive of amplification of the mutant allele (patients 11, 19). The highest ratio favoring the mutant allele was seen in the archival sample from a patient also with a pre-treatment sample, which had a ratio favoring the wild-type allele. Two patients with V600E mutations had additional concurrent mutations, one with *CTNNB1* (beta-catenin) p.S45del and another with *MAP2K2* (MEK2) p.Q60P. The biopsy with the mutation in *MAP2K2* was taken at time of progression on trametinib (MEK inhibitor), prior to treatment with dabrafenib. Melanoma tumor samples, all taken from the same lesion, at multiple time points - archival, prior to treatment, and on-treatment with trametinib - were available for this patient and did not show the *MAP2K2* p.Q60P mutation, suggesting it arose after treatment with trametinib (Infante, Nathanson, unpublished data). This patient had progressive disease as their best response to treatment with dabrafenib.

Data were available from two on-treatment and 11 progression samples from ten patients (Table 2). Both of the on-treatment samples were from patients who also had progression samples. The ten patients had melanomas carrying the following mutations; *BRAF*^{V600E} (6), *BRAF*^{V600K} (2), *BRAF*^{K601E} (1), and *NRAS*^{Q61K} (1). The progression sample with the *NRAS* mutation did not have a concurrent *BRAF* mutation, so it is possible that the patient had more than one primary melanoma or that the *BRAF* V600E mutation was present but undetectable based on the sensitivity of the assay. Six patients had matched pre-treatment/archival and progression samples. In general, the ratio of mutant to wild-type allele was the same in both samples.

Association of baseline PTEN status with PFS

As we were particularly interested in the relationship of *PTEN* status to PFS upon treatment with dabrafenib, we evaluated it genetically with copy number analysis, using MLPA and/or aCGH, and sequencing of all exons. For this analysis, we focused on pre-treatment and archival samples; data from the pre-treatment sample was used preferentially for all analyses. Three patients had both archival and pre-treatment samples. One patient had an archival sample with amplified PTEN; the pre-treatment sample showed diploid PTEN. In one patient, a mutation in *PTEN* was observed in the pre-treatment, but not archival sample. This mutation was the only one observed in *PTEN*, p.P95L, and has been previously reported in association with cancer (27–29). For four samples, we obtained only mutational, but not copy number data; these samples were removed from further analysis. Thus, 34 patients had melanoma samples with PTEN data available for analysis (Table 2); patient characteristics are reported in Supplementary Table 1.

PTEN status was available for 11 progression and two on-treatment samples. Five progression samples had matched archival or pre-treatment samples; in all but one case the copy number status was the same in both samples. Of note, homozygous deletion of PTEN was more frequently observed in progression tumor samples (4/10) than in archival or pre-treatment samples (2/34), p=0.017.

The relationship between best overall response (BOR) and PFS were evaluated. No association was seen between BOR and PTEN status with patients with melanomas having wild-type or non-deleted/non-mutant PTEN status having a response rate (CR+PR) of 43% (10/23; 95% confidence interval [CI] 21–65.9%) versus those with deleted or mutant PTEN of 36% (4/11; 95% CI 3.4–69.3%), p=0.059 (Figure 1A). However, the patients with wild-

type or non-deleted/non-mutant PTEN status had a longer PFS (32.1 weeks, 95% CI 24.1–33) and those with deleted/mutant PTEN (18.3 weeks, 95% CI 9.1–24.3, p=0.059), p=0.059 (Figure 1B). As the *PTEN* status for four samples with exonic amplification could not be precisely determined to be either wild-type or deleted, as potentially they could indicate rearrangements leading to PTEN loss, as in prostate cancer (30), they were removed for a secondary analysis. The PFS in the 18 patients with wild-type PTEN status was 32.1 weeks (95% CI 24.1–39.4), longer than those with mutant/deleted PTEN, but this difference did not reach statistical significance (p=0.066).

PTEN immunohistochemistry was performed on a subset (n=17) of the samples with complete genetic data (Supplementary Table 2). The correlation between IHC data and genetic status was 75% (95% CI: 50.5–89.8); presence/absence of staining data was not predictive of PFS in this small subset of patients (data not shown).

aCGH data

aCGH data was generated on 34 samples from 26 patients. Twenty-four archival and pre-treatment samples were analyzed from 23 patients (one patient had both archival and pre-treatment samples). Eight patients had progression samples analyzed (two for which we also had on-treatment samples). Three patients had both archival or pre-treatment samples and progression samples. Overall, the copy number analysis (Figure 2A) was similar to that previously published for *BRAF* mutant cutaneous melanoma, with frequent copy number gains of 6p, 7q and 22q, and frequent losses of 6q, 9p, and 10q (31–34). We also examined the overall genomic profile of pre-treatment melanomas in relationship to PFS (Figure 2B), BOR (Supplementary Figure 1), and $BRAF^{V600E}$ vs. $BRAF^{V600K}$ mutation status (Supplementary Figure 2). The percentage of individual chromosome arms and total fraction of the genome that was altered in patients with PFS > 24 weeks (n = 12) was compared to those with PFS ≤24 weeks (n = 11). While the overall fraction of the genome gained or lost did not significantly differ between groups (p = 0.19 and 0.11, respectively), there were some notable qualitative differences. Most notably, when considering large alterations defined as those encompassing > 25% of the size of a chromosomal arm (indicative of broad instability), gains of 6p appeared more frequent in the patients demonstrating > 24 vs. ≤24 week PFS (58% vs. 27% respectively). Similarly, large losses of 11q appeared more frequent in those patients with PFS > 24 vs. ≤ 24 weeks (33% vs. 9% of patients respectively). Conversely, losses of 9p (25% vs. 55%), and 4q (0 % vs. 27%) were qualitatively more frequent in those with shorter PFS. While none of these differences achieved statistical significance (p > 0.05 in all cases), the overall rarity of such large genomic changes could suggest relationships with response in a subset of patients.

Specific genes from aCGH data

We selected 36 genes (Supplementary Table 3) implicated in melanoma pathogenesis for copy number variation (CNV) characterization. A correlation analysis between copy number gains and losses of various genes, both positive and negative (i.e. gain-gain, gain-loss) was done, and is shown in Supplementary Table 4. Correlation of CNV results to PFS demonstrated that copy gain of *CCND1* (p=0.009) and loss of *CDKN2A* (p=0.012) were independently predictive of shorter PFS. For visualization purposes of the association between copy number and outcome, the samples were divided by median copy number and Kaplan-Meier curves for PFS were generated (Figure 3A, B). For *CCND1*, patients whose tumors had greater than the median copy number (n=11) had a median PFS of 27 weeks (95% CI, 15–32 weeks), which was shorter (Hazard ratio [HR]=2.22) than those with a lower copy number (n=12, median PFS 33 weeks, 95% CI, 24–45 weeks). Patients with less than median copy number of *CDKN2A* (n=11) had a median PFS of 25 weeks (95% CI, 17–32 weeks), whereas patients with a higher copy number had a median PFS of 31 weeks

(95% CI, 15–56 weeks, HR=0.59). Of note, neither copy number of *CDKN2A* nor *CCND1* was associated with BOR. High level amplification of the BRAF locus (> 5 copies) was not observed in these 25 patients, and *BRAF* copy number did not significantly correlate with BOR or PFS (Figure 3c).

Discussion

To the authors' knowledge, this study represents the largest genetic and genomics based examination of human melanoma tissue from patients treated with BRAF inhibitors, specifically patients treated with dabrafenib on the phase I/II trial (11), to identify correlates associated with response. We focused on the genes encoding the proteins of the MAPK (Ras/Raf/MEK/ERK) and PI3K/Akt pathways, as they are the principal signaling pathways shown to be crucial in melanoma initiation and progression (6, 35). Cell cycle regulatory proteins, such as p16, CKD4 and cyclin D1, also have been shown to play important roles in melanoma, and those genetic and genomic aberrations involving these also were specifically interrogated (36–38). We also took a more comprehensive un-biased approach, using aCGH, to identify additional genomic aberrations which might be associated with response.

All baseline (pre-treatment and archival) samples had confirmed V600 mutations in *BRAF*, a study eligibility requirement. High level copy (over five) amplification of *BRAF* was not observed in any of the samples. A few samples demonstrated relative increases in the ratio of the *BRAF* V600 mutant to wild-type allele, either in the pre-treatment samples, or at the time of progression; most samples demonstrated equivalent or increased wild-type allele. Of note, two of the three samples from patients with progressive disease as best response had copy number gain of *BRAF*, however one was from a progression sample without a matching pre-treatment specimen. Although our numbers are too small to draw any definitive conclusion from, they are consistent with prior findings suggesting that increased copy number of *BRAF* is associated with progressive disease in some patients who are treated with BRAF or MEK inhibitors (15, 39). Importantly, preclinical studies suggest that this mechanism of resistance may potentially be overcome with increased doses of selective BRAF inhibitors (15).

We performed detailed analysis of PTEN genetic status in association with response to BRAF inhibition, as several pre-clinical studies have suggested that PTEN loss contributes to intrinsic BRAF resistance (23, 24). Our data suggest that PTEN loss is associated with a shorter PFS in patients treated with BRAF inhibitors, although is not predictive of best overall clinical response. This finding is not unexpected, as degree of response is not necessarily correlated with duration with targeted agents. Interestingly, PFS correlated with overall survival in the phase 3 study of vemurafenib, the only study able to use OS as a primary endpoint (40). It has been suggested that PTEN loss, as measured through immunohistochemistry (IHC), is a negative prognostic factor for melanoma, independent of BRAF inhibition (41, 42). PTEN loss also is found more frequently in brain than lung or liver metastases, which are associated with worse prognosis (35). However, genetic analysis of PTEN status has not been previously assessed as a predictive or prognostic marker in melanoma, and has not been correlated with immunochemistry analyses. Although our data suggest that analyses using genetic methods may ultimately be better predictors of outcome than immunochemistry measurements, analyses of larger cohorts of patients with parallel genetics and proteomic analysis are needed to make definitive conclusions.

Copy number analysis of 36 genes previously observed to be altered in melanoma was performed on samples from 23 dabrafenib-treated patients. Among those genes, lower copy number of *CDKN2A* and higher copy number of *CCND1* were significantly associated with shorter PFS. The regions containing these genes also were implicated in our overall

(unbiased) analysis of aCGH data, as *CCND1* is located on 11q and *CDKNA* on 9p. Both deletion/loss of *CDKN2A* (p16) and amplification/overexpression of *CCND1* (cyclin D1) previously have been implicated as poor prognostic markers in melanoma (43–47). Thus, these genetic changes may function as either prognostic or predictive markers in response to BRAF inhibition. Amplification of *CCND1* also has been associated with intrinsic resistance to BRAF inhibition in pre-clinical studies (25, 48). Other genes which have been implicated in intrinsic resistance to BRAF inhibition, including *Rb1* loss, *MET* and *SRC* amplification, did not emerge as associated with PFS in our analysis (24, 25).

Although this study contains a large set of melanoma tissue samples from patients treated with dabrafenib, samples were not available from all patients enrolled in the clinical trial, potentially limiting the generalizability of our results. However, the characteristics of the patients included in the correlative studies, as reviewed in Table 1, did not differ from those on the trial as a whole (data not shown). We were not able to determine whether the genetic alterations identified as correlates of clinical outcome were predictive, prognostic or both, and prior clinical and pre-clinical data support both interpretations of the data. Some tissue samples collected at progression lacked a paired baseline specimen, limiting our interpretation of the genetic and genomic findings in the progression samples. Several studies have demonstrated intertumoral heterogeneity using massively parallel sequencing (49, 50). Based on our own studies using massively parallel sequencing in a similar sample set (data not shown), low level point mutations (<5–10% allele frequency), such as in *NRAS*, could have been missed. In addition, copy number changes (other than high level amplification) are more difficult to interpret without matched germline samples, particularly for capture based methods. Thus, it is less likely that massively parallel sequencing would have provided greatly improved sensitivity in this context. Finally, as we mainly focused on known genetic alterations and genomic aberrations associated with melanoma, it is possible that we did not identify novel predictors of clinical outcome in this data set.

In summary, we identified *PTEN* loss, *CDKN2A* deletion and *CCND1* amplification as associated with decreased PFS upon treatment with dabrafenib. An exploratory analysis (data not shown) showed that carriage of two, or all three together, was associated with a trend towards worsening PFS. Although the interpretation of this analysis was limited by the small number of patients in each group, this approach should be considered for larger sample sets in the future. Ultimately, if validated in future patients, genetic analysis of *PTEN*, *CDKN2A*, and *CCND1* may be used to identify patients who should be considered for frontline combinatorial approaches, particularly utilizing agents that are activated by aberrations in these genes. Thus, these data suggest where potential combination therapies in conjunction with dabrafenib might be most effectively targeted. These studies also emphasize the continued importance of trials with associated biopsies on a large number of patients, so that we may gain insights into predictors of clinical outcome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors wish to thank Madhavi Vaddi at the Penn Molecular Profiling Facility and Dr. Quian-Chen Yu at the Histology Core Facility for excellent technical assistance at the University of Pennsylvania. Services provided in the Penn Molecular Profiling and Histology Core Facilities are supported by the Abramson Cancer Center core grant P30CA016520. They also would like to thank the staff of Sydney West Cancer Trials Centre, and the surgical, dermatology and Anatomical Pathology staff of Westmead Hospital and Melanoma Institute Australia. This work was performed in the U.T. MD Anderson Cancer Center Clinical and Translational Research Center (CTRC) and was supported by the Center for Clinical and Translational Sciences, which is funded by National Institutes of

Health Clinical and Translational Science Award UL1 RR024148 and by the National Institutes of Health Cancer Center Support Grant (CCSG) award CA016672 to MD Anderson Cancer Center.

Grant Support

Supported by research grants from GlaxoSmithKline, Inc., Melanoma Research Alliance Team Science Award to KLN and MAD, ASCO Career Development Award to MAD, Cancer Institute New South Wales Fellowship program to GL.

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Statement of translational relevance

Multiple studies have evaluated acquired mutations in melanomas from patients who progress on BRAF inhibitor therapies. The current study focuses on pre-treatment predictors of outcome when treated with the BRAF inhibitor dabrafenib. Common genetic mutation and genomic aberrations in melanoma were examined, with those in *CDKN2A*, *CCND1*, and *PTEN* found to correlate with the duration of PFS. Interestingly, these genetic changes have been previously implicated as associated with outcome in natural history studies of melanoma, and pre-clinically, in response to treatment with BRAF inhibition, so we cannot discriminate if they are predictive or prognostic markers. Our data suggest genetic analysis of *PTEN*, *CDKN2A*, and *CCND1* may be used to identify patients who should be considered for frontline combinatorial approaches, particularly utilizing agents targeting pathways activated by aberrations in these genes. This study also emphasizes the continued importance of trials with associated biopsies on a large number of patients, so that we may gain insights into predictors of clinical outcome.

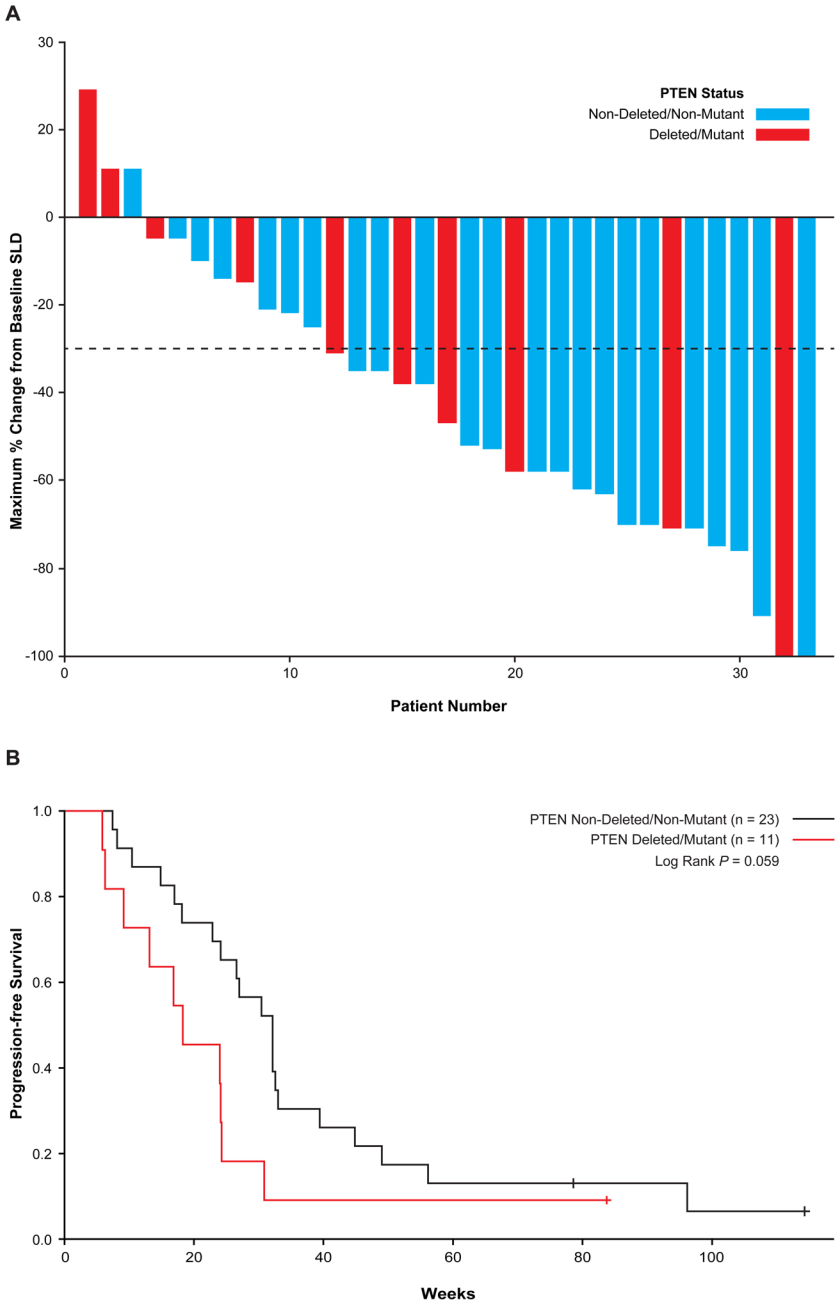

Figure 1. Association with PTEN genetic status with response and progression free survival upon treatment with dabrafenib

A. Waterfall plot. One patient with stable disease was not included because tumor percent change was unknown. B. Median PFS in patients with tumors with mutations or deletions of PTEN (red line) vs. all others (black line).

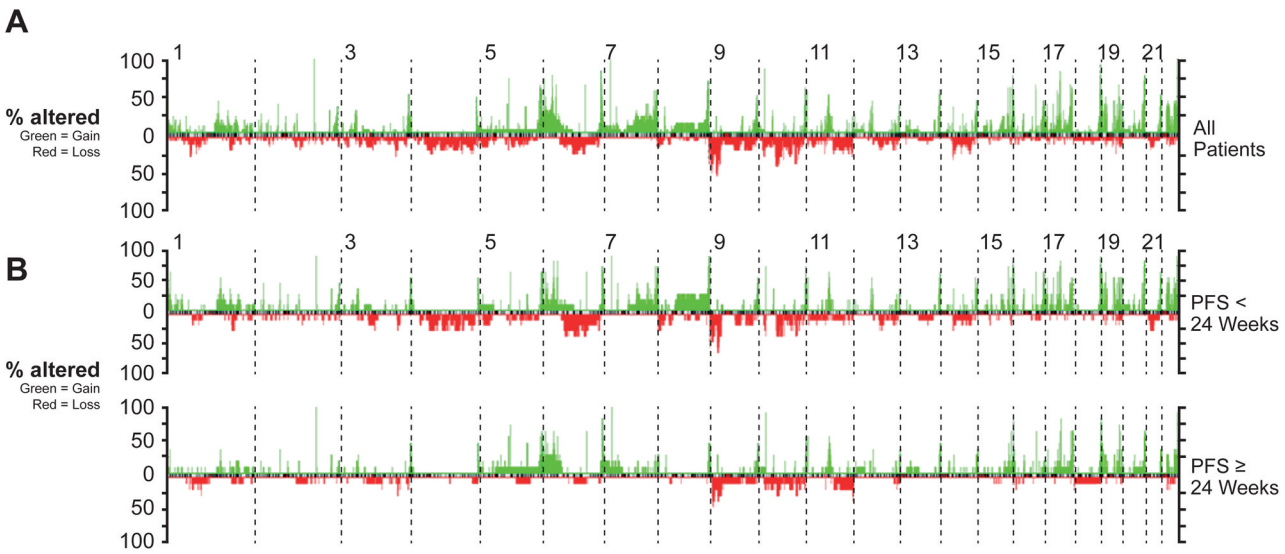

Figure 2. array based Comparative Genomic Hybridization analysis of melanoma samples

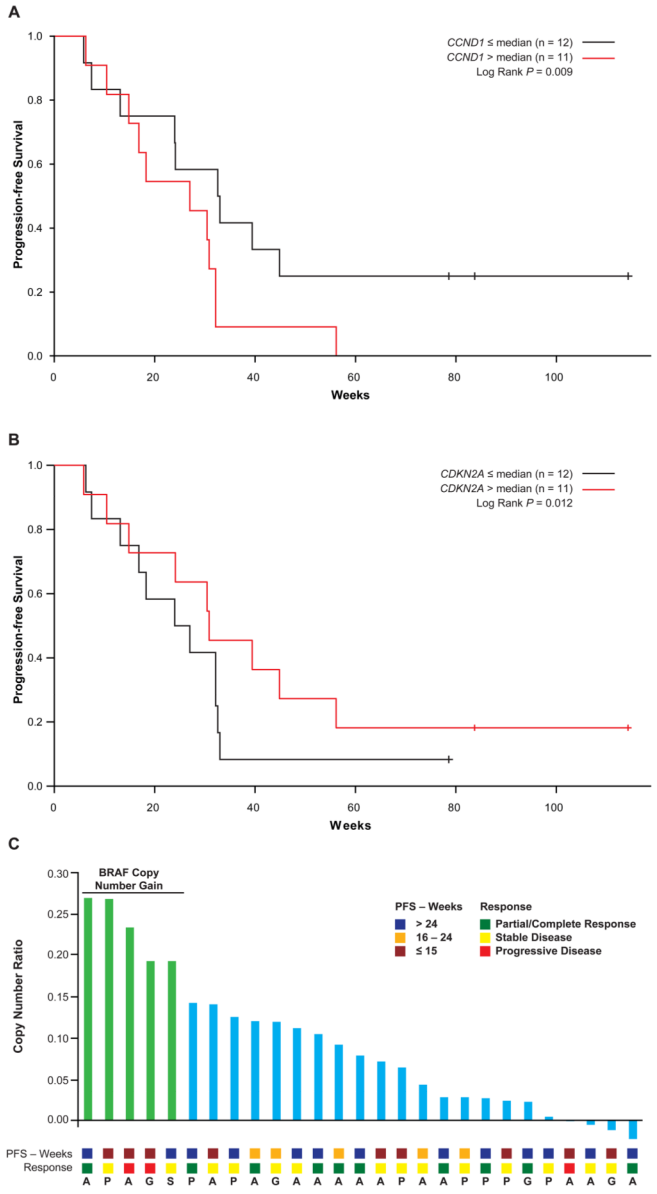

Figure 3. Association between gene copy number and outcome

For visualization of the association between copy number and outcome, the samples were divided by median copy number and Kaplan-Meier curves for PFS were generated A. *CCND1* B. *CDKN2A* C. BRAF copy gain and correlation with PFS, response and time of sample collection (A – archival; P – pre-treatment; S – on-treatment; G – progression)

Table 1

Patient description

Patient characteristic		Total (n = 41)*
Dabrafenib starting dose	< 150 mg BID	17 (41%)
	150 mg BID	15 (37%)
	> 150 mg BID	9 (22%)
BRAF V600 mutation	V600E	34 (83%)
	V600K	7 (17%)
Distant metastasis	M1a/M1b	7 (17%)
	M1c	34 (83%)
ECOG performance	0	28 (68%)
	1	12 (29%)
	2	1 (2%)
LDH level	< 1xULN	19 (46%)
	1–2xULN	13 (32%)
	> 2xULN	7 (17%)
	Missing	2 (5%)
Number of prior systemic therapies	0	17 (41%)
	1	12 (29%)
	≥ 2	12 (29%)

* Only patients with baseline (pre-treatment or archival) samples are included

Table 2

Mutational status using targeted assays and copy number analysis of PTEN

Patient #	Sample type	BRAF	WT/mutant (T:A) Ratios	PTEN Mutation Status and Copy #	Additional mutations
1	Pre-treatment	V600E	1:1	WT/no CN*	
1	Progression	V600E	1:1	WT	
2	Progression	K601E	ND	Homo-Del†	
3	Pre-treatment	V600E	1.5:1	WT	
4	Archival	V600K	1.5:1	Hemi-Del‡	
4	Progression	V600K	2:1	WT	
5	Archival	V600E	2:1	AF	
6	Archival	V600E	1:1	Dup Ex 1[a]	
7	Archival	V600E	3:1	WT	
7	On-treatment	V600E	1:1	Hemi-Del	
7	Progression	V600E	3:1	WT	
8	Archival	V600E	1:1	Hemi-Del	
9	Archival	V600E	1:1	WT	*CTNNB1* S45del
10	Pre-treatment	V600E	1.5:1	Homo-Del	
10	Progression	V600E	2:1	Homo-Del	
10	Progression	V600E	3:1	Hemi-Del	
11	Archival	V600K	1:3	Amplified	
11	Pre-treatment	V600K	1.5:1	WT	
12	Archival	V600E	1.5:1	WT	
13	Archival	V600E	3:1	Amp/Del[β]	
14	Archival	V600E	1:1	WT/no CN	
14	Pre-treatment	V600E	1.5:1	WT	
14	Progression	V600E	1:1	WT	
15	Archival	V600E	1.5:1	WT	
16	Archival	V600E	1:1	WT	
16	Pre-treatment	V600E	2:1	Mutant	
17	Archival	V600E	1.5:1	WT/no CN	
18	Archival	V600K	1.5:1	WT	

Patient #	Sample type	BRAF	WT/mutant (T:A) Ratios	PTEN Mutation Status and Copy #	Additional mutations
19	Pre-treatment	V600K	1:1.5	WT	
19	On-treatment	V600K	2:1	WT	
19	Progression	V600K	1:1.5	WT	
20	Archival	V600E	1:1	Hemi-Del	
21	Pre-treatment	V600E	2:1	WT	
22	Archival	V600E	2:1	WT	
23	Archival	V600E	1:1	WT	
24	Archival	V600E	1:1	Hemi-Del	
25	Archival	V600E	1.5:1	WT	
26	Pre-treatment	V600K	2:1	Homo-Del	
27	Archival	V600K	1:1	Dup Ex 1	
28	Archival	V600E	2:1	Hemi-Del	
29	Archival	V600E	2:1	Hemi-Del	
30	Archival	V600E	1.5:1	Amp/Del	
31	Archival	V600E	3:1	WT/no CN	
32	Archival	V600E	1:4	AF[γ]	
33	Archival	V600E	1:2	Hemi-Del	
34	Progression	V600E	1:1	Homo-Del	
35	Progression	V600E	3:1	Homo-Del	
36	Archival	V600E	2:1	Hemi-Del	
37	Progression	WT	ND	WT	*NRAS* Q61K
38	Pre-treatment	V600E	1:1	WT	*MEK2* Q60P
39	Archival	V600E	1:1	WT	
40	Archival	V600E	1:1	WT	
41	Archival	V600E	2.5:1	WT	
42	Archival	V600E	5:1	Hemi-Del	
43	Pre-treatment	V600E	2:1	AF	
44	Archival	V600E	2:1	WT	
45	Archival	V600E	3:1	AF	

[*] WT/No CN – Sequence wild-type, copy number could not be obtained;

[†] Homo-Del – homozygous deletion;

‡Hemi-Del – hemizygous deletion;

αDup Ex 1 – duplication of exon 1;

βAmp/Del – duplication of exon 1, deletion of other exons;

γAF - assay failed