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Targeted Next Generation Sequencing Identifies Clinically Actionable Mutations in Patients with Melanoma

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Abstract

Somatic sequencing of cancers has produced new insight into tumorigenesis, tumor heterogeneity, and disease progression, but the vast majority of genetic events identified are of indeterminate clinical significance. Here we describe a NextGen sequencing approach to fully analyze 248 genes, including all those of known clinical significance in melanoma. This strategy features solution capture of DNA followed by multiplexed, high-throughput sequencing, and was evaluated in 31 melanoma cell lines and 18 tumor tissues from patients with metastatic melanoma.

Mutations in melanoma cell lines correlated with their sensitivity to corresponding small molecule inhibitors, confirming, for example, lapatinib sensitivity in *ERBB4* mutant lines and identifying a novel activating mutation of *BRAF*. The latter event would not have been identified by clinical sequencing and was associated with responsiveness to a *BRAF* kinase inhibitor. This approach identified focal copy number changes of *PTEN* not found by standard methods, such as comparative genomic hybridization (CGH). Actionable mutations were found in 89% of the tumor

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tissues analyzed, 56% of which would not be identified by standard-of-care approaches. This work shows that targeted sequencing is an attractive approach for clinical use in melanoma.

INTRODUCTION

Clinical sequencing is already an essential part of melanoma treatment. Mutations in *BRAF* and *KIT* have both high frequency (45% and 4% respectively (Hodis et al. 2012; Krauthammer et al. 2012)) and clinical implications in melanoma (Flaherty et al. 2010; Kim et al. 2008). Numerous other oncogenic mutations, including *NRAS*, and *PIK3CA* (18% and 3% of melanoma) could join the list of clinically actionable mutations, as ongoing clinical trials progress (Ascierto et al. 2013). Additional therapies are also in development for driver mutations that infrequently occur in melanoma (e.g. *EZH2*, *RET*, *MEK1*). Bringing these therapies to bear on melanoma treatment will require rapid identification of tumor genotype across a large, but defined subset of genes.

Recent whole exome sequencing studies have demonstrated substantial power and sensitivity to discover new genes with significantly elevated mutation rates in melanoma. Two such studies recently identified mutations of *RAC1* and *ARID2* as potential pathogenic events in melanoma, among others, (Hodis et al. 2012; Krauthammer et al. 2012). Studies of melanoma whole exomes, such as The Cancer Genome Atlas project, are powered to identify genes mutated at even lower frequency. Whole exome analysis, however, suffers from several drawbacks. Current methods cost ~\$1,000 per sample for sequencing alone, as well as additional costs of the capture methods. As exome sequencing is currently not amenable to a high level of multi-sample pooling (multiplexing), it also incurs high cost in both personnel and sequencing machine time. Perhaps most problematic, whole exome and whole genome studies require computationally intensive bioinformatics analysis and data storage consuming significant resources and specialist interpretation. This problem has been described as “The \$1,000 genome, [and] the \$100,000 analysis” (Mardis 2010). Lastly, even after a list of high confidence somatic mutations has been generated and annotated from exomic data, the vast majority of such events will be of unknown significance.

More targeted approaches exist that can potentially alleviate the above issues. These options include PCR-based resequencing or in-solution hybrid capture techniques with a more focused target list. These approaches reduce costs and complexity at each stage. In the case of PCR-based resequencing, reduction of the DNA sequence target to a much smaller scale (< 1 megabase, Mb) permits more rapid sequencing and library generation, much higher levels of multiplexing, and more simplified bioinformatics analysis. Hybrid capture technologies, by comparison, retain a slower library generation process, but similarly allow increased multiplexing for reduced sequencing requirements and per-sample costs. Hybrid capture has two principal advantages over PCR-based approaches: it permits analysis of a larger target (several MB), and, with concurrently sequenced matched normal control, can be used to identify copy number events (Chinnaiyan & Palanisamy 2010; J. Li et al. 2012; Sathirapongsasuti et al. 2011; Timmermann et al. 2010). The latter is of particular relevance in melanoma, where deletion of tumor suppressor genes (e.g. *CDKN2A*, *STK11* and *PTEN*) plays a critical role in tumor pathogenesis.

To develop a more cost effective sequencing pipeline we designed a targeted hybrid capture approach for use in solid tumors including melanoma. This panel was designed to find all known genetic events of clinical relevance for these cancers. The approach employed is flexible and customizable to addition of new genes as their roles in cancer are better characterized and clinical correlates are defined, and can make use of germline sequencing information when available. Our captured list increased from 75 genes in version 1 to 248 genes in version 6. We first employed this assay on a panel of melanoma cell lines, and identified novel genetic events that correlated with increased sensitivity to corresponding small molecule inhibitors. We subsequently performed targeted exon capture sequencing in 18 tumor tissues obtained from patients with metastatic melanoma, including 4 tumor tissues that were prospectively collected along with matched peripheral blood. Actionable events detected in the prospective samples were disclosed to treating physicians after validation in a clinical laboratory improvement amendment (CLIA) assay.

RESULTS

Targeted Capture and Sequencing Genotyping of Melanoma Cell Lines and Tumors

We performed target resequencing of genes with a reported role in melanoma and other solid tumors by employing a multiplexed, in-solution capture in 31 melanoma cell lines and 18 patients. Variants were identified by comparison to matched normal specimens for the prospective samples, or with the Unified Genotyper single sample mutation calling pipeline (DePristo et al. 2011; McKenna et al. 2010) for the retrospectively collected tumor tissue from patients with advanced melanoma and various melanoma cell lines (see methods). Calls from retrospective samples without a matched normal were subjected to further filtering to remove likely germline variants. This informatics approach was employed to identify possible somatic variants with clinical relevance in melanoma treatment, biology, and prognosis.

By applying version 1 (V1) of the assay in 31 melanoma cell lines, we identified 237 single nucleotide variants (SNV) in coding regions of 59 genes (Table 1 and Supplementary Tables 1 and 2) and 15 insertion-deletion (indel) variants in 10 genes. Of the SNV calls, 64 were synonymous (S) and 173 nonsynonymous (NS), including 9 resulting in a stop codon gain, for a nonsynonymous-to-synonymous (NS:S) ratio of 2.70. This was higher than that seen in previously reported whole-exome study of melanoma cell lines (Stark et al. 2012), likely due to a higher degree of selection on this gene subset. Given our target sequence size of 446 kb in 31 lines, this is an overall somatic mutation rate of 18 somatic mutations per Mb per line (inner quartile range 8.97 to 26.9), consistent with the high mutation rate previously described in melanoma (Hodis et al. 2012; Pleasance et al. 2010; Stark et al. 2012).

To demonstrate the clinical utility of our sequencing approach we subsequently used V2 of the assay (Supplementary Table 1) to analyze 14 retrospectively collected melanoma tumor tissues. For these samples, matched germ line DNA was not available, and therefore likely germline events were filtered using bioinformatic approaches (see methods). By employing this approach we identified 68 SNV and 4 indel variants in coding regions of 52 genes. Of the SNVs, 50 were coding and 18 noncoding for a ratio of 2.78 and an average 25 somatic mutations per Mb per tumor (inner quartile range 12.7 to 42.4).

Finally, by employing V6 of the assay (Supplementary Table 1), we analyzed 4 tumors prospectively collected from patients with metastatic melanoma for which a matched normal tissue was available. SNVs were called by a more robust approach, relying on sequencing of the matched normal to control for germline variants. We identified 105 coding variants, with a range from 2 to 64 somatic mutations per patient.

Internal and External Validation

We previously sequenced *NRAS*, *KIT*, *BRAF*, *MET* and *CDKN2A/B* in the panel of 31 melanoma cell lines ((Shields et al. 2007) and not shown). These results were confirmed using the V1 of our next generation sequencing (NGS) assay. A normal primary fibroblast cell line processed in parallel with the melanoma cell lines resulted in only one synonymous variant called as an SNV, validating that our approach has a low rate of false positives. In the four melanomas with a matched normal, we identified seven mutations, out of which five were successfully validated using a CLIA-certified assay. Although it is possible that our failure to confirm these two mutations may reflect sequencing errors of our NGS assay, more likely, we believe the observed discordance reflects lower sensitivity of the CLIA-certified assay (e.g. Sanger sequencing), especially in the presence of stromal contamination (Nollau & Wagener 1997). These results suggest the assay is highly accurate with regard to the detection of SNVs in cell lines and primary tumors.

In comparison with published melanoma datasets (COSMIC (Forbes et al. 2011) and two studies (Hodis et al. 2012; Krauthammer et al. 2012)), somatic single nucleotide mutation frequencies and profiles were similar for both melanoma cell lines and tumors (Fig. 1A,B). For example, *BRAF* and *NRAS* mutations were noted in 52% and 21% respectively of all samples (cell lines and patient samples). Canonical *BRAF* V600E and *NRAS* Q61R mutations were present at a range of estimated allele frequencies in patient derived samples (from 16% to 91%). This suggests robust detection of mutations in the presence of substantial aneuploidy or stromal contamination. In addition, single base pair mutations in cell lines and unmatched tumor samples exhibited C → T mutation bias (Fig. 1C,D). This mutation profile has been reported in whole genome (Plesance et al. 2010) and exome sequencing (Hodis et al. 2012; Krauthammer et al. 2012) in melanoma, suggesting common ultraviolet light-induced mutagenesis. These data suggest targeted NextGen sequencing yields mutation frequencies and spectrum that are similar to large-scale whole genome and exome sequencing strategies.

Exemplary Events not Found by Standard Analyses

Apart from hot-spot mutations of RAS/RAF/KIT detected by standard molecular pathology approaches, the assay was able to identify a diverse set of genetic aberrations not found using standard clinical sequencing. For example a 5 codon in-frame deletion mutation of *BRAF* in the Mel537 cell line was discovered (*BRAF* del486-491) (Fig. 2A). This mutation exhibited remarkable structural similarity (Fig. 2B) to activating in-frame deletions of the *EGFR* kinase found in non-small cell lung cancer (Jackman et al. 2006). Consistent with this mutation being an activating event, this line co-clustered with *NRAS* and *BRAF* mutant tumors, but not with *RAS/RAF* wildtype tumors, in an RNA expression analysis (Supplementary Fig. 1). Additionally this line exhibited increased levels of phospho-ERK

that decreased with treatment with a *BRAF* inhibitor, PLX-4032 (Fig. 2C,D), in contrast to the paradoxical increase in phospho-ERK that occurs in *NRAS* mutant tumors (Fig. 2C, VMM39, see also (Carnahan et al. 2010; Halaban et al. 2010; Heidorn et al. 2010)). Compared to lines harboring the classical V600E *BRAF* mutation, Mel537 was comparatively less sensitive to both *BRAF* (PLX-4032) and *MEK* inhibitors (trametinib or GSK1120212 (Stones et al. 2013)) (Fig. 2C-F). This observation may be driven by altered downstream signaling in the *BRAF* del486-491 mutant, altered binding of drug substrates, or by additional mutations in the Mel537 cell line not related to the *RAS-RAF-ERK* signaling cascade. These results suggest that the *BRAF* del486-491 mutation is a non-canonical mechanism to activate *ERK* and downstream targets in melanoma, and is associated with moderate sensitivity to *BRAF* and *MEK* inhibitors.

Consistent with a prior report (Prickett et al. 2009; Stark et al. 2012), we noted several mutations in diverse regions (Fig. 3A) of the *ERBB4* gene in melanoma cell lines and tumors. Melanoma cell lines harboring mutations of this kinase exhibited a trend ($p=0.1$) towards enhanced sensitivity to the EGFR-inhibitor, lapatinib, in accordance with prior findings (Fig. 3B) (Prickett et al. 2009). While these mutations appear common (observed in 10% of melanoma primaries and cell lines assessed in this sample), the dispersion of these mutations throughout the gene makes their identification difficult by classical (Sanger) sequencing. Other potential clinically actionable events were observed in both melanoma cell lines (Supplementary Table 2) and clinical samples (Table 2) and included previously described as well as novel mutations in *PIK3CA* and *KIT*.

Tumor Suppressor Loss

In addition to detecting mutations in oncogenes, we employed our NextGen assay to detect genetic aberration in tumor suppressor genes that play important roles in melanoma progression (Dankort et al. 2009; Liu et al. 2012), overall prognosis (Nathanson et al. 2011), and response to small molecule inhibitors (Trunzer et al. 2013). To estimate tumor suppressor inactivation in the melanoma cell line panel, data from NextGen sequencing were analyzed to identify complete deletion of targeted exons. For this analysis, we identified exons with no sequencing coverage in a subset of melanoma cell lines with otherwise substantial coverage ($>30x$) in other lines. These calls were validated by CGH array data where available.

All NextGen-based deletion calls were consistent with CGH arrays, and *vice-versa*, with the exception of highly focal deletions of *PTEN* noted in two melanoma cell lines that were called in sequencing but were undetectable by CGH (Fig. 4A). These were regions with complete loss of coverage of individual exons of *PTEN* in the WM2664 and SKMEL24 lines (Fig. 4B). These findings were in keeping with the total absence of *PTEN* protein observed in these lines by ourselves and others ((Stahl et al. 2003) and not shown).

Melanoma cell lines with larger deletions spanning the *PTEN* gene were apparent as loss of sequencing coverage of all exons, while the majority of lines showed intact *PTEN* genes with coverage of all exons. When these genetic aberrations were combined with SNV/indel calls, the observed rates of *CDKN2A*, *PTEN* and *TP53* mutation were 44%, 29%, and 24%

respectively (Fig. 4C), consistent with prior analyses of melanoma cell lines (Forbes et al. 2011; Stark et al. 2012) and TCGA (see e.g. cBioPortal (Cerami et al. 2012)).

Combined SNV and copy number analysis of NextGen sequencing yielded a high total rate of less common tumor suppressor gene (TSG) mutations in our clinical samples as well (Table 2). Mutations in *APC*, *ATM*, *BRCA1*, *BRCA2*, *PTEN*, *RBI* or *TET1* were seen in 10 out of 14 completed patients (71%) lacking matched normal. We additionally identified copy number changes in both target and off-target regions by comparing tumor coverage to that of the matched normal, where available. When restricting the analysis to captured genes, two tumors had copy number changes reaching significance (adjusted $p < 0.10$). These were in *CCND2* (5.4 fold copy number vs. normal) and *PTEN* (0.3 fold copy number vs. normal), and both are commonly mutated in melanoma (Forbes et al. 2011), TCGA (cBioPortal (Cerami et al. 2012)). Collectively, our results show the ability of targeted capture exome NGS to detect various genetic aberrations, both mutation and copy number changes, in common as well as infrequently mutated genes, which may provide further insights about their role in prognosis and treatment decisions.

Assessment of Clinical Actionability and Result Reporting

To assess the potential clinical applicability of this targeted sequencing approach, we identified patients with somatic mutations that might influence clinical care (Table 2). These mutations were associated with either a potential treatment (small molecule inhibitors) or with use in prognosis and diagnosis (Table 3). Therapeutic indications were mutations associated with sensitivity to agents i) approved for use in melanoma, ii) approved for use in a different tumor type or iii) in human clinical trials for melanoma. Of the 18 patient-derived tumors, 16 patients (89%) carried mutations that could influence treatment decisions, including possible enrollment into a clinical trial. These included *BRAF* activating mutations (V600E and D594E) as well as mutations in *ALK*, *ERBB4*, *KIT*, and *PIK3CA*. Even excluding *BRAF* mutations found by standard clinical testing, 9 patients exhibited mutations that could influence therapy. This ‘actionability’ rate is comparable to that recently reported by another group in a large study of multiple cancer types including melanoma (Frampton et al. 2013).

Patients with metastatic melanoma who consented to have their tumor sequenced for actionable somatic mutations underwent NextGen sequencing using our assay as part of an ongoing clinical trial (NCT01457196). Somatic mutations deemed clinically actionable by the above criteria could be reported to a physician after validation using a CLIA-certified assay. In the four melanomas analyzed, we found between 0 and 6 actionable mutations per tumor (Table 3). One tumor had the *BRAF* V600E mutation alone. Two other tumors had several mutations, including one in *MAP3K1*, recently reported to have tumor suppressor activity in melanoma (Stark et al. 2012), as well as *ERBB4* and *PIK3IR*. Mutations in five genes (*ABL*, *BRAF*, *MET*, *PDGFRA*, and *PDGFRB*, Table 2 underlined) from 3 patients were confirmed by a CLIA-certified method and reported in the clinical record. These results indicates that this low-cost, capture-based sequencing can be performed in clinical real-time to provide actionable information beyond currently performed clinical sequencing.

DISCUSSION

In this report, we describe the development and performance of a customized, capture-based NextGen assay to identify somatic genetic aberrations, both mutations and copy number changes, in genes associated with cancer development, progression, prognosis, and response to treatment. Identification of such genetic aberrations may influence clinical care of patients with melanoma and other cancers. This approach can be easily modified to include additional genes whose role in cancer is being better established. Using this approach, we demonstrated in a limited number of tumor tissues obtained from patients with advanced melanoma that such patients harbor a high frequency of actionable genetic aberrations beyond *BRAF*, *NRAS* and *KIT*. We observed actionable events both for oncogenes outside of regions commonly subjected to sequencing in the current clinical setting (e.g. “non-canonical” mutations of *BRAF*) and of druggable oncogenes not traditionally considered to drive melanoma formation (e.g. *PIK3CA* and *ERBB4*). For three cases, events identified using this approach were validated using CLIA-approved methods and reported to patients and caregivers within the context of a clinical trial.

While whole exome/genome analyses have their merit for scientific discovery, we believe that the use of highly targeted sequencing panels is presently the most cost-effective approach for use in clinical decision-making. In addition to cost-efficacy, highly targeted sequencing can produce greater depth of sequencing coverage over the target, allowing the identification of mutations with relatively low minor allele frequency (<10%) within a sample. This is essential for samples with stromal contamination, which is likely to be a common feature of clinical samples, or in the presence of genetic heterogeneity within the tumor. Such an approach will be valuable in upcoming clinical trials that attempt to: identify in real-time patient subsets that may exhibit primary resistance to small molecule inhibitors (Trunzer et al. 2013), understand the genetic patterns of resistance to small molecule inhibitors (Nazarian et al. 2010; Shi et al. 2012), and provide guidance for rational treatment combinations applicable only to select patient subgroups. This approach leverages the information from large discovery-driven projects (Hodis et al. 2012; Krauthammer et al. 2012) and TCGA while allowing significantly reduced costs through multiplexing. Additional multiplexing is likely to be possible as sequencing technologies continue to advance. Of note, we have now extended the assay to formalin-fixed paraffin embedded (FFPE) samples, allowing for greater utility in common clinical care. DNA quality from FFPE in general is adequate for capture based NextGen sequencing, yielding results comparable to those obtained using frozen material (not shown and (Frampton et al. 2013)).

Using this approach, we identified an unusual mutation (del486-491) in *BRAF* in a cell line that would not be identified by standard-of-care approaches. Although we are not aware of a prior identification of this *BRAF* mutation in melanoma, the del486-491 mutation has been previously reported in an ovarian cell line (Hanrahan et al. 2012). The ability of this mutation to lead to activation of ERK and its moderate sensitivity to BRAF and MEK inhibitors suggests this mutation merits clinical assessment. We believe this mutation may be more common than appreciated, having escaped prior identification because it is not in a commonly analyzed exon (12 as opposed to 15 and 11) and is a 15 bp deletion, requiring approaches dedicated toward identifying these events. Targeted sequencing successfully

identified *BRAF* and *NRAS* mutations observed in melanoma, but also discovered more rare events in these genes. This approach also readily identified canonical, activating mutations of other druggable oncogenes, many of which are the target of ongoing clinical trials (e.g. PIK3CA).

The ability to uncover many simultaneous mutations within a melanoma affords new opportunities for multiple targeted therapies. However, the principles of such treatments remain unclear. Which mutations, if any, should take priority for targeting, or should multiple oncogenic mutations always be targeted with multiple therapies? Though it seems likely that multiple therapies will more effectively target resistance pathways, as with dual *BRAF*/MEK inhibition in melanoma, multiple pathway targeting has also lead to increased toxicity (e.g. as seen with combinations of MEK and PI3K inhibitors). Most difficult is the rarity of any one particular assemblage of mutations, given the number of possible mutations involved. In the near term, the personalization of cancer therapy remains experimental.

Finally, capture offers a platform for determination of genetic aberrations in tumor suppressors genes, allowing simultaneous assay of SNVs, indels, and copy loss in genes such as *ERBB4*, *STK11*, *RBI*, *CDKN2A*, and *PTEN*. Inactivation of these genes is common in melanoma, and likely will strongly affect tumor biology. For example, *RBI/CDKN2a* status correlates with sensitivity to CDK4/6 inhibitors (Wiedemeyer et al. 2010), whereas loss of *ERBB4* is associated with lapatinib sensitivity (Figure 4 and ((Prickett et al. 2009)), which is currently being tested in a clinical trial (NCT01264081). Our results identifying microdeletions within the *PTEN* gene are of particular interest, since such events were not detected by CGH and could play important roles in targeted therapy. Recent improvements in bioinformatic techniques enabling comparison of patient germline to neoplastic tissue for CNV detection will aid future analysis of targeted tumor suppressor sequencing in a clinical setting. The ability to affordably and reliably assay TSG status could aid prognosis, subtyping and therapy in melanoma.

METHODS

Clinical Material

Snap-frozen primary tumor tissues were obtained as part of institutional review board (IRB)-approved protocols at the Lineberger Comprehensive Cancer Center and the University of North Carolina. Samples from four prospective patients were collected under an ongoing clinical trial LCCC1108 (NCT01457196), whereas samples from 14 retrospective patients were collected under IRB#09-0989. For the four prospective patients, a matched normal tissue (whole blood), was also acquired. DNA was isolated with the Puregene DNA Purification Kit (Gentra Systems) or DNAeasy (QIAGEN).

Sequencing Library Preparation and Capture

Custom adapters were designed to match Illumina PE adapters with an additional 4 nucleotide ‘barcode’ sequence located internal to the first and second read sequencing (Supplementary Fig. 2). Barcode sequences were designed to require errors in at least 2

locations to be misidentified, allowing for error correction with the use of paired reads tolerant to up to 3 errors.

Sample workflow is detailed in Supplementary Figure 3A. Libraries were constructed per the SureSelect protocol (Agilent) with custom adapters replacing the Illumina PE adapters. Samples were quantified by bioanalyzer prior to capture, and pooled in equimolar quantity with 10 libraries to a pool in the case of cell lines and 8 libraries to a pool in the case of melanoma tumor samples to a final quantity of 500ng. Four samples for which a matched normal was available were prepared with the SureSelect XT library preparation kit (Agilent) by manufacturer protocols. Capture and post capture amplification for all samples proceeded by SureSelect protocol. Samples were sequenced on the GAII or HiSeq machine in a variety of formats (see Supplementary Table 3).

Bioinformatics Analysis of Unmatched Samples

Bioinformatics workflow is detailed in Supplementary Figure 3B. Custom Java applications were used to assign multiplex libraries to their source sample, after which samples were mapped using the Burrows-Wheeler Aligner (Keniry & Parsons 2008) to the hg18 reference genome. The Genome Analysis Toolkit GATK (version 1.0.5506)(H. Li & Durbin 2009) was used for quality score recalibration, indel realignment, and SNV and indel calling. SNV and indels were then annotated for appearance in prior 1,000 genomes (DePristo et al. 2011; McKenna et al. 2010) and dbSNP version 130 (Consortium 2012) to filter likely germline mutations and identify affected transcripts using the ANNOVAR tool (Sherry et al. 2001). Mutations with any prior detection rate in 1,000 genomes samples as annotated by ANNOVAR were ignored in further analysis, with exception of the NRAS Q61R mutation, which appears in dbSNP 130, as it is a known oncogenic event. Analysis of duplicate reads counts, base quality calls, and read mapping percentages were performed at appropriate steps as quality control. One line sequenced, 1205LU, was proved to be murine in origin, and was excluded from downstream analysis. Two cell lines believed to be distinct were genetically identical (SKMEL78 and SKMEL88), and were treated as a single line in analysis.

Bioinformatics Analysis of Matched Samples

Bioinformatics analysis of tumor samples for which DNA extracted from a same-patient normal tissue was available followed a similar IRB-approved protocol, but somatic variants were called using VarScan (Koboldt et al. 2009) and annotated using the ANNOVAR utility(Wang et al. 2010). Coding somatic variants with “Moderate” or “High” predicted effects were reported. For copy number analysis, each gene targeted to capture is tested for significantly different coverage compared to a normal reference using the DESeq algorithm (Anders & Huber 2010).

Gene Expression Microarray

Previously published gene expression microarray data sets (Carson et al. 2012) were filtered for the top 10% most variable genes (standard deviation of \log_2 ratio of expression vs. reference) and median centered by gene. Probe sets were clustered by unsupervised hierarchical clustering using uncentered correlation as the similarity metric via the Gene

Cluster 3.0 (Carson et al. 2012) tool and visualized with Java TreeView (de Hoon et al. 2004).

Array Based Identification of Copy Number Variation

For array-based determination of copy number in cell lines we used the Agilent 180K SurePrint G3 Custom CGH+SNP arrays (catalog # G4884a). Calls were determined by circular binary segmentation with the DNACopy package through Bioconductor. Calls were filtered for those with a log₂ ratio < -0.5 and p value < 0.01. These were further analyzed for deletions in *APC*, *CDKN2A*, *MSH6*, *TP53*, *RBI*, and *PTEN* tumor suppressors.

Sequencing Based Identification of Copy Number Variation in Cell Lines

Coordinated analysis of these same genes by sequencing utilized the clonality of cultured cell lines to identify additional deletion events. Deletion regions of the genome were those showing zero coverage by high-throughput sequencing reads. As coverage is expected to be low except in regions of capture, we restricted our analysis to exons of *APC*, *CDKN2A*, *MSH6*, *TP53*, *RBI*, and *PTEN* with average coverage > 30x in control cell lines. Genes with zero coverage in a sample with no otherwise notable mutations were marked as deleted by sequencing determined exon loss.

Chemosensitivity Assays

Cells were plated onto 96-well assay plates (Costar #3903) at different cell densities, depending on each cell line's growth rate, in 100 µl of media per well. Cells were then treated with dilutions of drugs or vehicle (DMSO) in triplicate 18 hours after plating. Drugs used included Lapatinib (GlaxoSmithKline), PLX 4032 (Roche), and trametinib (GSK1120212, GalaxoSmithKline). Cell viability was assessed at 72 hours after plating using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) and a Synergy2 microplate reader (BioTek). Raw data was imported into GraphPad Prism 5.0 and IC₅₀ calculated using non-linear regression analysis of the log inhibitor concentration versus luminescence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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SIGNIFICANCE

Gene sequencing is an essential part of treatment for metastatic melanoma, but current clinical strategies are limited to a few exons of only two genes. Here, we develop a scalable NextGen sequencing assay to identify point mutations and copy number events affecting all actionable genes associated with advanced melanoma. Using this approach, we identify actionable events in 90% of patients, which we disclose to caregivers after validation using a CLIA-approved assay. Importantly, we find that over half of tumors harbor actionable mutations that would not be detected by standard-of-care clinical sequencing, including a novel activating BRAF mutation.

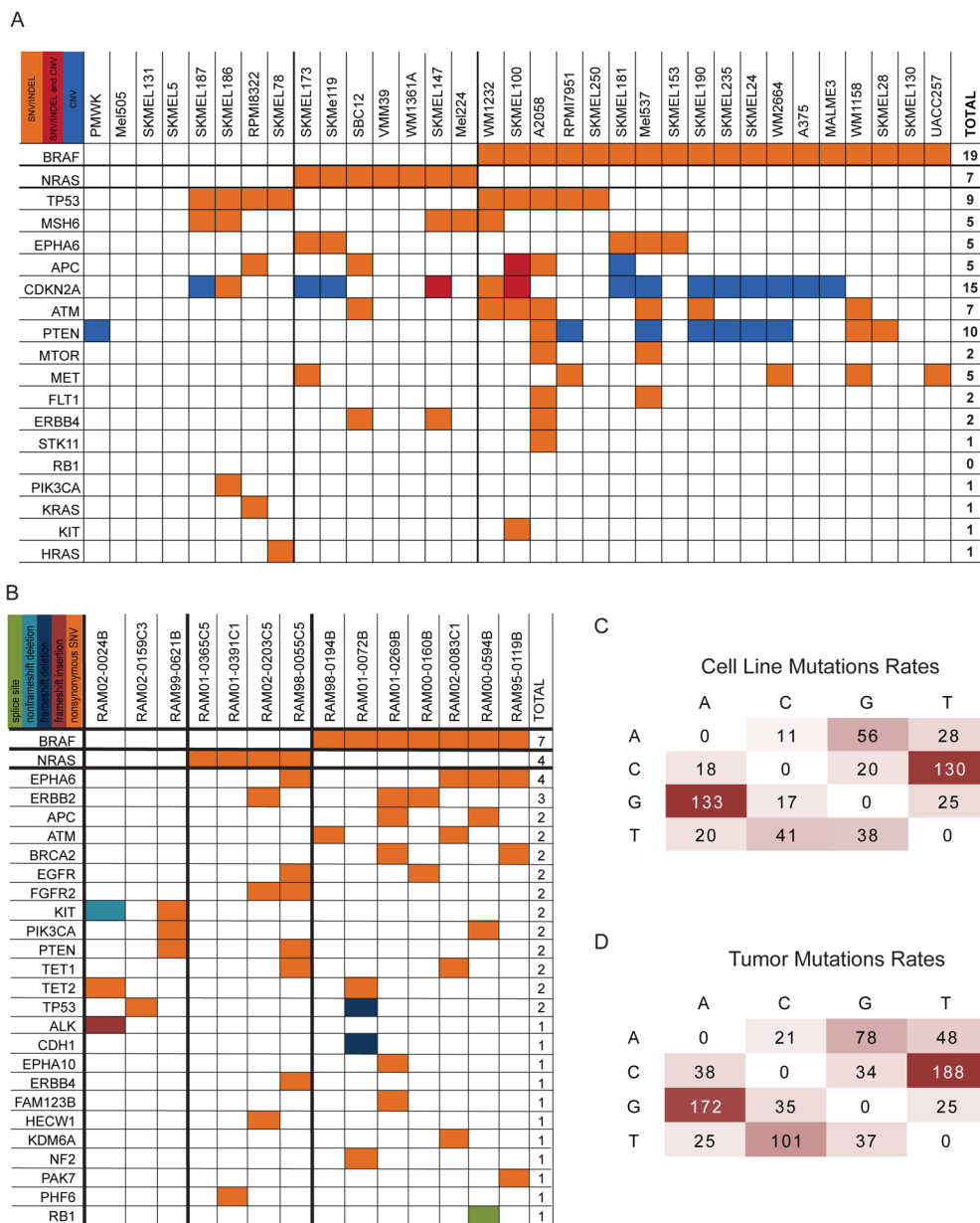


Figure 1. Aggregate Analysis of Mutation Calls

(A) Co-occurrence of mutations in melanoma cell lines including SNVs, CNV calls from SNP array, and both. (B) Co-occurrence of mutations in metastatic cutaneous melanoma showing KIT mutations in BRAF and NRAS wild type lines. (C) Putative somatic mutation rates in melanoma cell lines and (D) putative somatic mutations in tumors.

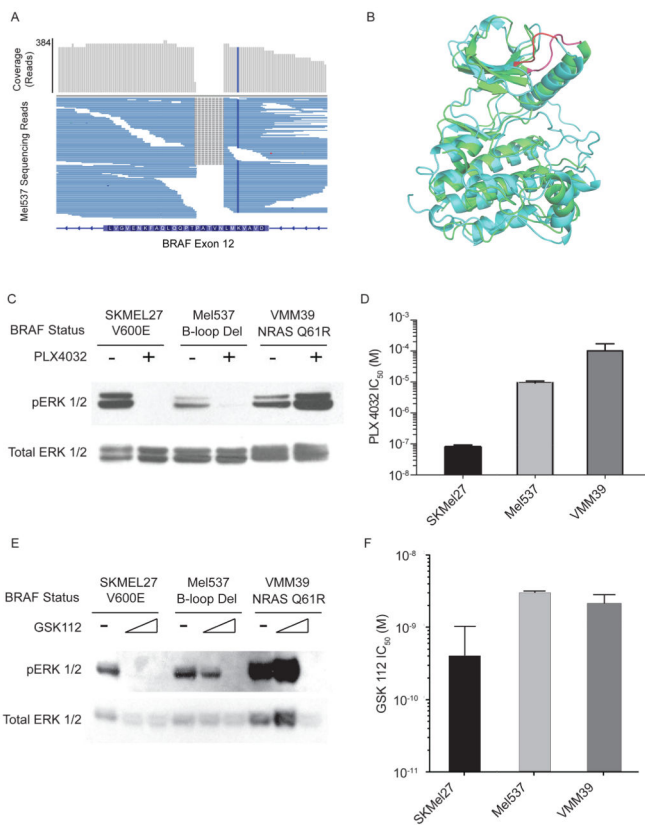
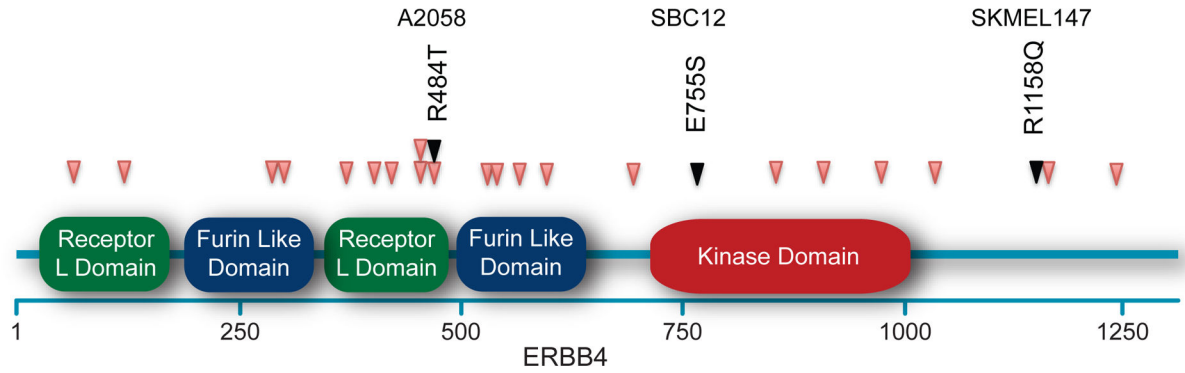


Figure 2. Non-Frameshift BRAF Mutation Sensitive to PLX4032

(A) Mel537 cell line aggregate read depth and mapped reads show homozygous non-frameshift deletion mutation in exon 12 of BRAF. (B) Structural alignment of inhibitor bound BRAF structure (CYAN) to inhibitor bound EGFR (GREEN) showing structural homology of the BRAF exon 12 deletion (RED) and know activating EGFR exon 19 deletions (PINK). (C) ERK and activated ERK (pERK) blots in serum-starved lines with and without PLX4032 treatment. (D) PLX4032 sensitivity of SKMEL27 (BRAF V600E), Mel537, and VMM39 (NRAS Q61R). (E) ERK and activated ERK (pERK) blots in serum-starved lines with and without MEK inhibition by GSK112. (F) GSK112 sensitivity of SKMEL27, Mel537 and VMM39.

A



B

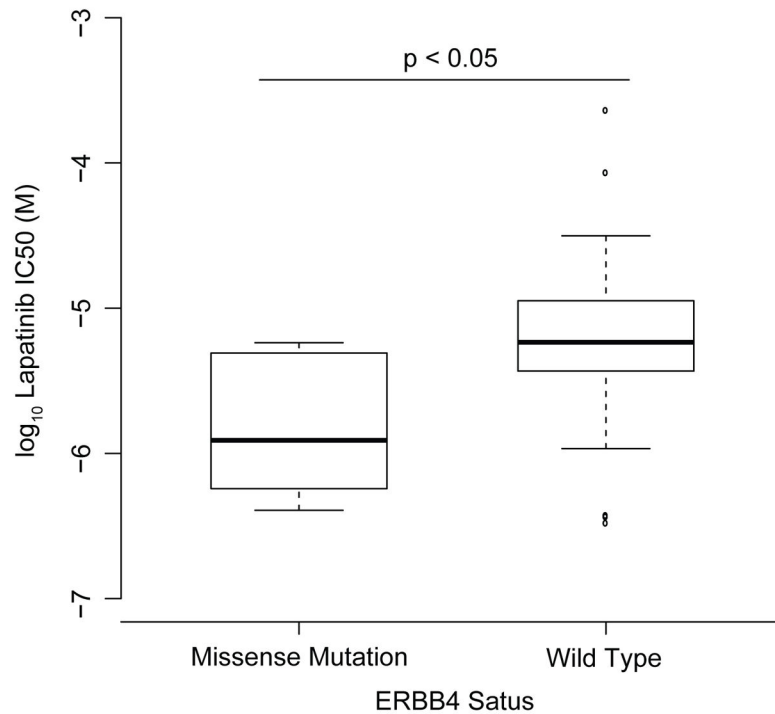


Figure 3. ERBB4 Mutation Predicts Lapatinib Sensitivity

(A) Previously reported mutations in ERBB4 (PINK) and in three cell lines (BLACK) span multiple domains of the gene. (B) Lapatinib sensitivity in three ERBB4 mutant lines was increased compared to ERBB3 wild type lines ($p = 0.1$).

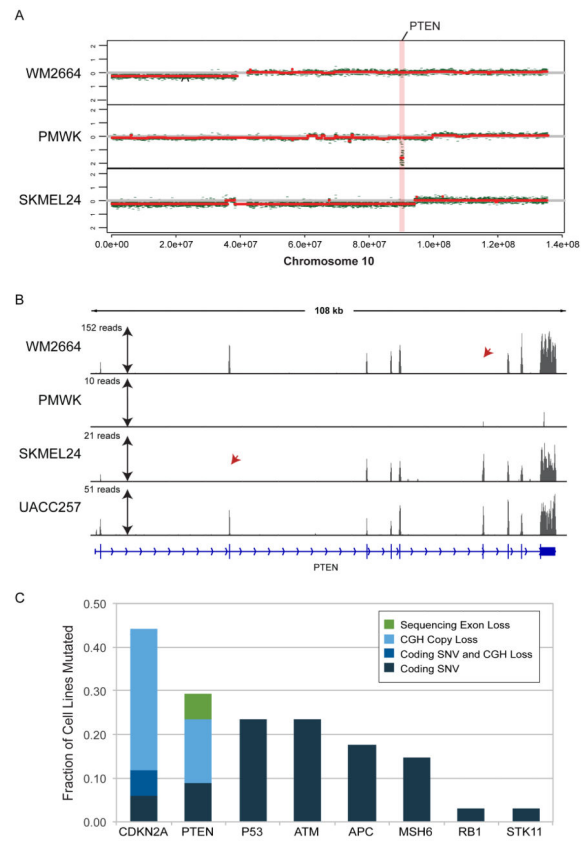


Figure 4. Sequencing Detects Focal Deletions at High Resolution
 (A) CNV calls using SNP microarrays. (B) Sequencing coverage of PTEN exons in WM2664, PMWK, SKMEL24 and UACC257 with very low coverage in a PTEN deletion line (PMWK) as well as isolated exons in other lines (red arrowheads). (C) Aggregated mutation rates in selected tumor suppressor genes in cell lines using CGH array and sequencing.

Table 1
Selected Mutations in Melanoma Cell Lines and Patients

Selected genes from melanoma cell lines and patient tumors with potential activating mutations.

	<u>Sample</u>	<u>Gene</u>	<u>Mutation</u>	<u>Previously Observed?</u>
Cell Line	SKMEL100	KIT	Y362F	No
	SBC12	PIK3CA	K678E	No
	RPMI8322	KRAS	G12V	Yes
	SKMEL78/88	HRAS	G12V	Yes
Patient Tumor	RAM99-0621B	KIT	L255Q	No
	RAM00-0594B	PIK3CA	R88Q	Yes
	RAM99-0621B	PIK3CA	E545K	Yes

Table 2

Targeted Resequencing Identifies Clinically Actionable Mutations

Clinical characteristics of 14 patients with advanced melanoma whose tumors underwent NGS. Mutation calls and mutational profiles, with potentially actionable mutations are highlighted in red. Somatic mutations validated by CLIA-certified testing and reported to physicians are underlined.

Pt No	Age	Race	Sex	Non Synonymous Single Nucleotide and Indel Variations	Survival (Months)
1	87	W	M	BRAF^{V600E} EGFR^{V1047M} ERBB2^{A386D} MET^{E168D}	0.8
2	38	W	M	APC^{P2813S} BRAF^{V600E} EPHA6^{R182H,G231E} , PIK3CA^{R88Q} , RB1^{SpliceSite}	11+
3	69	W	M	BRAF^{D594E} BRCA1^{R1300G} NF2^{S184N} TET2^{Y867H,P1723S} TP53^{T77del} CDH1^{S599_600del}	71
4	57	W	M	APC^{S1344F} ATM^{D1853V} BRAF^{V600E} BRCA2^{T598A,G602R} EPHA10^{R618H} ERBB2^{S819P} FAM123B^{S1024L} KDM6A^{T726K}	37
5	28	W	M	ATM^{P1054R} MSH6^{R1068X} NRAS^{Q61R}	12
6	31	W	M	ATM^{E498Q,P604S} BRAF^{V600E}	17
7	70	W	M	IDH1^{L189V} NRAS^{G13R} PHF6^{A41V}	74
8	41	W	M	ALK^{L1031fs} KIT^{S556_558del} TET2^{V1718L}	9
9	70	Afr	F	ATM^{T2030V} BRAF^{V600E} EPHA6^{S4A} FAM123B^{F159L} KDM6A^{T581A,L615V} TET1^{E995D}	17
10	37	As	F	KDM6A^{T726K} PTCH1^{A741V} TP53^{V31I}	21

Pt.No	Age	Race	Sex	Non Synonymous Single Nucleotide and Indel Variations	Survival (Months)
11	73	W	F	ERBB2 ^{P625L} FGFR2 ^{S130F} HECW1 ^{P328S} NRAS ^{Q61R}	16
12	24	W	F	BRAF ^{V600E} BRCA2 ^{P877S} EPHA6 ^{R588K} PAK7 ^{E248K}	204
13	38	W	M	EGFR ^{R255Q} EPHA10 ^{A955T} EPHA6 ^{L1102H} ERBB4 ^{G107E} FGFR2 ^{I235L} NRAS ^{Q61R} PTEN ^{R130P} TET1 ^{T1421A}	31
14	72	W	F	KIT ^{L255Q} PIK3CA ^{E545K} PTEN ^{P95L}	4
M1	-	-	-	EPHB6 ^{P163FS} TSC2 ^{FS}	N/A
M2	-	-	-	MET ^{G554R} KIT ^{D812H,D816H} ABL1 ^{T391I} PDGFRA ^{G592R} NF1 ^{Q1785*,Q1255*} CTNNB1 ^{P119L} IKZF1 ^{E195K} CDH1 ^{K520E} MAP3K1 ^{R1500I} ...	N/A
M3	-	-	-	BRAF ^{V600E} MLL ^{K3663*} NTRK3 ^{E756K}	N/A
M4	-	-	-	MET ^{E355K,P1013S} PDGFRB ^{P148S} KDR ^{G282E,S373F} EGFR ^{SpliceSite} TP53 ^{W107*} NOTCH4 ^{P599L} FGFR2 ^{E160K} HSP90AA1 ^{T317A} ERBB4 ^{G741E} ...	N/A

Table 3
Genes and Associated Clinical Action

Genes discovered in patient tumors with clinical actionability, along with the associated action and therapy.

<u>Gene</u>	<u>Category</u>	<u>Action</u>
ABL	Approved Therapy	Imatinib, Dasatinib, Nilotinib, Ponatinib
ALK	Approved Therapy	Crizotinib
BRAF	Approved Therapy	Vemurafenib
ERBB4	Clinical Trials	Lapatinib
KDR	Approved Therapy and Clinical Trials	Nonspecific kinase inhibition: Sunitinib, Sorafenib, Pazopenib
KIT	Approved Therapy	Dasatinib
MET	Approved Therapy	Crizotinib, Carbozantinib
NOTCH4	Clinical Trials	Gamma Secretase Inhibitors
NRAS	Future Clinical Trial	MEK/ERK inhibitors
PDGFRA	Clinical Trials	PDGFR inhibitors (e.g. Crenolanib)
PDGFRB	Clinical Trials	PDGFR inhibitors (e.g. Crenolanib)
PIK3CA	Clinical Trials	PI3K Inhibitors
PTEN	Future Clinical Trial	PI3K Inhibitors