A high throughput panel for identifying clinically-relevant mutation profiles in melanoma.

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Abstract

Success with molecular-based targeted drugs in the treatment of cancer has ignited extensive research efforts within the field of personalised therapeutics. However, successful application of such therapies is dependent on the presence or absence of mutations within the patient’s tumor that can confer clinical efficacy or drug resistance. Building on these findings, we developed a high throughput mutation panel for the identification of frequently occurring and clinically-relevant mutations in melanoma. An extensive literature search and interrogation of the Catalogue of Somatic Mutations in Cancer (COSMIC) database identified over 1000 melanoma mutations. Applying a filtering strategy to focus on mutations amenable to the development of targeted drugs, we initially screened 120 known mutations in 271 samples using the Sequenom MassARRAY® system. A total of 252 mutations were detected in 17 genes, the highest frequency occurred in BRAF (n=154, 57%), NRAS (n=55, 20%), CDK4 (n=8, 3%), PTK2B (n=7, 2.5%) and ERBB4 (n=5, 2%). Based on this initial discovery screen, a total of 46 assays interrogating 39 mutations in 20 genes were designed to develop a melanoma-specific panel. These assays were distributed in multiplexes over 8 wells using strict assay design parameters optimized for sensitive mutation detection. The final melanoma-specific mutation panel is a cost-effective, sensitive, high throughput approach for identifying mutations of clinical relevance to molecular based therapeutics for treatment of melanoma. When used in a clinical research setting, the panel may rapidly and accurately identify potentially effective treatment strategies using novel or existing molecularly targeted drugs.
Introduction

Melanoma is a highly aggressive malignancy and accounts for the majority of all skin cancer-related deaths [1]. The high mortality rate for advanced stage metastatic melanoma is largely due to the ineffectiveness of currently approved systemic treatment strategies [2]. For example, use of traditional chemotherapeutic approaches has shown poor response rates; less than 10% of patients demonstrate a clinical response to standard treatment with dacarbazine and less than 5% patients survive beyond 5 years [3]. Recently, novel effective molecular targeted therapies for the treatment of metastatic melanoma have emerged.

One class of molecular-based drugs of current interest for treating metastatic melanoma are RAF inhibitors. RAF mutations represent highly desirable targets because approximately 60% of melanoma patients have constitutively active MAPK signalling due to mutations in BRAF [4]. Although initial investigations using the RAF inhibitor sorafenib showed no significant increase in survival compared to chemotherapy [5], the recent development of more selective BRAF inhibitors has reignited research into this class of drug [6]. Recently, a phase 3 randomised clinical trial of PLX4032 (vemurafenib) showed improved rates of overall and progression-free survival in patients with BRAF V600E mutations when compared to dacarbazine [7].

Interestingly, the use of PLX4032 in tumors not harbouring the V600E mutation has shown adverse biological endpoints including increased cell division and proliferation both in vitro and in vivo [8-10]. Furthermore, mutations occurring in MEK, a downstream effector of BRAF in the MAPK signalling pathway, have been shown to confer resistance to PLX4032 treatment [11, 12]. Additionally, durable responses have yet to be achieved with PLX4032, suggesting that a number of mechanisms can lead to acquired drug resistance [11-15]. Thus screening for multiple mutations will be necessary for the development of effective treatment strategies.
Targeted approaches treating melanoma patients harbouring aberrations other than BRAF mutations are also being investigated. One approach was highlighted in a phase II trial of Imatinib Mesylate in which a clinical response was observed in a melanoma patient whose tumor harboured a KIT mutation [16]. A number of case reports have recently highlighted the efficacy of Imatinib treatment in KIT-mutated melanoma patients, and more recently, significant clinical responses in a subset of patients in a phase II clinical trial [17, 18].

Due to the recent successes of a number of targeted drugs in the treatment of metastatic melanoma, we sought to develop a melanoma-specific mutation panel to facilitate rapid identification of somatic mutation events relevant to targeted treatments. An extensive literature search was performed, including interrogation of the COSMIC database, to identify oncogenic mutations in melanoma. A confirmation panel of 120 highly ranked mutations was screened for prevalence in 271 melanomas before a final melanoma-specific mutation panel of 39 amino acid alterations was derived. Mutations selected for the final panel were ‘clinically-relevant’ in regards to having either documented evidence or potential clinical significance for targeted therapeutics.
Materials and Methods

Cell line and tumor samples
The first cohort of samples consisted of 61 cutaneous or nodal melanoma metastases previously described [19, 20] as well as a panel of 43 stage III (AJCC) melanoma early passage cell lines established at the Queensland Institute of Medical Research (unpublished). In addition to this, 16 matched tumor samples were included to assess the similarity of mutation profiles with the associated cell line. Eleven previously described uveal melanoma cell lines (provided by Dr. Jerry Nierderkorn, UT Southwestern Medical Centre, Dallas, Texas), were included to identify mutations in this subtype of melanoma [21]. A second cohort comprised 98 fresh-frozen stage III and IV metastases, from the Melanoma Institute Australia [22], and thirty melanoma cell lines (of which nine represented additional clones) established as previously described [23]. Cell lines with corresponding matched tumors were profiled to confirm authenticity using an AmpFISTR Profiler Plus PCR amplification kit (Applied BioSystems) and analysed on a 3100 Genetic Analyzer (Applied Biosystems).

Cell Culture and DNA extraction
The panel of 43 stage III melanoma cell lines was cultured in filter-sterilised RPMI1640 supplemented with 10% heat inactivated foetal calf serum (55°C for 30 min), 100 U/ml penicillin and 100 ug/ml streptomycin at 37°C in 5% CO2. Genomic DNA was extracted using QIAGEN QIAamp Blood Maxi Kits according to the manufacturer’s instructions (Qiagen Pty Ltd., Clifton Hill, Victoria, Australia). Tumor DNA was extracted from 20-30 mg of fresh-frozen tumor using QIAamp DNA mini kits (Qiagen) with on- column RNAse digestion. Briefly, tissue was pulverized in liquid nitrogen then incubated with ATL buffer (Qiagen) and proteinase K for 96 hr at 56°C.
Mutation detection

Mutation detection was performed using the Sequenom MassARRAY® system following standard protocols (Sequenom, San Diego). In brief, 20 ng of genomic DNA were used in a PCR reaction and cleaned post-amplification with shrimp alkaline phosphatase. A single base pair extension reaction using iPLEX Pro chemistry was performed, resin-treated to remove contaminants and spotted onto SpectroCHIP II arrays. Mutant and wild type alleles were then discriminated via mass spectrometry using the Sequenom MassARRAY® Analyser 4 platform. Mutations were detected using a minimum 10% threshold of the mutant allele peak and were all manually reviewed. The panel of stage III melanoma cell lines was also analysed using the Sequenom OncoCarta® v1.0 panel, which consists of 238 mutations covering 17 oncogenes [24].

Assay Design Parameters

As the confirmation panel’s purpose was to identify frequently occurring mutation events from a large list of mutations, standard iplex parameters (Assay designer 4.0, Sequenom) were used with a maximum multiplexing level of 24 assays per well for cost-effective, high throughput screening. The final melanoma-specific mutation panel, in contrast, used strict assay design parameters optimised for sensitive mutant allele peak detection. A maximum multiplexing level of 12 assays per well with increased penalty scores for hairpin, self-dimerisation, heterodimerization and false priming of the extension primer were applied. In addition, if possible, mutant allele peaks were designed as the first detected allele peak of an assay in order to reduce potential noise from salt adducts. Further details of the confirmation panel are provided in supplementary Table 1. The final melanoma-specific mutation panel (MelaCARTA v1) is available from Sequenom Inc.
Statistical Analysis

To assess the accuracy and sensitivity of the final melanoma-specific mutation panel compared to the OncoCarta® panel, the mutant allele frequencies obtained with each panel were compared. A two-tailed paired students T-test was used to determine whether any differences were statistically significant.
Results

Mutation detection using the OncoCarta® mutation panel v1.0

To identify frequently occurring mutations to be included in the melanoma-specific mutation panel, 43 stage III melanoma cell lines were screened using the Sequenom OncoCarta® panel. Mutations were detected in 80% of cell lines (34 of 43). BRAF and NRAS mutations were mutually exclusive, with mutations occurring in approximately 60% (25 of 43) and 20% (9 of 43) of cell lines respectively. Four BRAF-mutated samples had an additional mutation in CDK4, PIK3CA or MET. Table 1 lists mutations and allelic ratios in the stage III melanomas. Ten mutations in five genes from the OncoCarta® panel were included in the final melanoma-specific mutation panel, thus eliminating excessive and potentially unnecessary genotyping not relevant for melanoma.

Mutations identified from the COSMIC database and detailed literature search

The COSMIC database is a comprehensive resource of somatic mutations occurring in cancer, curated from data in scientific literature and large scale tumor genomic resequencing efforts [25]. Using this resource, a comprehensive list of mutations occurring in metastatic melanoma was compiled along with associated population mutation frequency data (Supplementary Table 2 lists mutations and references).

At the time of database accession, the majority of mutations in this list derived from a resequencing study of 518 kinases in 210 diverse cancers including 6 melanomas [26]. Most mutations in these samples were found in MZ7-mel and CP66-mel (476 and 258 mutations respectively out of 900 mutations), which were deemed to have a ‘mutator phenotype’ and were thus excluded from our analysis as most of the mutations are likely ‘passenger’ events. After removing duplicate and synonymous changes, 87 mutations from the remaining 4 samples were included for consideration in the confirmation stage. An additional 76 documented mutations in COSMIC, not related to these 4 samples, were also considered.
For comprehensive coverage of mutations in melanoma, an extensive literature search was performed using PubMed to identify mutations not included in COSMIC. Mutations in ERBB4, MITF, GNAQ, GNA11, MEK, and the MMP family were identified. The combination of approaches identified 314 mutations that were considered for inclusion in the confirmation phase (Supplementary Table 2).

Filtering strategy to reduce mutations screened in the confirmation panel

To reduce the number of mutations genotyped in the confirmation phase, mutations were ranked according to criteria based on technology platform capabilities, clinical significance and translational value. High priority mutations include those that occur frequently in the population; occur frequently at single amino acid positions; are relevant to targeted drugs currently available or in clinical development; or are responsible for tumorigenesis through the disruption of important biological pathways. For this reason, mutations that had a reported mutation frequency of >1%, occurred in genes with kinase domains, introduced charge changes in the amino acid sequence and that did not occur in genes mutated in a typical fashion associated with inactivation of tumor suppressor genes were preferentially selected. This strategy reduced the list from 314 mutations to 110 highly ranked mutations in 42 genes. Figure 1 shows a summary of the mutation filtering process. The confirmation panel was used to determine the significance of these mutations in a large number of melanoma cell lines and tumors.

Mutations identified in the confirmation panel

The confirmation panel detected 247 mutations in 271 samples (Table 2). The highest frequency of mutation was in BRAF (n=154 or 57%), NRAS (n=55 or 20%), CDK4 (n=8 or 3%), PTK2B (n=7 or 2.5%) and ERBB4 (n=5 or 2%). BRAF mutations were mutually exclusive to NRAS mutations and the majority occurred at codon 600, mostly resulting in a
valine to glutamate substitution, i.e. V600E (137/271 or 50.5%). The most frequently occurring NRAS mutation (52/271, 19%) was at amino acid 61 (2 x Q61H, 21 x Q61K, 9 x Q61L and 20 x Q61R).

Twenty-seven samples had more than one mutation; the majority were a BRAF mutation in association with another mutation (23/27, 85.1%). Samples with ERBB4 mutations did not segregate with BRAF (2/5) or NRAS mutations (1/5). Interestingly, of the 3 samples with mutations in MEK, one (D28) also had a BRAF V600K mutation.

Eleven melanoma cell lines of uveal origin were screened. Consistent with previous reports [27, 28], mutations in GNAQ (3/11, 27%) and GNA11 (2/11, 18%) were mutually exclusive. With one exception, TB1, a cutaneous melanoma metastasis, mutations of GNAQ and GNA11 were identified only in tumors of uveal origin. Three uveal melanomas (27%) had mutations in BRAF, including cell line OCM1, which has been documented [29, 30]. No mutations were detected in approximately 18% of all samples (48/271). A complete list of results is provided in Supplementary Table 3.

All mutations identified in the confirmation panel were included in the final version of the melanoma-specific mutation panel (Table 3). Forty-six assays interrogating 39 mutations in 20 genes were designed into 8 wells using strict assay design parameters optimised for sensitive mutation allele detection. The final version of the melanoma-specific mutation panel was re-tested on all samples and all mutations identified in the confirmation panel were re-identified.

Replication and concordance between panels/mutant to normal allele peak ratios

To assess the accuracy of the final melanoma-specific mutation panel compared to the OncoCarta® panel, the mutant allele frequencies obtained with each panel were compared.
There was complete concordance between the mutations identified, and overall, the mutant allele ratios did not significantly differ \((p=0.118, \text{ Students paired T test})\) (Table 1). A wide range of mutant allele peaks was detected in the complete cohort of samples, ranging from 5% to 100%. Tumor samples generally had lower mutant allele peaks than cell lines (Figure 2). This effect is likely due to the presence of contaminating stromal cells in the tumor specimens.

Several cell lines in the series tested had either additional clones or matched tumor samples that were also screened with the melanoma-specific mutation panel. Cell lines with duplicate clones had 100% concordance in mutations identified \((n=21)\). Fourteen of 16 cell lines \((87.5\%)\) had comparable mutation profiles when compared to their matched tumor samples. The remaining two cell lines simply differed in mutant to normal allele frequency ratios from their parent tumors (Supplementary Table 4).
Discussion

Recent success in molecular-based targeted therapies has provided new avenues for the treatment of a wide variety of cancers based on the mutational profiles of the tumor. This approach is exemplified by the clinical efficacy observed in recent trials using RAF and tyrosine kinase inhibitors [7, 17, 18]. The success of this strategy relies heavily on the stratification of patients based on the spectrum of mutations harbored within their tumor. For effective use in a clinical research setting the identification of mutations that confer drug sensitivity and absence of those that confer drug resistance in an accurate, high throughput and timely manner is highly desirable. Here we describe the development of a melanoma-specific mutation panel that can rapidly and accurately identify clinically-relevant mutations in metastatic melanoma.

A very high concordance between the melanoma-specific panel and OncoCarta® panel was observed when comparing mutation and allele frequencies, indicating the re-multiplexing process required to generate the melanoma-specific panel did not affect its reproducibility. High concordance was also observed between matched tumor and cell line samples; only 2 of 16 samples had slight differences in mutation profiles, possibly indicating clonal selection during cell line generation. Alternatively, the disparity may result from stromal contamination in the tumor samples leading to an observed difference in mutant normal allele ratios. One benefit of using the Sequenom MassARRAY® system for mutation analysis is the quantification of mutant to wild type allele ratios, a key feature for estimating mutant sub-populations of cells. The platform allowed detection of samples with mutant allele frequencies at 10%. However, one mutation was reliably detected as low as 5% (MM595-Tumor) with the inclusion of a number of duplicates. A skewing towards lower mutant allele peaks was observed in excised human tumors when compared to cell lines, consistent with stromal contamination. Sensitive mutation detection is an important feature in a clinical
setting as a high degree of admixture of cell populations within a tumor has the potential to
disguise mutation events critical to choice of therapy.

In an attempt to reduce cost while also maintaining high throughput analysis, a confirmation
panel of highly ranked mutations was initially screened in 271 melanoma samples in order to
identify frequently occurring mutations. As the majority of mutations selected for the
confirmation panel have only been identified in single tumors, screened in small numbers of
samples, and not replicated in independent cohorts of samples, it is possible a number of
these events may be patient-specific mutations or passenger mutations not responsible for
melanoma development. This is demonstrated by a high proportion of mutations identified in
a large-scale sequencing screen of the protein tyrosine kinase gene family [31] that were not
identified here in 271 melanoma samples during the confirmation stage (n=50 or 85%).

The final melanoma-specific panel consisted of mutations identified within the confirmation
stage or that have direct clinical significance to current or novel molecular-based targeted
therapeutics. The clinical utility of these mutations is highlighted in Figure 3. Not surprisingly,
a high rate of BRAF V600E mutations was observed in patients for whom BRAF inhibitor
treatment would represent an appropriate treatment strategy. Although not all mechanisms
of RAF inhibitor resistance can be detected using the melanoma-specific mutation panel,
such as upregulation of COT or receptor tyrosine kinases [13], a number of interesting
mutations that could potentially confer resistance to inhibitors of the MAPK pathway were
identified, as detailed below.

An early report investigating mechanisms of resistance to inhibitors of the MAPK pathway
identified a P124L MEK mutation from a resistant metastatic tumor in a patient treated with a
MEK inhibitor, AZD6244, which was not present before treatment [11]. Ex-vivo functional
analysis revealed the mutation was responsible for acquired resistance of AZD6244
treatment and likely explained drug resistance in this patient. Interestingly, the P124L mutation showed cross-resistance to PLX4720 in-vitro, indicating MEK mutations may be clinically-relevant to RAF inhibitor treatment. Screening for the MEK P124L mutation with the melanoma-specific mutation panel revealed mutations in 3 samples, one of which had an additional BRAF V600K mutation (D28). This suggests that MEK P124L mutations occur in a small proportion of patients with metastatic melanoma and possibly represents an alternative mechanism to the activation of the MAPK pathway apart from commonly observed BRAF and NRAS mutations.

Although some clinical trials of recent BRAF inhibitors have enrolled only melanoma patients with BRAF V600E mutations, there is evidence of positive drug response for V600K mutated tumors in-vitro and in-vivo [7, 32, 33]. If V600K mutated tumors are susceptible to modern BRAF inhibitors, as is suggested by results of an early phase clinical trial of GSK2118436 [34], screening for additional clinically-relevant mutations within the tumor through mutation panels may be important for overall response to selected therapies. The sample with both BRAF V600K and MEK P124L is an interesting example in which screening only for the BRAF mutation would not have identified the ideal treatment strategy: a BRAF inhibitor would likely be ineffective due to the co-occurrence of a MEK mutation.

Recently, mutations in GNAQ and GNA11 at amino acid residues R183 and Q209 have been identified in up to 83% of uveal melanomas [27, 28]. Mutations in GNAQ and GNA11 occurred here only in cell lines of uveal origin except for a GNA11 R183C mutation identified in a cutaneous, superficial spreading melanoma with a nodular component (TB1). As far as we are aware, this is the first documented GNA11 mutation in a non-uveal melanoma. From a clinical aspect, initial reports indicate that tumors with mutations in GNAQ and GNA11 are likely to be responsible for the activation of the MAPK pathway and may potentially be susceptible to treatment with MEK inhibitors [27]. However, GNAQ and GNA11 mutant uveal
cells showed mild sensitivity to MEK inhibitors and complete resistance to BRAF inhibitor treatment *in vitro* [35].

An emerging treatment regimen in KIT-mutated melanomas is through use of tyrosine kinase inhibitors such as Imatinib [17]. TB349 was the only sample harboring a KIT mutation (W557R) in our series. The low rate of KIT mutations observed in this dataset is possibly due to insufficient coverage provided by the melanoma-specific mutation panel, compared with the wide variety of mutations reported to activate this gene [36]. However, the most frequently documented mutations identified in KIT in melanoma were included in the final panel. Acral and mucosal melanomas are the subtypes most likely to harbor mutations in KIT (10-15% of cases); however, the frequency of KIT mutations in melanomas occurring in chronically sun-exposed anatomic sites is probably much lower than reported in earlier studies [37]. This interpretation is supported by our results because while our cohort contained only 2 acral and 1 mucosal melanomas, one third of the melanomas in the MIA subset arose from primary melanomas with high sun damage scores or from chronically sun-exposed body sites.

Platelet-derived Growth Factor Receptor A (PDGFRA) mutations occur in a mutually exclusive pattern to KIT in 5-10% of gastro-intestinal stromal tumors (GIST) and is a therapeutic target for Imatinib [38]. The most frequent mutation of PDGFRA in GIST occurs at D842V and shows both *in-vitro* and *in-vivo* resistance to Imatinib; however it is important to note that alternative mutations in PDGFRA are Imatinib responsive and represent a viable treatment strategy [38-40]. Although early reports failed to identify PDGFRA mutations in melanoma [41, 42], recent studies indicate mutations in PDGFRA do occur but represent rare events [31, 43, 44]. The melanoma-specific mutation panel revealed a PDGFRA E996K mutation in MM648 that had been previously detected in another sample and may represent a mutation hotspot [43]. Although a rare event in melanoma, the identification of PDGFRA
mutations warrants further investigation because of its likely prediction of Imatinib sensitivity in this subset of patients.

Screening of the protein tyrosine kinase gene family in cutaneous metastatic melanoma revealed that novel mutations of ERBB4 occurred in 19% of patients and led to increased kinase activity \textit{in vitro} \cite{42}. Although a majority of these mutations were tested in the confirmation panel (12 of 20 mutations), R393W and E452K were the only mutations in ERBB4 identified in the series of samples. This suggests that a majority of ERBB4 mutations may be rare or patient-specific mutations, or alternatively occur throughout the entire gene instead of in localised regions. However, the identification of E452K in 3 samples in this study suggests that E452 is a likely mutation hotspot in ERRB4, occurring in a small proportion of melanomas. This has clinical significance as \textit{in-vitro} analysis revealed ERRB4 E452K-mutated cell lines had 10-250-fold enhanced sensitivity to Lapatinib, a tyrosine kinase inhibitor of the HER2 growth receptor pathway \cite{42}. Although the significance of ERBB4 in the development and progression of melanoma has yet to be confirmed \textit{in-vivo}; ERBB4 mutations represent rational therapeutic targets for ERBB receptor inhibitors in a subset of patients.

Cyclin-dependent kinase 4 (CDK4) is an important kinase responsible for cell cycle regulation and is a high penetrance melanoma predisposition gene mutated in a small number of families’ worldwide \cite{45}. In addition, a small number of CDK4 somatic mutations have been identified in melanoma tumors and cell lines; the majority of which result in a cysteine or histidine substitution of an arginine residue at position 24 \cite{46}. Mutations occurring at this position abrogate the binding of inhibitor p16 and lead to constitutive kinase activity of CDK4 and cell cycle progression \cite{47}. The melanoma-specific mutation panel revealed 8 samples with either R24C or R24H mutations in CDK4 and was the most
commonly mutated gene after BRAF and NRAS. Interestingly, all of these samples also harboured BRAF V600E mutations.

Although the CDK4 mutations identified here have not been determined as somatic events in the samples tested, the recurrence of these mutations suggests an important role in melanoma progression and possible clinical significance in a small number of patients. This subset of tumors may be susceptible to a CDK4 inhibitor strategy of which a number of drugs are currently under development, or in clinical trials. Evidence of this approach was observed in a phase I clinical trial of a CDK modulator UCN-01 which resulted in a partial response in a patient with metastatic melanoma [48]. Although a larger phase II trial of UCN-01 in a cohort of 17 patients with metastatic melanoma did not reveal significant disease progression; the spectrum of mutations occurring within these patients was not investigated [49]. It is interesting to speculate about the possibility of CDK4 inhibitor drugs such as UNC-01 being most effective in tumors harbouring CDK4 mutations, although this has yet to be investigated experimentally. Lastly, the presence of CDK4 mutations in BRAF V600E mutated cell lines did not confer resistance to BRAF inhibitors \textit{in-vitro}; however one report investigating a combination approach of CDK4/MEK inhibitors in BRAF V600E/p16 negative cell lines showed a significant increase in apoptosis compared to a respective mono-therapeutic strategy [50]. Further investigation into the clinical significance of CDK4 mutations and pharmacological treatment strategies in metastatic melanoma patients is warranted.

In conclusion, we describe here the development and validation of a melanoma-specific mutation panel that can rapidly and accurately determine the molecular profiles of tumors in a cost-effective and high throughput manner. Utilising a mass spectrometry approach on the Sequenom MassARRAY® system is not only advantageous in terms of minimizing price and maximizing speed but also provides a highly flexible format that will facilitate the addition of
novel mutations influencing therapeutic patient response as they are identified. This will be of particular importance with the application of next generation sequencing in identifying mutations associated with the development of melanoma or drug resistant mutations acquired during treatment. The melanoma-specific mutation panel also allows for sensitive allele detection important for the analysis of tumor samples where contaminating stromal cells may affect the mutant wild type allele ratio. Finally, the panel can quickly identify mutation profiles in patient samples and may be useful in a clinical setting to determine effective treatment strategies using molecular-based targeted drugs.
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References


    KIT mutations in ocular melanoma: frequency and anatomic distribution. Mod Pathol
    2011; 24: 1031-5

    Germline mutations in the p16INK4a binding domain of CDK4 in familial melanoma.

    A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human

47. Coleman, K.G., B.S. Wautlet, D. Morrissey, J. Mulheron, S.A. Sedman, P. Brinkley,
    et al., Identification of CDK4 sequences involved in cyclin D1 and p16 binding. J Biol

    al., Phase I trial of 72-hour continuous infusion UCN-01 in patients with refractory

49. Li, T., S.D. Christensen, P.H. Frankel, K.A. Margolin, S.S. Agarwala, T. Luu, et al.,
    A phase II study of cell cycle inhibitor UCN-01 in patients with metastatic melanoma: a
    California Cancer Consortium trial. Invest New Drugs 2010; in press.

50. Li, J., M. Xu, Z. Yang, A. Li, and J. Dong, Simultaneous inhibition of MEK and CDK4
Table 1: Comparison between mutations identified using the OncoCarta and melanoma-specific mutation panels. WT%: percentage wild type allele, MUT%: percentage mutant allele. Dashes identify samples with no mutations.

<table>
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<th>OncoCarta Mut %</th>
<th>Melanoma-specific WT %</th>
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Table 2: Summary of mutations detected with the melanoma-specific mutation panel.

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<th>GENE</th>
<th># samples</th>
<th>Percentage (%)</th>
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<tr>
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<td>1.10</td>
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<tr>
<td>GNA11</td>
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<td>CTNNB1</td>
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<tr>
<td>KRAS</td>
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<td>Wild Type</td>
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Table 3: Mutations included in final version of the melanoma-specific mutation panel (39 in total). Asterisks indicate multiple amino acid changes detected.

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<th>Gene</th>
<th>Mutation (AA)</th>
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<td>KIT</td>
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<tr>
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<td>KIT</td>
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Figure Legends

**Figure 1:** Filtering process for selecting mutations for the melanoma-specific panel. From a list of approximately 1000 mutations, the panel was narrowed down to 39 mutations to allow for cost-effective, high throughput screening. Mutations that occurred at high frequency and with clinical significance were prioritised for inclusion in the final melanoma-specific mutation panel.

**Figure 2:** Mutant allele frequencies detected between cell line and tumor samples. The mutant allele frequency of samples is grouped on the X axis in 20% increments while the Y axis shows the overall percentage of samples.

**Figure 3:** Commonly affected genes and pathways in melanoma. A number of mutations in these genes are included in the melanoma-specific mutation panel (highlighted in green) and have therapeutic targets that have been or are currently under clinical investigation (denoted under gene name). Oval boxes represent oncogenes while rectangular boxes represent tumor suppressor genes.
Figure 1: Filtering process for melanoma-specific mutation panel

Compiled a list of mutations occurring in melanoma from 3 different sources:

1. Mutations from literature reports
   - GNA11: Van Raamsdonk et al. NEJM, 2010
   - MEK: Emery et al. PNAS, 2009
   - MITD: Cronin et al. Pigm Cell Mel Res, 2009
   - MMP: Palavalli et al. Nat Genet, 2009
   - MMP: Bleeker et al. Hum Mutat, 2009

2. COSMIC Database
   - Sample Mutations
     - LB2515-MEL: 16
     - COLO-829: 32
     - MZ7-MEL: 57
     - CP66-MEL: 61

3. Mutations identified using Sequenom OncoCarta Panel
   - BRAF, NRAS, KIT, CDK4, PIK3CA, MET

Silent mutations removed. Duplicate mutation data removed/merged.

Mutations selected for final screening according to them:
- occurring in 1% of all samples tested
- occurring in genes encoding kinases
- causing a charge change in the substituted amino acid
- not occurring in known tumor suppressor genes

Any mutation not identified in the samples screened were removed. Mutations with known clinical and functional significance included.
Figure 2

Overall percentage of samples tested vs Mutant Allele Frequency within an individual sample for Cell Line and Tumour.

- Cell Line: Solid line
- Tumour: Dashed line

The graph shows the distribution of mutant allele frequencies with the highest percentages occurring at lower frequencies, with a peak around 0.6 for Cell Line and around 0.4 for Tumour.

[Graph showing the distribution of mutant allele frequencies with peaks at 0.6 for Cell Line and 0.4 for Tumour]
Figure 3

Affected proliferation, cell division, apoptotic, invasive and metastatic pathways

GNAQ
GNA11
AZD6244?

PLX4032
Sorafenib

PLX4032
Sorafenib

Imatinib

RAS

BRAF

PTEN

GNAQ
GNA11
AZD6244?

MEK

AZD6244

RAS

ERBB4

Lapatinib

RAS

KIT

MET

PDGFRA

Imatinib?

BRAF

MEK

ERK

AKT

PTEN

CDK4

CDKN2A

UCN-01?

RB1

PDGFRA

Figure 3 on June 25, 2017. © 2012 American Association for Cancer Research. mct.aacrjournals.org Downloaded from
A high throughput panel for identifying clinically-relevant mutation profiles in melanoma

Ken Dutton-Regester, Darryl Irwin, Priscilla Hunt, et al.

Mol Cancer Ther Published OnlineFirst March 1, 2012.

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