PP6C Hotspot Mutations in Melanoma Display Sensitivity to Aurora Kinase Inhibition

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Running Title: PP6C mutations in melanoma

Key Words: melanoma, PP6C, Aurora Kinase

Financial Support: Supported by the NYU Cancer Institute Center Support Grant (5P30CA016807-27) and the Marc Jacobs Campaign to support melanoma research. LBG is the Saul J. Farber Associate Professor of Medicine.

Conflict of Interest: None

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Word Count: 2492

Figure Count: 3
ABSTRACT

Recent whole genome melanoma sequencing studies have identified recurrent mutations in the gene encoding the catalytic subunit of serine/threonine phosphatase 6 (PPP6C/PP6C). However the biochemical, functional, and clinical ramifications of these mutations are unknown. Sequencing PP6C from melanoma patients (233 primary and 77 metastatic specimens) with extended prospective clinical outcome revealed a large number of hotspot mutations in both primary and metastatic melanoma patients. Despite minimal association between stage and presence of PP6C mutations in primary patients, a subpopulation of cells within each tumor did contain PP6C mutations, suggesting PP6C mutation is an early, but non tumor-initiating event in melanoma. Among primary patients with PP6C mutations, patients with stop mutations had significantly shorter recurrence-free survival compared to patients without stop mutations. In addition, PP6C mutations were independent of commonly observed BRAF and NRAS mutations. Biochemically, PP6C mutations could be classified as those that interact with PP6C regulatory subunits and those that do not. Mutations that did not bind to PP6C regulatory subunits were associated with increased phosphorylation of Aurora kinase, a PP6C substrate, and mitotic defects. However, both classes of PP6C mutations led to increased sensitivity to Aurora kinase inhibition. Together, these data support for the first time that PP6C mutations are molecularly, biochemically, and clinically heterogenous.

Implications: PP6C mutations have distinct functional and clinical consequences in melanoma, and confer sensitivity to Aurora A kinase inhibitors.
INTRODUCTION

Genomic tumor sequencing has increasingly identified tumor specific genes and is altering the approach to cancer investigation. Recent whole exon sequencing studies in melanoma have identified several recurrently mutated genes, including the serine/threonine phosphatase PP6C (1-2). PP6C encodes for the catalytic subunit of the PP6 serine threonine phosphatase complex and specifically associates with three regulatory proteins, PP6R1, PP6R2, and PP6R3, which bridge PP6C with its substrate proteins (3). PP6C has been reported to play an important role in several cancer relevant pathways, including the prevention of aneuploidy via the regulated dephosphorylation of the essential mitotic kinase, Aurora A (4). Although PP6C mutations have been predicted to result in a loss of PP6C activity and to be a driver mutation in melanoma, these non-biased discovery studies, by their very nature, were performed on a limited number of patients and did not focus on the biological or clinical consequences of PP6C mutations in melanoma. With the increasing appreciation that even tumor specific mutations may simply be passenger mutations without functional implications (5-6), we sought to characterize the biochemical, functional and clinical role of PP6C mutations in melanoma using a large representative tumor cohort linked to extensive prospectively collected clinical data.
MATERIALS AND METHODS

Study population and tissue sequencing of PP6C, BRAF, and NRAS. Clinicopathological information linked to biospecimens from prospectively enrolled patient with melanoma, as previously described (7), was utilized to randomly identify 91 stage I, 71 stage II, 70 stage III primary melanoma patients. We also identified 77 stage IV metastatic patients in which we characterized PP6C mutations, but analyzed for clinical outcomes separately. For primary patients, the median overall survival was 9 years (95% CI: 6.7-16.5 years). Chromatograms, obtained by Sanger sequenced (8) using primers available upon request, were scanned manually for mutations, defined as a chromatogram peak greater than 5%, seen on both forward and reverse sequences absent in the reference protein derived from PP6C variant 2 mRNA (NM_002721).

Cell culture and generation of PP6C mutant cell lines. U20S, MEL501, 293T cells, and additional melanoma cell lines (gift of E. Hernando) were maintained as previously described (9). PP6C was depleted by pLKO.1 shPP6C (TRC0000002764, Sigma). PP6C, PP6 regulatory subunits, and PP6C mutants were generated by standard PCR or PCR mutagenesis with cloning primers available upon request, cloned into the selectable retrovirus plasmid pQXCIN, and virus was generated and cells infected as previously described (9).

Immunoblots, immunoprecipitation and immunofluorescence. To assess Aurora A Kinase phosphorylation, mitotic cells were collected as (4) and immunoblots were performed as previously described (9). Fluorescent secondary antibodies were used and intensities of bands were determined with a LiCor Odyssey imaging system. Antibodies used include anti-Tubulin (Sigma), Flag (Sigma), Phospho-Aurora A (Thr288) and Aurora A mouse antibodies (Cell Signalling Technology). For immunoprecipitations, Flag tagged regulatory PP6 regulatory subunits and PP6C or PP6C mutants were transfected into 293T cells with calcium-phosphate, and PP6Rs were immunoprecipitated using anti-FLAG beads (9). For immunofluorescence cells were synchronized with a double thymidine block, fixed with cold methanol for 10 minutes, and imaged as previously reported (10).

Assessing sensitivity of PP6C mutant cells to Aurora Kinase Inhibitors. Cells were treated with 400 nM of the Aurora Kinase inhibitors, VX-680 (Tozasertib), MLN8237 (Alisertib), Danusertib, and ZM447439 (Selleck Chemicals) for 72 hours and viability was assessed using MTT Cell Proliferation Assay Kit (Life Technologies).
Serial dilutions were piloted to select a concentration minimally toxic in control melanoma cell lines over 72 hours; much greater toxicity was noted with a five to ten-fold increase in drug concentrations.
RESULTS

PP6C mutations are recurrent and occur in the presence and absence of BRAF and NRAS mutations.

We sequenced PP6C in 233 primary and 77 metastatic melanoma specimens. There were 39 PP6C mutations in 25 primary melanoma patients and 11 in eight metastatic patients. 30 of these patients had corresponding germ line DNA samples which, when sequenced, confirmed PP6C mutations to be a somatic event. A majority of mutated tumors had one PP6C mutation, but eight primary patients and two metastatic patients had greater than one PP6C mutation (Supplemental Table 1). There was no association between stage and presence of PP6C mutations in primary patients, suggesting that the mutation of PP6C can be an early event in the development of melanoma (Supplemental Table 1). Mutations clustered in distinct regions of the gene, and 8 mutations were recurrent (Figure 1A). The most common recurrent mutation was PP6C R264C, which occurred in 6/25 (24%) primary patients. There were seven primary patients with a single stop mutation and two metastatic patients with stop mutations (including one with two stop mutations). These stop mutations were recurrent and located within a nine amino acid segment from amino acid 205 to 214 (Figure 1A). When combined with previously described mutations (1-2), 42 amino acids have been described to be mutated in melanoma, 14 of which are recurrent (Supplemental Table 1).

We also sequenced all tumors for both BRAF and NRAS. BRAF V600E mutations were found in 102/233 (44%) primary patients and 40/77 (52%) metastatic patients. NRAS Q61 mutations were found in 56/233 (24%) primary patients and 18/77 (23%) metastatic patients, consistent with previously recorded frequencies (8). In primary patients 14/24 (58%) of the tumors with PP6C mutations were wild-type for BRAF and 4/8 (50%) metastatic patients with PP6C mutations were wild-type for BRAF. In primary patients with PP6C mutations, 7/24 (29%) were wild-type for both BRAF and NRAS and in metastatic patients, 2/8 (25%) were wild-type for both BRAF and NRAS. We found no association between PP6C mutation and BRAF/NRAS mutations in either primary tumors or in metastatic tumors (Supplemental Table 2). Together these data suggest that recurrent PP6C mutations are not a rare event in melanoma, cluster in distinct regions of the PP6C protein, and occur regardless of BRAF/NRAS mutational status.
Mutations resulting in a premature stop codon associate with a poor prognosis. PP6C mutations did not associate with most known prognostic variables except tumor thickness in patients with primary tumors (Supplemental table 3). In the 233 sequenced stage I-III primary tumors, 25 were PP6C mutated. Strikingly 6/7 (86%) of patients with premature stop codons in PP6C recurred and developed visceral metastases, including two patients who presented in stage I, one patient who presented in stage II, and three patients who presented in stage III. Those patients with stage I-III with tumors harboring premature stop codons progressed significantly more rapidly than those with other PP6C mutations (Log-rank p-value=0.044) (Fig. 1B) and there was a trend for worse overall survival (Log-rank p-value= 0.06). These data indicate a clinical heterogeneity amongst PP6C mutations and suggest that PP6C stop mutations may confer a distinct clinical prognosis.

PP6C mutations are heterozygous and occur in a subpopulation of tumor cells. We next sought to understand some of the biological consequences of PP6C mutations. We first examined the percentage of PP6C mutated alleles found in each tumor, as a high ratio of mutated allele to wild-type allele, or a loss of heterozygosity (LOH) associated at the PP6C allele, is more consistent with loss of function mutations. We observed a wide range of PP6C mutated:wild-type allele ratios in tumors, but almost all ratios were below 50% (Fig 2A and Supplemental Table 4). In 22 tumors with single PP6C mutations, this ratio ranged from 6% to 50% and averaged 24%, and in the 11 tumors with multiple PP6C mutations the aggregate mutated:wild-type allele ratios ranged from 25% to 130% with an average of 51%. In those PP6C mutated tumors which also harbored BRAF or NRAS mutations, the BRAF/NRAS mutated:wild-type allele ratio averaged ~50% as expected for an activating mutation, thus excluding normal tissue contamination in the sequenced macrodissected section. Allele burdens of select mutations were confirmed by Sequonome (Mass Spec) sequencing (Fig 2B).

Although any mutated:wild type allele ratio below 50% indicates that the mutation is found in a subpopulation of tumor cells, we next sought to distinguish whether this subpopulation contained homozygous or heterozygous PP6C mutations. We screened 25 melanoma cell lines and identified two cell lines with PP6C mutations (WM853 with PP6C R177L and SKMEL100 with PP6C R264C). When we isolated RNA from these
cell lines, generated cDNAs, and sequenced these, we found a 1:1 ratio of mutated PP6C and wildtype PP6C in both cell lines. We then established single cell clones from one of these cell lines, and again found an allele burden of 50% in each clone (Fig 2C). Because sequencing was performed on reverse transcribed mRNA isolated from these cells, these studies eliminate the possibility that a non-mutated PP6C allele is epigenetically silenced in these cells. The lack of homozygous mutations in melanoma suggests that either PP6C haploinsufficiency plays a role in melanoma, or that PP6C mutations may exhibit activating and/or dominant negative functions.

Distinct PP6C mutants are unable to bind PP6C regulatory subunits. To further explore the biochemical consequence of PP6C mutations we assessed the ability of 12 PP6C mutants, selected by their frequency, conservation of involved amino acids, and distribution throughout the PP6C gene, to form a functional PP6 complex. When PP6C mutants and FLAG-tagged PP6C regulatory subunits were co-transfected into 293T cells, mutations could be grossly divided into two distinct groups: those that bound regulatory subunits, and those that did not (Fig 3A). Binding of specific PP6C constructs was consistent among the three regulatory subunits (PP6R1, PP6R2, and PP6R3) (data not shown). Interestingly, the common PP6C R264C mutant was able to bind all regulatory subunits normally. Thus a subclass of PP6C mutations, though not all, disrupts regulatory subunit binding.

Distinct PP6C mutations increase levels of active phosphorylated Aurora A and sensitizes cells to Aurora A Kinase inhibition. Next we examined the functional implications of these two distinct classes of PP6C mutants. We elected to focus on the well established role PP6C plays on the dephosphorylation of the Aurora Kinase A to regulate mitosis, which has recently been reported to be disrupted by the PP6C H114Y mutation (4, 11). To first study the intrinsic activity of each mutant, we generated cell lines in which we expressed individual PP6C mutants while concomitantly depleting endogenous PP6C stores using a shRNA targeting the 3’UTR of PP6C, a sequence not found in our PP6C constructs. The depletion of PP6C led to an increase of Aurora A kinase phosphorylation in mitotic cells (Fig 3B). This increased phosphorylation was reversed to the level of unmanipulated control cells with the expression of wild-type PP6C. We then studied
the Aurora A kinase phosphorylation status in cells expressing only mutant PP6C. Of the 12 mutations studied, five resulted in increased Aurora A Kinase phosphorylation to an extent similar to what was seen when PP6C was depleted, despite the expression of mutant PP6C to levels similar to wild-type PP6C (Fig 3B). Mutants predicted to lead to a kinase dead PP6C (D53N) led to increased Aurora A kinase phosphorylation, as did all the mutants that did not bind regulatory subunits (Supplemental Table 5). Intriguingly, the one mutant which bound regulatory subunits but led to increase Aurora A Kinase phosphorylation was PP6C R264C. Consistent with aberrant Aurora A Kinase activity, we noted a significant increase in micronuclei in cells expressing mutants that led to elevated Aurora A Kinase phosphorylation, when compared to other mutant expressing cells (Fig 3C).

Because many PP6C mutations are found at 1:1 allele ratio (or below) with wild-type PP6C, we then assessed Aurora A Kinase phosphorylation in cells expressing PP6C mutants in the presence of wild-type PP6C. Despite the presence of wild-type PP6C, expression of PP6C mutants still led to elevated Aurora Kinase phosphorylation, both in the melanoma cell line MEL501 and U2OS cells (Fig 3D and data not shown), suggesting that some PP6C mutants not only lack the ability to form an active phosphatase complex and dephosphorylate Aurora A Kinase, but also act as dominant negatives to suppress PP6C activity from wild-type PP6C.

Aurora Kinases are often hyperactivated in cancer, and Aurora Kinase inhibitors are being investigated therapeutically in a number of malignancies. We chose a selective Aurora A Kinase (Alisertib) a pan-Aurora inhibitor (Tozasertib and ZM-447439) and an inhibitor with a IC50 for Aurora Kinases that is 2 fold lower than for other kinases (Danusertib). After assessing a wide range of concentrations, we found that 400 nM of these four unique Aurora A Kinase inhibitors had limited toxicity in control MEL501 cells or in cells expressing wild-type PP6C but resulted in a marked decrease in viability in cells expressing mutated PP6C (Fig 3E). This sensitivity of MEL501 cells expressing PP6C mutants to Aurora A Kinase inhibition was observed even with those mutants that did not lead to increase Aurora Kinase Activity, suggesting these mutants may alter other pathways which can affect mitosis/proliferation and render cells more sensitive to Aurora Kinase inhibitors.
DISCUSSION

Although PP6C is mutated in 10% of melanoma, our data indicate, for the first time, that PP6C mutations in melanoma are heterogeneous, with varied impact on the biochemical and functional activity of PP6C, and with diverging clinical impact. We find that while PP6C mutations occur across all stages of melanoma, they are present within a subpopulation of tumor cells and thus unlikely to drive melanoma transformation. Indeed, we have noted that the over-expression of several PP6C mutants does not confer anchorage independent growth to immortalized melanocytes (data not shown). The presence of intra-tumor heterogeneity is a well known phenomenon in many cancers, including melanoma (12-13).

Novel approaches in melanoma therapy will likely develop from a better understanding of its molecular biology. For example, although BRAF mutational status has not been shown to have significant prognostic relevance, and activated BRAF is not a tumor initiating mutation, patients with BRAF mutated tumors are initially highly responsive to BRAF inhibition (13-14). In fact our data indicate that the presence of some PP6C mutations indicate a poor prognosis which, if confirmed, may help inform decisions regarding adjuvant therapy for early stage melanoma. Aberrant phosphorylation of PP6C substrates, including H2AX and IκBε (15-17), in PP6C mutated tumors may also lead to new targeted approaches. Our data suggests that the PP6C substrate Aurora A Kinase may be one such target. Potential therapeutic implications of PP6C mutations are strengthened by the observation that PP6C mutations occur in BRAF wild-type patients, a population with limited targeted therapeutic options. Of note, our identification of PP6C mutations in BRAF/NRAS wild-type cells differs from previous reports (1-2), presumably because these previous studies primarily sequenced tumor derived short term cultures (susceptible to clonal selection) from advanced staged, previously treated patients, the majority of whom had BRAF/NRAS mutations. The lack of either a positive or negative association between BRAF/NRAS and PP6C mutations suggests that the pathway(s) affected by PP6C mutations differ from those affected by BRAF/NRAS, though this demands formal investigations.

The clustering of PP6C mutations, and specifically the high recurrence of the PP6C R264C point mutation, are suggestive of activating mutations, as seen with classical oncogenes. This would be consistent with the
observation that PP6C is over-expressed in glioblastomas (18). However, we also found PP6C mutations in its catalytic domain, as well as several mutations which disrupt PP6 complex formation and blunt phosphatase activity, suggesting that some PP6C mutations may result in a loss of function, typical for a tumor suppressor. Some of the “loss of function” mutations which lead to PP6C proteins which cannot bind to regulatory subunits lead to increased Aurora A Kinase phosphorylation even in the presence of wild-type PP6C, indicating that these PP6C mutations may act as dominant negatives to suppress the activity of endogenous wild-type PP6C. Still other mutants are able to bind PP6C regulatory subunits and have no effect on Aurora A Kinase phosphorylation, although they appear to still activate a pathway that sensitizes cells to Aurora A Kinase inhibition. Thus there appears to be a marked heterogeneity amongst PP6C mutations. It is well established that different mutations in a single gene can result in distinct phenotypes. For example, there is a strong genotype-phenotype correlation in Von-Hippel-Lindau gene mutations such that the location of the mutation site determines the type of syndromes that arise (19). Similarly, both activating and inactivating mutations in PTPN11, encoding the SHp2 phosphatase, can lead to similar but non-identical, clinical syndromes (20). Because of the significant heterogeneity amongst PP6C mutants, the in depth study of individual PP6C mutations may provide further insights into melanoma biology and lead to better prognostication and therapies in melanoma.
References


Figure legends

**Figure 1.** PP6C is recurrently mutated in melanoma. A. 310 melanomas were sequenced for PP6C. Somatic mutations are represented in closed circles, with mutations resulting in premature stop codon in open circles. B. Recurrence free survival in stage I-III primary patients with PP6C stop mutations, PP6C wild-type, and PP6C non-stop mutations.

**Figure 2:** PP6C allele burden in BRAF/NRAS mutated melanoma. A. Allele burdens of PP6C and BRAF/NRAS in tumors with one PP6C mutation or B. >1 PP6C mutation. B. Mass Spec analysis of a select mutation. C. Chromatogram of PP6C R264C mutation in single cell clones from SKMEL100.

**Figure 3:** PP6C mutations are biochemically and functionally heterogenous, and sensitize cells to Aurora Kinase inhibitors. A. PP6C or PP6C mutants, and PP6R3 were co-transfected into 293T cells, PP6R was immunoprecipitated and the intensity of PP6C co-immunoprecipitated, assessed by fluorescence, is indicated as % input. Gels are representative of three biological replicate experiments. B. U2OS cells were depleted of PP6C and either empty vector, wild-type, or mutated PP6C was expressed. Mitotic cells were harvested and Aurora A Kinase phosphorylation was assessed, as described in the text. Intensity of phosph-Aurora A Kinase, determined by fluorescence, is indicated as fold (compared to control cells). Experiments were repeated three times and representative gels are shown. C. Cells were stained with DAPI and micronuclei and were assessed and quantitated. * = p<0.05 by Student’s T test. D. Aurora A Kinase phosphorylation was assessed in MEL501 cells expressing wild-type or mutant PP6C. E. MEL501 cells were infected with wild-type or mutated PP6C, treated with DMSO or the Aurora Kinase inhibitors indicated at 400 nM for 72 hours, and viability assessed by MTT assay. Experiments were performed in duplicates, with average + standard error displayed. * = p< 0.05 by Student’s T Test.
A. % mutant allele/wild-type allele

| PP6C mutant allele ratio | BRAF/NRAS mutant allele ratio | PP6C mutant allele ratio | BRAF/NRAS mutant allele ratio |

B. 

C. 

Fig 2
Molecular Cancer Research

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Mol Cancer Res Published OnlineFirst December 12, 2013.

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