Integrated Molecular and Clinical Analysis of AKT Activation in Metastatic Melanoma

Michael A. Davies,1,2 Katherine Stemke-Hale,2 E. Lin,3 Carmen Tellez,8 Wanleng Deng,1 Yennu N. Gopal,1 Scott E. Woodman,4 Tiffany C. Calderone,5,6 Zhenlin Ju,3 Alexander J. Lazar,7 Victor G. Prieto,7 Kenneth Aldape,7 Gordon B. Mills,2 and Jeffrey E. Gershenwald5,6

Abstract

Purpose: Activation of the phosphoinositide-3-kinase (PI3K)-AKT pathway has been implicated in melanoma based primarily on the prevalence of mutations in PTEN and NRAS. To improve our understanding of the regulation and clinical significance of the PI3K-AKT pathway in melanoma, we quantitatively measured the levels of phosphorylated AKT, its substrate GSK3α/β, and its negative regulator PTEN in clinical metastases. Results were compared with mutational status, clinical outcomes, and sites of metastasis.

Experimental Design: DNA and protein were isolated from dissected frozen melanoma metastases (n = 96). Activating mutations of BRAF, NRAS, AKT, PIK3CA, and KIT were detected by mass spectroscopy genotyping. Phosphorylated AKT (Ser473 and Thr308), P-GSK3α/β, and PTEN protein expression were measured by reverse-phase protein array. A panel of human melanoma cells lines (n = 58) was analyzed for comparison.

Results: BRAF-mutant tumors had higher levels of P-AKT-Ser473 (P = 0.01), P-AKT-Thr308 (P = 0.002), and P-GSK3α/β (P = 0.08) than NRAS-mutant tumors. Analysis of individual tumors showed that almost all tumors with elevated P-AKT had low PTEN levels; NRAS-mutant tumors had normal PTEN and lower P-AKT. Similar results were observed in melanoma cell lines. Stage III melanoma patients did not differ in overall survival based on activation status of the PI3K-AKT pathway. Brain metastases had significantly higher P-AKT and lower PTEN than lung or liver metastases.

Conclusions: Quantitative interrogation of the PI3K-AKT pathway in melanoma reveals unexpected significant differences in AKT activation by NRAS mutation and PTEN loss, and hyperactivation of AKT in brain metastases. These findings have implications for the rational development of targeted therapy for this disease. (Clin Cancer Res 2009;15(24):7538–46)

Melanoma is the most aggressive form of skin cancer. It is estimated that 68,720 patients will be diagnosed with melanoma, and 8,650 patients will die of this disease, in 2009 (1). Clinical trials of chemotherapy, immunotherapy, and biochemotherapy have failed to significantly improve outcomes in melanoma (2). Thus, new therapeutic approaches are needed. Somatic mutations in BRAF, a serine-threonine kinase that is a component of the RAS-RAF-mitogen-activated protein kinase (MAPK) signaling pathway, are detected in ~50% of melanomas (3). These mutations result in constitutive activation of BRAF, with resultant activation of MAPK in vitro (4). Unfortunately, the pan-RAF inhibitor sorafenib failed to improve outcomes in patients with metastatic melanoma (5). It is possible that the lack of efficacy was due to insufficient inhibition of the RAS-RAF-MAPK pathway, as is suggested by the recent promising, albeit preliminary, results with the more potent, selective inhibitor of mutant BRAF, PLX4032 (6). However, there is also evidence that activating mutations in BRAF alone cannot fully
explain the pathogenesis of this disease. For example, although benign nevi have no malignant potential, they harbor BRAF mutations at a rate similar to melanomas (7, 8). Mutant BRAF also failed to transform melanocytes without additional genetic lesions (9, 10). Thus, other molecules or pathways likely complement the activation of the RAS-RAF-MAPK signaling pathway in melanoma. An improved understanding of these pathways may lead to more effective therapeutic strategies.

One candidate pathway is the phosphatidylinositol 3-kinase (PI3K)-AKT cascade. NRAS, which is located upstream of BRAF in the RAS-RAF-MAPK signaling cascade, is mutated in ∼15% of melanomas (3). Similar to mutant BRAF, mutant NRAS activates the RAS-RAF-MAPK cascade. In contrast to BRAF, NRAS can also activate PI3K (11). Activation of the PI3K-AKT pathway has also been implicated in melanoma by loss-of-function mutations in PTEN, which have been detected in 10% to 30% of melanomas (12, 13). As PTEN is a lipid phosphatase that antagonizes PI3K activity, and thus inhibits PI3K-AKT signaling, loss of PTEN results in activation of the PI3K-AKT pathway in several tumor types, including melanoma (14, 15). PTEN mutations are often observed in melanomas harboring BRAF mutations, whereas NRAS mutations do not occur with either BRAF or PTEN mutations (16, 17). Thus, the combination of BRAF and PTEN mutations may mimic the effect of mutant NRAS. In addition, ∼5% of melanomas have activating mutations in PIK3CA, the catalytic subunit of the most commonly expressed form of PI3K, AKT1, or AKT3 (18–20).

Although multiple genetic events implicate the PI3K-AKT pathway in melanoma, few studies have directly assessed the activation status of this pathway in metastases, and have generally been done using nonquantitative methods (21–24). Quantitative assessment of protein expression facilitates objective comparisons between molecular and clinical characteristics and mathematical approaches to assess interactions. Direct, quantitative approaches may be particularly relevant for assessing the role of PTEN in melanoma, as PTEN expression can be lost in the absence of detectable mutations, and its functional status is more accurately assessed by quantitative measures of expression than by immunohistochemical (IHC) analysis (13, 25–27). Reverse-phase protein arrays (RPPA) are powerful tools that allow for quantitative analysis of protein expression and activation using small amounts of protein (28). Previously, we used RPPA to analyze the activation of the PI3K-AKT pathway in breast cancer (29). That study showed an expected inverse relationship between PTEN protein levels and phosphorylated (activated) AKT (P-AKT) levels in breast cancer but a surprising lack of elevated P-AKT in most tumors with PIK3CA mutations. The nonequivalence of different mutations in the PI3K-AKT pathway is also supported by concurrent mutations of PIK3CA and PTEN in endometrial cancers, and concurrent RAS and PIK3CA mutations in colon cancers (30, 31). Thus, there is a strong rationale for quantitatively analyzing the activation status of AKT in melanoma, particularly as numerous agents that target this pathway are in clinical development (32).

In this study, we used RPPA to perform a quantitative analysis of AKT activation in dissected, well-annotated melanoma clinical specimens and cell lines. We analyzed associations between AKT activation and mutational status, clinical outcomes, and anatomic sites of metastasis. Surprisingly, we observed significantly greater activation of AKT with loss of PTEN expression than with NRAS mutation in both tumors and cell lines. Our data also implicates activation of AKT in melanoma brain metastases.
normalized to the median value of each protein. The raw data were then divided by the relative-loading factor to determine load-corrected values. Logarithmic values for each protein were mean centered to facilitate concurrent comparisons of different proteins.

**Western blot analyses.** Western blot analyses were done using standard methods (29). Membranes were probed with antibodies against total AKT, P-AKT-Ser473, P-AKT-Thr308, PTEN (Cell Signaling); AKT1, AKT 3 (Upstate Biotechnology); AKT2, and ERK2 (Santa Cruz Biotechnology).

**Immunohistochemical analysis.** IHC analysis was done on 5-μm sections cut from formalin-fixed, paraffin-embedded tumor blocks (35). Slides were probed with an antibody against P-AKT-Ser473 (1:25 dilution; Cell Signaling). When indicated, IHC analysis to confirm the presence of melanoma was done with a cocktail of antibodies (“Pan-mel”) against three antigens present in melanoma (HMB45, Mart1, anti-tyrosinase) as described previously (36). All IHC analyses were reviewed by an experienced dermatopathologist (AIL).

**Mutation detection.** Extraction of DNA from tumors and cell lines was done as previously described (20). Activating mutations in BRAF, NRAS, AKT1, AKT2, AKT3, PIK3CA, and KIT (Supplementary Table S3) were detected by mass spectroscopy genotyping (20, 37).

**Statistical analysis.** Student’s t test was used to compare protein expression levels between groups. Protein-protein correlation coefficients were determined by the Pearson correlation method, and the significance of the interactions was determined by the t statistic using R software.9 Unsupervised hierarchical clustering of mean-centered protein expression values was done using Cluster 2.1 and Treeview software.10 Overall survival (OS) was defined as either the time between surgical accession (date the analyzed specimen was harvested) and last follow-up or death or the time between the original date of melanoma diagnosis and last follow-up date or death. Central nervous system (CNS) disease-free survival was defined as the time between the date of surgical accession and the date of diagnosis of CNS involvement, censored to the last negative CNS imaging. The Kaplan-Meier product-limit method was used to generate survival curves, and the log-rank test was used to assess the difference in survival rates between groups. Univariate and multivariate Cox proportional hazards regression models were used to assess the hazards ratios. All computations were done using SAS (SAS Institute) and S-plus version 8.0 (Insightful Corp.) software.

**Results**

**Technical assessment of RPPA analysis of melanoma samples.** Tumor-enriched isolates from 96 frozen melanoma samples, including 6 cutaneous tumors, 70 regional metastases (55 lymph node, 15 soft tissue), and 20 distant metastases (10 brain, 5 liver, and 5 lung), were analyzed by RPPA. We found a positive correlation between P-AKT-Ser473 levels and levels of its known substrates P-GSK3α/β (r = 0.675; P < 0.001) and P-TSC2 (r = 0.549; P < 0.001). Levels of PTEN, a negative regulator of AKT activation, negatively correlated with levels of P-AKT-Ser473 (r = -0.574; P < 0.001) and P-AKT-Thr308 (r = -0.634; P < 0.001). Similar protein-protein correlations were observed for P-AKT-Ser473 levels in proteins isolated from melanoma cell lines grown in vitro (P-GSK3α/β, r = 0.46; P-TSC2, r = 0.63; PTEN, r = -0.552; P < 0.001 for each).

To test the technical reproducibility of the analysis, eight lysates were printed on separate regions of each RPPA slide, and we determined the Pearson correlation coefficients for levels of P-AKT-Ser473, P-AKT-Thr308, P-GSK3α/β, P-TSC2, and PTEN for the matched samples (average r = 0.992). In addition, cell lysates were prepared at different times using independent shears from 10 tumors (r = 0.851 for pairs). Taken together, these results confirm that the phosphoproteins of interest were maintained during the preparation of samples and that the RPPA results are reproducible.

**AKT activation in human melanoma tumors and its correlation with activating mutations.** Tumors were assessed for the presence of activating BRAF, NRAS, AKT, PIK3CA, and KIT mutations by mass spectroscopy–based genotyping (Table 1). Thirty-nine (40.6%) of the tumors harbored a BRAF mutation, 18 (18.8%) harbored an NRAS mutation, and 37 (38.5%) were wild-type (WT) for BRAF and NRAS. One tumor with an activating BRAF mutation also had an AKT1-E17K mutation, whereas another BRAF-mutant tumor had an AKT3-E17K mutation. No PIK3CA mutations were detected. Two tumors had KIT mutations (K462E and K560D); neither tumor had a concurrent BRAF or NRAS mutation. Two tumors with indeterminate results were classified as “unknown mutation” and were excluded from the subsequent analysis.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Site</th>
<th>n</th>
<th>BRAF n (%)</th>
<th>NRAS n (%)</th>
<th>WT n (%)</th>
<th>UKN n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Local</td>
<td>Skin</td>
<td>6</td>
<td>2 (33.3)</td>
<td>0</td>
<td>4 (66.7)</td>
<td>2 (2.9)</td>
</tr>
<tr>
<td></td>
<td>LN</td>
<td>70</td>
<td>30 (42.9)</td>
<td>14 (20.0)</td>
<td>24 (34.3)</td>
<td>2 (2.9)</td>
</tr>
<tr>
<td></td>
<td>Soft Tissue</td>
<td>55</td>
<td>23 (41.8)</td>
<td>9 (16.4)</td>
<td>21 (38.2)</td>
<td>2 (3.6)</td>
</tr>
<tr>
<td></td>
<td>Lung</td>
<td>15</td>
<td>7 (46.7)</td>
<td>5 (33.3)</td>
<td>3 (20.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>20</td>
<td>7 (35.0)</td>
<td>4 (20.0)</td>
<td>9 (45.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>10</td>
<td>6 (60.0)</td>
<td>1 (10.0)</td>
<td>3 (30.0)</td>
<td></td>
</tr>
<tr>
<td>Distant</td>
<td>Lung</td>
<td>5</td>
<td>1 (20.0)</td>
<td>2 (40.0)</td>
<td>2 (40.0)</td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>Liver</td>
<td>5</td>
<td>0</td>
<td>1 (20.0)</td>
<td>4 (80.0)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: n, number; UKN, unknown; LN, lymph node.

9 http://www.r-project.org
10 http://rana.lbl.gov/EisenSoftware.html
To determine the relationship between mutation status and AKT activation, we measured the levels of P-AKT-Ser473 and P-AKT-Thr308 in the tumors by RPPA (Fig. 1A). Surprisingly, the BRAF-mutant tumors had significantly higher levels of P-AKT-Ser473 (\(P = 0.01\)) and P-AKT-Thr308 (\(P = 0.002\)) than NRAS-mutant tumors. Consistent with increased AKT activity, BRAF-mutant tumors also showed a trend toward higher levels of P-GSK3\(\alpha/\beta\) (\(P = 0.08\)) than NRAS-mutant tumors. There was no significant difference in the levels of total AKT or GSK3\(\alpha/\beta\) between BRAF- and NRAS-mutant tumors (Supplementary Fig. 1).

Fig. 1. RPPA analysis of AKT activation in melanoma clinical specimens and correlation with activating mutations. A, average expression levels of P-AKT-Ser473 (blue), P-AKT-Thr308 (red), P-GSK3\(\alpha/\beta\) (yellow), and PTEN (green) in BRAF-mutant or NRAS-mutant tumors or those that are WT for both genes. *, \(P < 0.05\) for BRAF versus NRAS; **, \(P < 0.01\) for BRAF versus NRAS; +, \(P < 0.05\) for WT versus NRAS. B, expression of P-AKT-Thr308 in individual BRAF- or NRAS-mutant tumors or those that are WT for both genes (○). Each circle represents one tumor and is colored to scale by the level of PTEN expression in the tumor (green, low; red, high). Tumors with AKT1(○), AKT3(●), and KIT(▵) mutations are indicated. C, expression of P-AKT-Ser473. D, expression of P-GSK3\(\alpha/\beta\).

Fig. 2. Comparison of RPPA results with IHC analysis of P-AKT. IHC staining for P-AKT-Ser473 on formalin-fixed, paraffin-embedded tissues from melanoma lymph node metastases. Black circle outlines the tumor deposit in each lymph node. A, tumor with high P-AKT-Ser473 by RPPA. B, tumor with low P-AKT-Ser473 by RPPA.
Fig. S1). BRAF-mutant tumors also had significantly lower levels of PTEN than NRAS-mutant tumors ($P = 0.005$; Fig. 1).

To further evaluate AKT activation according to mutation status, we assessed relative levels of P-AKT in the individual tumors (Fig. 1B-D). In these figures, each tumor was colored proportionally to the level of PTEN expression. Although P-AKT levels for many of the BRAF-mutant tumors were comparable with levels in NRAS-mutant and most WT tumors (Fig. 1B and C), a subset of the BRAF-mutant tumors had markedly elevated P-AKT; all of these tumors with markedly elevated P-AKT had low PTEN. In contrast, all NRAS-mutant tumors had relatively high PTEN and low P-AKT levels. Some WT tumors had elevated P-AKT; of these, almost all had relatively low PTEN.

For the two tumors with KIT mutations, we observed high P-AKT and P-GSK3α/β. Although P-GSK3α/β was elevated in most tumors with low PTEN, relatively high P-GSK3α/β levels were also noted in some tumors that did not have low PTEN levels. This may be due to the activity of kinases other than AKT that can phosphorylate GSK3α/β, including P90 RSK, P70 S6K, PKC isoforms, and PKA (38).

Fig. 3. AKT activation in human melanoma cell lines. A, RPPA analysis of levels of P-AKT-Ser473 in cells following serum starvation for 24 h. Cells are grouped on the basis of mutations in BRAF and NRAS. Cell lines with AKT3 (A) and KIT (▵) mutations are indicated. B, levels of P-AKT-Thr308. C, Western blotting of human melanoma cells grown in serum-replete medium. Mutations in BRAF (B), NRAS (N), and KIT (▵) are indicated, as are secondary alterations in the AKT pathway (A, AKT3-E17K; P, loss of PTEN expression). The same cell lysate (WM46) was run in the first lane of the first gel and the last lane of the second gel to ensure comparable immunoblot results. Loading was confirmed by ERK2 staining.

had low levels of P-AKT and P-GSK3α/β. Although P-GSK3α/β was elevated in most tumors with low PTEN, relatively high P-GSK3α/β levels were also noted in some tumors that did not have low PTEN levels. This may be due to the activity of kinases other than AKT that can phosphorylate GSK3α/β, including P90 RSK, P70 S6K, PKC isoforms, and PKA (38).

We performed IHC analyses of two tumors with high and low P-AKT-Ser473 levels, as assessed by RPPA, using formalin-fixed paraffin-embedded specimens that were harvested during the same surgery as the frozen tumors analyzed by RPPA. The tumor with high levels of P-AKT-Ser473 (Fig. 2A) showed homogenous, strong staining for P-AKT, whereas the tumor with low P-AKT-Ser473 (Fig. 2B) had homogenously weaker staining. Normal lymphocytes showed almost no staining for P-AKT.

**AKT activation in melanoma cell lines.** We extended our analysis to a panel of 58 human melanoma cell lines, which offers the theoretical advantage of allowing examination of...
homogenous populations of tumor cells and the ability to finely control conditions during protein harvest. Forty-six (79.3%) cell lines had BRAF mutations, eight (13.8%) had NRAS mutations, and four (6.9%) had no detectable mutations in either BRAF or NRAS (Supplementary Table S1). Two cell lines with activating BRAF mutations had concurrent AKT3-E17K mutations and one WT cell line had a KIT-L576P mutation. BRAF-mutant cell lines had higher P-AKT-Ser473 (\(P = 0.01\), serum-replete; \(P = 0.03\), serum-starved) and P-AKT-Thr308 (\(P = 0.01\) and \(P = 0.07\)) levels than NRAS-mutant cell lines (Fig. 3A and B). The average P-GSK3α/β level was also higher in the BRAF-mutant cell lines, but this difference was not statistically significant (data not shown). As observed in the tumors, most cell lines with elevated P-AKT also had low PTEN expression. Western blotting of independent cell lysates generated under serum-replete conditions was done on representative cell lines, and the results confirmed the relative differences in P-AKT levels between cell lines with PTEN loss and those with NRAS mutations (Fig. 3C). Elevated P-AKT was not observed in one of the cell lines with loss of PTEN (WM1552). When probed for the individual isoforms of AKT (AKT1, AKT2, AKT3), this cell line had low levels of AKT3; in contrast, the other cell lines with PTEN loss and elevated P-AKT had high levels of AKT3. This correlation between reduced P-AKT levels and a specific reduction in AKT3 expression is consistent with previous reports suggesting that this AKT isoform may be critical in melanoma (39, 40).

**AKT activation and clinical outcome.** To assess the prognostic significance of the AKT pathway in melanoma, we examined clinical outcomes in patients with regional metastases (i.e., regional lymph node or soft tissue). Fourteen patients who had already developed distant metastases before the collection of regional metastatic specimens were excluded from the survival analyses.

By univariate analysis, overall survival (OS) did not vary according to the levels of P-AKT-Ser473, P-AKT-Thr308, or PTEN, using each protein as a continuous variable (data not shown). To assess the prognostic significance of the coordinate regulation of these proteins, we dichotomized patients as “P-AKT High/PTEN Low” or “P-AKT Low/PTEN High” based on clustering.
analysis of the RPPA results for these proteins (Fig. 4A). There were no significant differences in OS between these groups, regardless of whether survival was calculated from the date of surgery and tumor specimen acquisition (Fig. 4B) or initial melanoma diagnosis (Fig. 4C).

**Potential impact of anatomic sites of melanoma on AKT activation.** As an exploratory study of the impact of anatomic site of involvement on signaling pathways, we analyzed the small set of resected distant metastases included in the analysis of mutational status versus AKT activation. Overall, there was no significant difference in P-AKT levels between the distant metastases (brain, liver, and lung; n = 20) and the regional metastases (lymph node and soft tissue; n = 70; data not shown). However, among the distant metastases, there was a significant difference in the activation of AKT among the different anatomic sites of involvement (Fig. 5A). Compared with liver and lung metastases, brain metastases showed higher levels of P-AKT-Ser473 (P = 0.002 versus liver; P = 0.008 versus lung), P-AKT-Thr308 (P = 0.04 and P = 0.01), and P-GSK3α/β (P = 0.10 and P = 0.003). The brain metastases also had low PTEN and elevated P-AKT levels. The brain metastases with elevated P-AKT included one tumor with a KIT-K462E mutation, three tumors with BRAF mutations, and two tumors with no known mutations. IHC analysis of representative formalin-fixed paraffin-embedded (FFPE) tissue from a brain metastasis with elevated P-AKT by RPPA analysis showed robust staining for P-AKT in the tumor and a lack of staining in normal brain tissue, supporting that this difference is unlikely to be due to nontumor elements in the specimens (Supplementary Fig. S3). In contrast, a FFPE lung metastasis from the same patient did not express P-AKT in the tumor or stroma by IHC (Supplementary Fig. S3). Among the patients with regional metastases, there was a trend, although not statistically significant, toward an increased risk of CNS metastasis for patients with the P-AKT High/PTEN Low signature compared with patients with the P-AKT Low/PTEN High signature (log-rank P = 0.18; Supplementary Fig. S4).

**Discussion**

Activation of AKT has been implicated in melanoma by the prevalence of NRAS and PTEN mutations and by IHC studies. We have quantitatively measured P-AKT in melanoma metastases to better define its activation status in patients with systemic disease, and to explore its relationship with mutations and clinical factors.
NRAS mutations, which occur in ~15% of melanomas, do not occur with either BRAF or PTEN mutations (16, 17, 41). This lack of redundancy suggests that NRAS mutations are sufficient to activate both the PI3K and the MAPK pathways. Consistent with the reported pattern of PI3K mutations, we found very low levels of PI3K in a subset of the BRAF-mutant tumors and in some WT tumors, but relatively high levels of PI3K in all NRAS-mutant tumors. Moreover, almost all tumors with low PI3K had high P-AKT. Surprisingly, RPPA analysis showed that NRAS-mutant tumors had P-AKT levels that were much lower than those in tumors with PI3K loss. In fact, P-AKT levels in NRAS-mutant tumors were comparable with those in tumors with no identified activating mutation. The validity of this finding is supported by the nearly identical results we observed in melanoma cell lines.

Although multiple models have implicated PI3K as a critical mediator of mutant NRAS–mediated signaling, there is limited direct evidence that NRAS mutations activate AKT in melanoma. Although the introduction of mutant NRAS into human melanoma cells is associated with acutely elevated P-AKT levels (42), our data suggest that melanomas develop compensatory changes over time that abrogate this effect. Our findings are consistent with a recent IHC study that found no significant increase in P-AKT levels in NRAS-mutant primary melanomas compared with BRAF-mutant or WT primary melanomas (43). PTEN genetic status or protein expression was not included in that study. Interestingly, we previously observed a similar apparent discrepancy in the effects of PI3K mutations. In contrast to the elevated P-AKT levels seen with acute expression of mutant PI3K, tumors and cell lines with somatic mutations in PI3K did not generally have elevated P-AKT (29, 44, 45). The lack of elevated P-AKT in melanomas with NRAS mutations does not preclude the possible contribution of other PI3K-regulated proteins in mediating the effects of mutant-NRAS in melanoma. However, our data suggests that treatments with selective efficacy in the setting of AKT hyperactivation may be most appropriate for melanomas with PTEN loss and not for melanomas with NRAS mutations (44, 46).

In this study, neither P-AKT nor PTEN expression levels were associated with OS in patients with regional melanoma metastases. Recent studies that examined the relationship of P-AKT levels to clinical outcome in melanoma have focused mainly on primary tumors (21, 43). In one immunohistochemistry-based study, the authors concluded that the presence of "strong" P-AKT was associated with poor OS (21). However, most of the observed differences in P-AKT levels and OS were related to patients with relatively thin primary tumors (tumor thickness <1.5 mm). Another study reported that lack of P-AKT actually correlated with worse outcomes in a small group of patients with primary tumors (43).

CNS involvement is a common manifestation of metastatic melanoma and is the cause of death in the majority of affected patients (47, 48). Preclinical models have previously implicated several proteins in the development of melanoma brain metastases, including transforming growth factor β and signal transducers and activators of transcription 3 (49, 50). However, these studies did not include a direct examination of distant metastases from different sites from patients. Such studies are generally challenging because distant parenchymal metastases are usually treated with systemic therapies, not surgical resection. In the small panel of distant metastases included in this study, we found that brain metastases had significantly higher P-AKT and lower PI3K expression than distant metastases from the lung and liver. Although it is possible that the increased P-AKT in brain metastases results from increased efficiency of circulating melanoma cells with activation of AKT to form metastases at this site in the body, we did not detect a significant difference in CNS involvement among stage III patients with High P-AKT/Low PI3K (Supplementary Fig. S4). These findings support the need for larger studies of distant metastases, including matched metastases from multiple sites in the same patient, to address the possibility of site-specific activation of different pathways. Such findings would support the need for the exploration of site-specific therapeutic approaches.

In summary, we have used RPPA to quantitatively analyze the activation of AKT in melanoma metastases and cell lines. This analysis was done with a relatively large number of carefully dissected and well-annotated clinical specimens and included analyses of reproducibility, known substrates and regulators of AKT, and confirmatory Western blot and IHC analyses. These findings have implications for the rational application of agents targeting the PI3K-AKT pathway in this disease. Importantly, this study shows the tremendous potential of prospective collection of well-annotated and carefully processed biospecimens and RPPA for the study of melanoma. When integrated with relevant molecular, clinical, and pathologic information, such studies have the potential to improve our understanding of the molecular pathogenesis of metastatic melanoma and lead to improved therapeutic approaches for this highly aggressive and poorly understood disease.

**Disclosure of Potential Conflicts of Interest**

M.A. Davies, commercial research grant, AstraZeneca.

---

**References**


Clinical Cancer Research

Integrated Molecular and Clinical Analysis of AKT Activation in Metastatic Melanoma

Michael A. Davies, Katherine Stemke-Hale, E. Lin, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-09-1985

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2009/12/21/1078-0432.CCR-09-1985.DC1
http://clincancerres.aacrjournals.org/content/suppl/2009/12/21/1078-0432.CCR-09-1985.DC2

Cited articles
This article cites 50 articles, 19 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/15/24/7538.full#ref-list-1

Citing articles
This article has been cited by 25 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/15/24/7538.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://clincancerres.aacrjournals.org/content/15/24/7538.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.