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Phosphatidylinositol-3-Kinase as a Therapeutic Target in MelanomaSaadia A. Aziz,¹ Michael Davies,³ Elah Pick,¹ Christopher Zito,¹ Lucia Jilaveanu,¹ Robert L. Camp,² David L. Rimm,² Yuval Kluger,⁴ and Harriet M. Kluger¹

Abstract Purpose: Phosphatidylinositol-3 kinases (PI3K) are critical for malignant cellular processes including growth, proliferation, and survival, and are targets of drugs in clinical development. We assessed expression of PI3K in melanomas and nevi, and studied associations between PI3K pathway members and *in vitro* response to a PI3K inhibitor, LY294002.

Experimental Design: Using Automated Quantitative Analysis, we quantified expression of p85 and p110 α subunits in 540 nevi and 523 melanomas. We determined the IC₅₀ for LY294002 for 11 melanoma cell lines and, using reverse phase protein arrays, assessed the association between levels of PI3K pathway members and sensitivity to LY294002.

Results: p85 and p110 α tend to be coexpressed ($P < 0.0001$); expression was higher in melanomas than nevi ($P < 0.0001$) for both subunits, and higher in metastatic than primary melanomas for p85 ($P < 0.0001$). Although phospho-Akt (pAkt) levels decreased in all cell lines treated with LY294002, sensitivity was variable. We found no association by *t* tests between baseline p85, p110 α , and pAkt levels and sensitivity to LY294002, whereas pS6 Ser²³⁵ and Ser²⁴⁰ were lower in the more resistant cell lines ($P = 0.01$ and $P = 0.004$, respectively).

Conclusions: Expression of p85 and p110 α subunits is up-regulated in melanoma, indicating that PI3K is a good drug target. Pretreatment pS6 levels correlated with sensitivity to the PI3K inhibitor, LY294002, whereas PI3K and pAkt did not, suggesting that full activation of the PI3K pathway is needed for sensitivity to PI3K inhibition. pS6 should be evaluated as a predictor of response in melanoma patients treated with PI3K inhibitors, as these drugs enter clinical trials.

Melanoma is the malignancy with the highest increase in incidence from 1950 to 2000, and the number of deaths from metastatic disease is increasing as well (1, 2). With the increase in incidence and lack of effective therapy once melanoma has metastasized, there is an immediate need to develop improved methods of treating patients with unresectable disease. Melanoma is usually resistant to standard chemotherapy; a number of chemotherapeutic and biological agents have activity in metastatic melanoma, albeit with disappointingly low response rates of <25% for any single agent or combination of agents, and none has improved overall survival when compared with

observation (3). As is the case with other malignancies, melanoma is a heterogeneous disease, and subsets of patients respond to systemic therapies, whereas others do not. Our understanding of mechanisms of resistance to different systemic therapy is limited, as is our ability to predict sensitivity, and new, well-tolerated agents and individualized approaches are required to improve outcome.

The phosphatidylinositol-3 kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway is activated by growth factors and other extracellular signals. PI3K activation affects many cellular processes including cell growth, proliferation, and survival. The alteration of components of this pathway, either through activation of oncogenes or inactivation of tumor suppressors, disrupts a signaling equilibrium and can thus result in malignant transformation, as reviewed by Dillon et al. (4). Activation of this pathway can occur through multiple mechanisms, including mutation, decreased expression of the tumor suppressor PTEN, mutation or amplification of PI3K, amplification of Akt, and activation of receptors or oncogenes upstream of PI3K.

The PI3Ks are a family of enzymes that phosphorylate the 3'-OH group on phosphatidylinositols. There are three classes of PI3Ks, and each has its own substrate specificity (5, 6). Class I_A PI3K, the type most widely implicated in cancer, is a heterodimer consisting of a p85 regulatory subunit and a p110 catalytic subunit. The p85 regulatory subunit mediates class I_A PI3K activation by receptor tyrosine kinases. p85 binds to phosphotyrosine residues on activated receptor tyrosine kinases. This binding serves both to recruit the p85-p110 heterodimer to the plasma membrane where its substrate

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Translational Relevance

Numerous phosphatidylinositol-3 kinase (PI3K) inhibitors are in clinical development. We studied expression of PI3K in a large cohort of melanomas and nevi. Levels of PI3K were higher in melanomas than nevi, and the p85 subunit was associated with disease progression, suggesting that PI3K is a valuable drug target in melanoma. In melanoma cell lines, we found variable sensitivity to the PI3K inhibitor, LY294002. We used reverse phase protein arrays to quantitatively study levels of PI3K pathway members. Levels of pS6 were significantly higher in the sensitive cell lines, whereas levels of all other PI3K pathway members studied, including phospho-Akt and PI3K, were not associated with sensitivity to the inhibitor. These results require confirmation in patients treated with PI3K inhibitors. Our data suggest that full PI3K pathway activation might be required for efficacy of PI3K inhibitors, and indicate that levels of pS6 might assist us in future patient selection for PI3K-targeted therapy.

resides and to relieve the basal inhibition of the p110 subunit by p85. The p110 α subunit is most commonly implicated in cancer, and has been shown to be essential for malignant transformation (7). Production of phosphatidylinositol-3,4,5-trisphosphate by PI3K initiates potent growth and survival signals. The kinases Akt and PDK1 directly bind to phosphatidylinositol-species at the 3' position and are thereby recruited to the plasma membrane. The phosphorylation of Akt at Thr³⁰⁸ by PDK1 and at Ser⁴⁷³ by an mTOR-Rictor-dependent process results in its full activation. Akt, in turn, phosphorylates several cellular proteins, including GSK3, FOXO transcription factors, MDM2, BAD, and others, to facilitate cell survival and cell cycle entry (Fig. 1; ref. 5). In addition, Akt activation results in activation of the mTOR/raptor complex, which regulates protein synthesis and cell growth (8).

The PI3K pathway is constitutively activated and/or up-regulated in various malignancies, and activation of this pathway plays a critical role in tumor progression (9, 10). PI3K activation is associated with resistance to chemotherapy and radiation therapy, as reviewed by Granville et al. (11), in numerous tumor types, including melanoma (12). The frequent activation of the PI3K/Akt/mTOR pathway in malignant cells has made components of this pathway attractive for therapeutic targeting. A number of PI3K inhibitors are available for preclinical research, including LY294002, wortmannin, PX-866, and others. The older compounds have remarkable antitumor activity in preclinical models, but their poor solubility and narrow therapeutic index have limited their clinical application. Newer PI3K inhibitors have entered phase I clinical trials, and activity of these agents should be assessed in diseases requiring new approaches, such as melanoma.

The PI3K pathway has been shown to be active in melanoma in preclinical models (13). Overexpression of Akt has been shown to convert radial growth melanoma to vertical growth melanoma (14). A number of preclinical studies have shown activity of agents that target this pathway in melanoma, including *in vitro* data generated by Smalley et al. (15) and Meier et al. (16), showing synergism between PI3K inhibitors

and MAP/ERK kinase inhibitors *in vitro*, and others have shown a similar phenomenon in mice (17). Derivatives of 4-morpholino-2-phenylquinazolines have been shown to specifically inhibit the p110 α PI3K subunit, resulting in growth inhibition in melanoma cells (18). A highly specific PI3K inhibitor, ZSTK474, was shown to be very active in B16 melanoma mouse models as a single agent (19). ZSTK474 has been shown to bind strongly to the ATP-binding pocket of PI3K, resulting in inhibition of downstream signaling components of this pathway, such as Akt and GSK3 β , causing a decrease in levels of cyclin D1. This drug was administered orally to mice, with minimal toxicity and resulted in remarkable decreases in tumor burden, with associated decreases in levels of phospho-Akt (pAkt). These latter results strongly support further clinical development of PI3K inhibitors for melanoma, alone or together with additional targeted therapies.

Despite the evident importance of the PI3K signaling in melanoma, very little is known about the frequency of expression of PI3K in this disease. Singh et al. (20) studied a small cohort of 35 nevi, 23 primary melanomas, and 31 metastatic melanomas for expression of the p85 and p110 subunits of PI3K, and found that few of the malignant lesions expressed either subunit, with only one visceral metastasis expressing the p85 subunit; 2 of 22 primary melanomas and 4 of 26 metastatic melanomas expressed the p110 subunit. Levels of pAkt, as a surrogate for PI3K activation, have been assessed in melanoma specimens; Dhawan et al. (21) showed an association between pAkt expression and disease progression in 29 patient samples. A relatively large study by Dai et al. (22) assessed expression of pAkt (Ser⁴⁷³) in 170 primary melanomas, 52 metastatic melanomas, and 70 nevi and found an association with disease progression and decreased survival. To the best of our knowledge, no large-scale quantitative studies have been conducted on patterns of PI3K expression in melanoma.

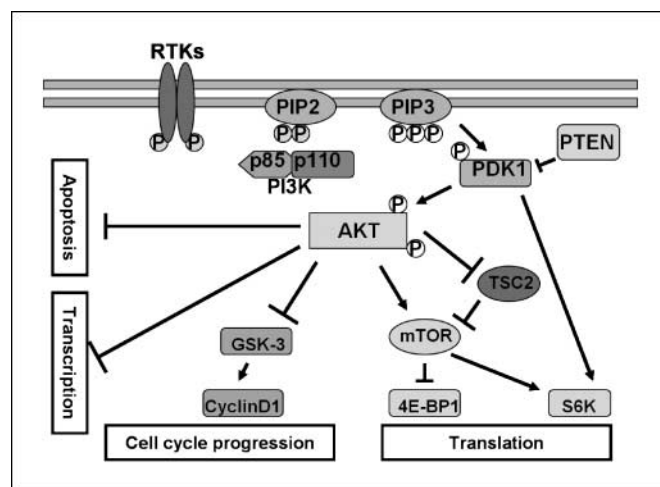


Fig. 1. Schema of the PI3K pathway. Growth factor receptors, integrins, activated Ras, or PTEN loss stimulate the PI3K/Akt/mTOR pathway. The products of PI3K activate Akt, which in turn controls key cellular processes by phosphorylating mediators of apoptosis, transcription, cell cycle progression, and translation. Arrows, activating phosphorylation; perpendicular lines, inhibitory phosphorylation.

Given the importance of PI3K in malignant progression and the current development of PI3K-targeting agents, we assessed its expression in a large cohort of melanomas and nevi, and showed a strong association with malignant progression. To obtain more accurate, objective expression measures, we used our method of automated, quantitative analysis (AQUA) of tissue microarrays. This method has been validated, and has proven to be more accurate than pathologist-based scoring of chromogenic stain (23). As is the case with some other targeted therapies, it is possible that response to PI3K-targeting drugs might be associated with expression levels of the target or downstream mediators, and quantitative assays need to be developed to predict response. Other markers that have both prognostic and predictive value in other diseases have significantly affected our ability to appropriately select therapeutic regimens for cancer patients. We hypothesized that levels of PI3K and/or pAKT would predict sensitivity to LY294002 treatment. We therefore assessed the association between levels of PI3K and PI3K pathway members in cell lines and sensitivity to LY294002. We found that levels of phosphorylated S6 (pS6) are most associated with sensitivity to LY294002, whereas levels of PI3K are not, indicating that full activation of the PI3K pathway is needed for cells to be sensitive to PI3K inhibition.

Materials and Methods

Tissue microarray construction. Tissue microarrays (TMA) were constructed as previously described (24). Cohorts of 230 primary melanomas, each measuring 0.6 mm in diameter, were spaced 0.8 mm apart on glass slides. For comparison of expression, specimens from a series of 293 metastatic patients were placed on the slides. Specimens and clinical information were collected with approval of a Yale Institutional Review Board. The specimens were resected from 1959 to 2000. The cohort has been described and validated in numerous publications (25, 26). Pellets of 15 melanoma cell lines were embedded as described (27), for normalization across slides. The benign nevus array contained 540 nevi as well as 40 melanomas and cell lines that were also present on the tumor array, used for controls and for normalization.

Immunohistochemistry. Staining was done for automated analysis of melanoma specimens as previously described (24, 28). Slides were incubated at 4°C overnight in a humidity tray with a primary antibody cocktail containing mouse anti-human PI3K p85, clone 4/PI3-Kinase, (BD transduction Laboratories) at a dilution of 1:50 with goat anti-rabbit IgG conjugated to Alexa 546 (Molecular Probes) to identify the S100 mask, or rabbit anti-human PI3K p110 α , clone C73F8, (Cell Signaling Technology), a dilution of 1:50 with goat anti-mouse anti-S100 IgG conjugated to Alexa 546. Goat anti-mouse or anti-rabbit horseradish peroxidase–decorated polymer (Envision; Dako Corporation) was used as a secondary reagent. The target was visualized with Cy5-tyramide (Perkin-Elmer). Coverslips were mounted using ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (Invitrogen).

Automated image acquisition and analysis. Images were acquired using our automated method, as described previously, and used in numerous prior melanoma papers (23, 29). Briefly, the S100-conjugated Alexa 546 defines the tumor compartment from stroma. Coalescence of S100 at the cell surface was used to localize cell membranes, and 4',6-diamidino-2-phenylindole was used to identify nuclei. The target signal, PI3K, was visualized with Cy5. The target signal pixels within the cytoplasm was normalized to the area of tumor mask and scored on a scale of 0 to 255 (the AQUA score).

Cell culture. Eight low-passage patient-derived melanoma cell lines were obtained from the Cell Culture Facility of the Yale Skin Disease

Research Core Center. Metastatic cell lines YUMAC (locally recurrent metastasis), YUSAC (distant soft-tissue metastasis), YULAC (distant soft-tissue metastasis), YUROB (distant soft-tissue metastasis), YUFIC (lymph node metastasis), YUGEN (brain metastasis), and YUSOC (in-transit cutaneous metastasis) were maintained in 15-cm dishes (Fisher Scientific) and Opti-MEM media (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and 1% antibiotic-antimycotic (penicillin, streptomycin, and amphotericin B; Invitrogen). The human primary melanoma cells, WW165, were maintained in Opti-MEM supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mmol/L 3-isobutyl-1-methyl-xanthine (Sigma Aldrich) and 1% antibiotic-antimycotic. Established cell lines including mel 501, mel 928, and mel 624 were obtained from Dr. Steven Rosenberg, Surgery Branch, National Cancer Institute (Bethesda, MD) and were maintained in RPMI 164 (Invitrogen) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic. Cells were incubated at 37°C in a humidified atmosphere of 95% air/5% CO₂.

Cell proliferation assays. At a density of 10⁵, cells were plated in triplicate in 6-cm plates with growth medium and allowed to adhere overnight. Cells were treated with trypsin and counted using a coulter counter (Beckman Coulter) for a pretreatment count. LY294002 (EMD Biosciences) in DMSO was used to treat the cells at a concentration of 5, 20, and 50 μ mol/L. DMSO was used in the control plates. Cells were trypsinized and counted after 72 h of treatment with drug or control. To determine the IC₅₀, we calculated linear regressions, and 50% inhibition was interpolated from the equation line.

Immunoblot analysis. After treatment with the LY294002 or control, cells were lysed in a buffer containing 20 mmol/L Tris-HCl (pH 8), 50 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP40, 30 mmol/L Na₃VO₄, 1 mmol/L phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail, Complete (Roche Diagnostics). Thirty micrograms of protein were separated using a SDS-PAGE by electroblotting onto a nitrocellulose membrane (Bio-Rad). Nonspecific blocking was conducted using 5% milk for 1 h at room temperature. The membrane was then incubated overnight at 4°C using the following primary rabbit anti-human antibodies: phosphorylated AKT Ser⁴⁷³, pS6 Ser^{235/236}, and p110 α at 1:1000 (Cell Signaling Technologies). A monoclonal mouse anti- β -actin antibody (Sigma Aldrich) was used at 1:10,000 for normalization of protein gel loading. Horseradish peroxidase–conjugated donkey anti-rabbit or anti-mouse IgG (JacksonImmunoResearch Laboratories, Inc.) was used as a secondary antibody. An enhanced chemiluminescence system (Pierce Biotechnology) was used to visualize proteins.

Reverse phase protein arrays. Reverse phase protein array (RPPA) methodology has been previously described (30). Briefly, cell lysates were normalized and diluted using 4 \times SDS buffer and lysis buffer to achieve a concentration of 1 μ g/ μ L and heated at 95°C for 10 min. The supernatants were serially diluted 2-fold with lysis buffer containing phosphatase inhibitors. Samples were printed on nitrocellulose-coated FAST slides (Schleicher & Schuele Bioscience, Inc.) using an Aushon 2470 arrayer (Aushon Biosystems, Inc.). To detect proteins, the RPPA slides were first blocked for endogenous peroxidase, avidin, and biotin activity. Each slide was probed with an antibody against a single protein, using conditions optimized for each antibody used. Probing was done for 4EBP1, phosphorylated 4EBP1 Thr³⁷/Thr⁴⁶, AKT, phosphorylated AKT Ser⁴⁷³ and Thr³⁰⁸, Cyclin D1, ERK2, GSK3, phosphorylated GSK3 Ser²¹/Ser⁹, phosphorylated mitogen-activated protein kinase Thr²⁰²/Tyr²⁰⁴, mammalian target of rapamycin, p110 α , p70S6K, phosphorylated p70S6K Thr³⁸⁹, p85, PDK1, phosphorylated PDK1 Ser²⁴¹, pS6 Ser²³⁵ and Ser²⁴⁰, TSC2, and phosphorylated TSC2 Thr¹⁴⁶². The manufacturers of the antibodies are listed in Supplementary Table S1. An anti-mouse or anti-rabbit secondary antibody was used for amplification. The antibody signal was then amplified using a DakoCytomation-catalyzed detection system. The probed slide was scanned, analyzed, and quantitated using Microvigen software (VigeneTech, Inc.) to generate a serial dilution-signal intensity super-curve for all samples on the slide. Each sample was then fitted to this

supercurve to generate logarithmic values representative of relative signal intensity. Differences in protein loading were determined using the median expression level for each sample across all antibodies used; protein values were divided by this factor. These corrected values were used for statistical analysis.

Statistical analysis. JMP version 5 and Statview were used to perform data analysis (SAS Institute, Inc.). The prognostic significance of the parameters was assessed for predictive value using the Cox proportional hazards model with overall survival as the end point. Associations with clinical and pathologic parameters were assessed by ANOVA. AQUA scores in primary and metastatic specimens and nevi were compared by unpaired *t* tests. Matlab software was used for clustering of the RPPA data, and associations between RPPA scores and sensitivity to LY294002 were assessed by unpaired *t* tests.

Results

PI3K expression in melanoma specimens. To assess the association between PI3K expression and melanoma tumor

progression, we stained large cohort TMAs with antibodies to the p85 and p110 α subunits. To account for intratumor heterogeneity in PI3K expression, two separate melanoma TMAs, each containing a core from a different area of tumor for each patient, were stained. Positive immunoreactivity of both subunits was predominantly observed in the cytoplasmic compartment, as shown in Fig. 2A and B, and thus, the nuclear compartment was not used for analysis. The regression for the scores of the two arrays for expression of p85 and p110 α was $R = 0.6$ and $R = 0.7$, respectively. AQUA scores of the melanomas ranged from 18.59 to 102.03 for p85 with a median score of 44.3, and from 4.94 to 81.55 for p110 α with a median score of 27.4. AQUA scores from companion slides were combined to give a single data set in the following manner: tumor spots were deemed uninterpretable if they had insufficient tumor cells, loss of tissue in the histospot, or an abundance of necrotic tissue. For patients who had two interpretable histospots, a composite score was formed by

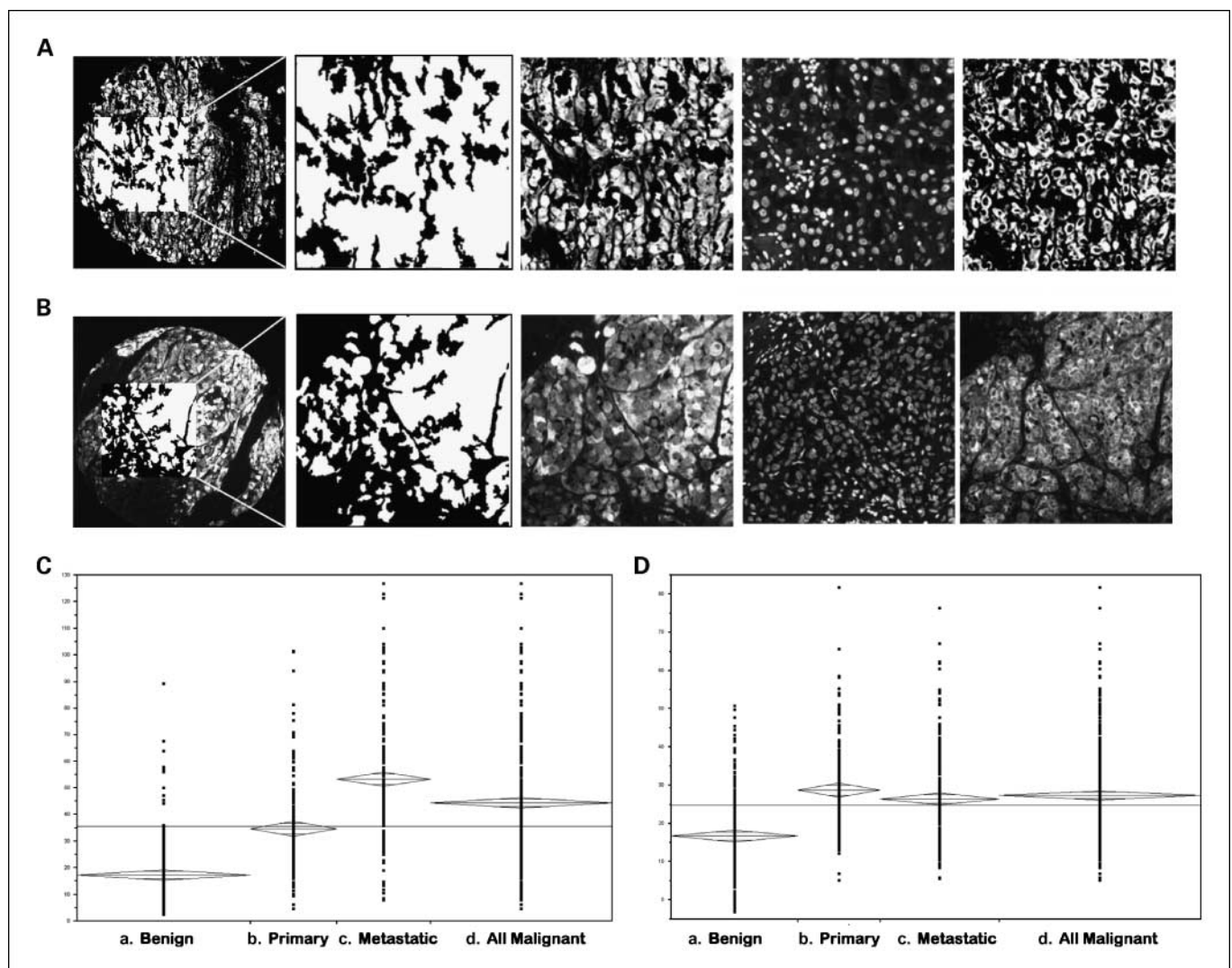


Fig. 2. A-D, AQUA of *in situ* protein levels (A, for p85; B, for p110 α): AQUA uses S100 to create a tumor or nevus mask (two panels on left, at $\times 10$ and $\times 40$). S100 staining was both nuclear and cytoplasmic (middle). 4', 6'-Diamidino-2-phenylindole defines the nuclear compartment (second from right) within the tumor mask. p85 (A) and p110 α (B) expression is measured within the cytoplasmic compartments, within the tumor mask (right), and each clinical case is assigned a score based on pixel intensity per unit area within the tumor mask. ANOVA was used to compare p85 (C) and p110 α (D) expression in benign nevi, primary, metastatic, and all malignant (primary and metastatic combined) specimens.

taking the average of the two scores. For patients with only one interpretable core, the single score was used. The resulting combined data set included 349 patients for p85 and 401 patients for p110 α . For the nevus arrays, we had scores for 339 spots for p85 and 252 for p110 α .

To assess coexpression of the p85 and p110 α subunits, we used Spearman's nonparametric rank correlation test, and found some coexpression of the two subunits ($\rho = 0.353$; $P < 0.0001$). We assessed the association between expression of the two subunits and melanoma tumor progression using unpaired t tests. Expression of p85 was significantly higher in malignant versus benign tissue cores ($P < 0.0001$), and expression was significantly higher in metastatic melanomas than in primary melanomas ($P < 0.0001$; Fig. 2C). Expression of p110 α was also significantly higher in malignant versus benign cores ($P < 0.0001$) but did not show a significant difference in metastatic lesions compared with primaries ($P = 0.06$; Fig. 2D). Unpaired t tests were used to assess the association between the expression of the PI3K subunits and other commonly used clinical and pathologic variables. High p85 expression was associated with advanced disease stage ($P < 0.0001$), higher Clark level ($P = 0.0126$), and absence of tumor infiltrating lymphocytes ($P = 0.0141$). We found no associations between the p110 α subunit expression and gender, age, disease stage, Breslow depth, Clark's level, presence of ulceration, or presence of infiltration lymphocytes. By Cox univariate analysis, no association was found between p85 or p110 α levels and survival in either the primary and metastatic groups.

In vitro activity of the PI3K inhibitor LY294002 in melanoma cell lines. Given the association between PI3K expression and melanoma tumor progression, we assessed the effect of PI3K inhibition on melanoma cell lines. A panel of 11 melanoma cell lines was treated using LY294002, a reversible pan-PI3K inhibitor. Cells were treated thrice in triplicate with concentrations ranging from 5 to 50 $\mu\text{mol/L}$. After a 72-h incubation, cells were trypsinized and counted, and the IC_{50} was calculated for each cell line, and averaged for the nine measurements. As shown in Table 1, the IC_{50} ranged from 16 to 42 $\mu\text{mol/L}$. The IC_{50} for 8 of the cell lines was 16 to 19 $\mu\text{mol/L}$, and for the remaining 3 cell lines, YUROB, YULAC, and mel 928, the IC_{50} was 38 to 42 $\mu\text{mol/L}$. For the remainder of the analyses, the latter three cell lines were labeled as "resistant," whereas the others were considered "sensitive."

Target inhibition of LY294002. LY294002 is a reversible inhibitor of PI3K. To verify target inhibition and assess the duration of inhibition, we studied levels of pAkt Ser⁴⁷³ as a

Table 1. IC_{50} of LY294002 on a panel of melanoma cell lines

Cell line	IC_{50} of LY294002 ($\mu\text{mol/L}$)
mel 501	18
YUSOC	17
YUGEN8	17
YUMAC	18
YULAC	42
mel 928	38
YUFIC	18
YUROB	39
YUSAC	16
mel 624	17
WW165	19

surrogate for PI3K activity, in both our resistant and sensitive melanoma cell lines. Levels of pAkt decreased in all cell lines with exposure to 50 $\mu\text{mol/L}$ of LY294002 for 1 hour, as shown in Fig. 3. The same analyses were done without serum for 18 hours, and no major differences were seen (Supplementary Fig. S1). We then studied the duration of pAkt down-regulation at different concentrations (5, 20, and 50 $\mu\text{mol/L}$) at different time points (0, 1, 4, and 24 hours) after LY294002 exposure. We used one resistant and one sensitive cell line (YULAC and YUSAC). We found that the degree of down-regulation of pAkt was somewhat more pronounced in the more sensitive cell line at 1 hour, yet in both cell lines, down-regulation of pAkt starts reversing as early as 4 hours after drug exposure for both cell lines, although declines in cell viability can be seen at later time points. These findings support the notion that resistance to LY294002 in YULAC is not likely to be due to poor penetration into the cell or quicker efflux from the cell, but rather to other molecular mechanisms (Supplementary Fig. S2). This is consistent with the fact that LY294002 is a reversible PI3K inhibitor.

B-RAF and N-RAS mutations in cell lines. Of the 11 cell lines, only YUFIC had a mutation in N-RAS (Q61R). V600K or V600E mutations in B-Raf were found in mel 501, YUGEN8, YUMAC, YULAC, mel 928, YUSAC, mel 624, and WW165. By χ^2 analysis, there was no association between presence of B-Raf mutation and sensitivity to LY294002 ($\chi^2 = 0.075$; $P = 0.8$).

Identification of predictors of sensitivity or resistance to PI3K inhibition by RPPA. To identify biomarkers that might be

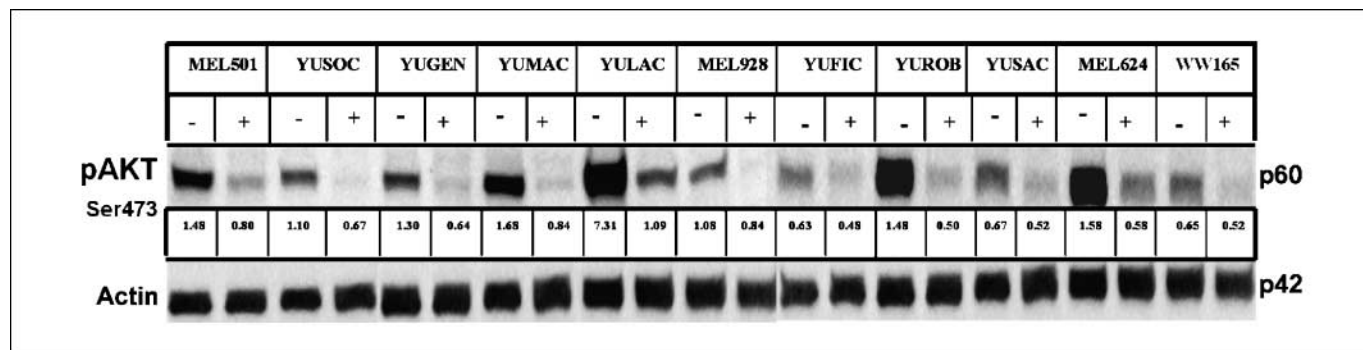


Fig. 3. Western blots showing levels of pAkt before and after exposure to 50 $\mu\text{mol/L}$ LY294002 for 1 h, normalized to β -actin loading. Levels of pAkt decreased in all cell lines, regardless of sensitivity to LY294002.

useful in the future for patient selection for PI3K inhibitors, we studied the basal expression and phosphorylation status of components of the PI3K pathway in our panel of sensitive and resistant cell lines by RPPA. We also included representative members of the mitogen-activated protein kinase pathway, as there is ample evidence of "cross talk" between these pathways. Markers studied and antibodies used are listed in Supplementary Table S1. We used RPPA using lysates from pretreatment cell lines, and assessed associations with drug sensitivity. All antibodies used were validated by Western blots, which confirmed a single band of the associated protein size, as described (30). Technical and biological replicates were included in the studies to confirm reproducibility of the results. Logarithmic regression analyses showed strong correlations ranging from 0.562 to 0.897 for p4EBP1 and pTSC2, respectively, for biological replicates, and 0.85 to 0.99 for technical replicates. RPPA results confirmed the correlation between the p85 and p110 α PI3K subunits ($R = 0.536$; $P = 0.02$) as seen with our AQUA analysis. To validate the RPPA results, we assessed protein levels of p110 α , pS6 Ser²³⁵, and pAkt Ser⁴⁷³ by densitometry using Western blots. Levels using these 2 methods correlated well ($\rho = 0.518$, $\rho = 0.655$, and $\rho = 0.730$, respectively).

RPPA scores were normalized for the loading for each lysate, and values for repetitions were averaged. Using Matlab software, we did unsupervised clustering, as shown in the heatmap in Fig. 4. The rows represent cell lines and the columns represent proteins, with red indicating high expression, whereas green indicates low expression. The more sensitive cell lines (labeled in black) tend to have higher levels of pS6 (labeled in red) than the more resistant cell lines. Associations between marker expression levels and sensitivity/resistance are shown in Table 2. A significant association was found between high levels of pS6 Ser²³⁵ Ser²⁴⁰ and increased sensitivity (low IC₅₀), by unpaired t test ($P = 0.01$ and $P = 0.004$, respectively). Interestingly, the associations between baseline levels of pAkt and viability were not statistically

Table 2. Association between biomarker level and IC₅₀ for LY294002 in 11 cell lines by t test

Protein	t statistic	P
4EBP1 pT37 and T46	0.366	0.734
4EBP1	0.077	0.941
AKT pS473	-1.130	0.365
AKT pT308	-0.134	0.905
AKT	0.222	0.844
Cyclin D1	-0.244	0.816
ERK2	-0.738	0.517
GSK3 pS21 and S9	0.316	0.766
GSK3	-1.763	0.148
Mitogen-activated protein kinase pT202 and Y204	-0.867	0.447
mTOR	0.573	0.600
P110 α	-0.773	0.464
p70s6K pT389	-0.208	0.840
p70s6K	-1.465	0.225
p85	0.952	0.366
PDK1 pS241	-1.025	0.347
PDK1	0.291	0.795
PTEN	-0.446	0.697
S6 pS235	3.030	0.010
S6 pS240	3.905	0.004
TSC2 pT1462	-0.539	0.637
TSC	-1.251	0.290

significant. Similarly, neither the p85 or p110 α subunit of PI3K was associated with sensitivity.

Discussion

In this work, we assessed expression of PI3K in a quantitative fashion in a large number of melanomas and nevi. The AQUA method gives objective, continuous measures of expression, rather than routinely used pathologist-based divisions of staining into nominal scores of 0, 1, 2 and 3, or "positive/negative." Our results were reproducible when using a second

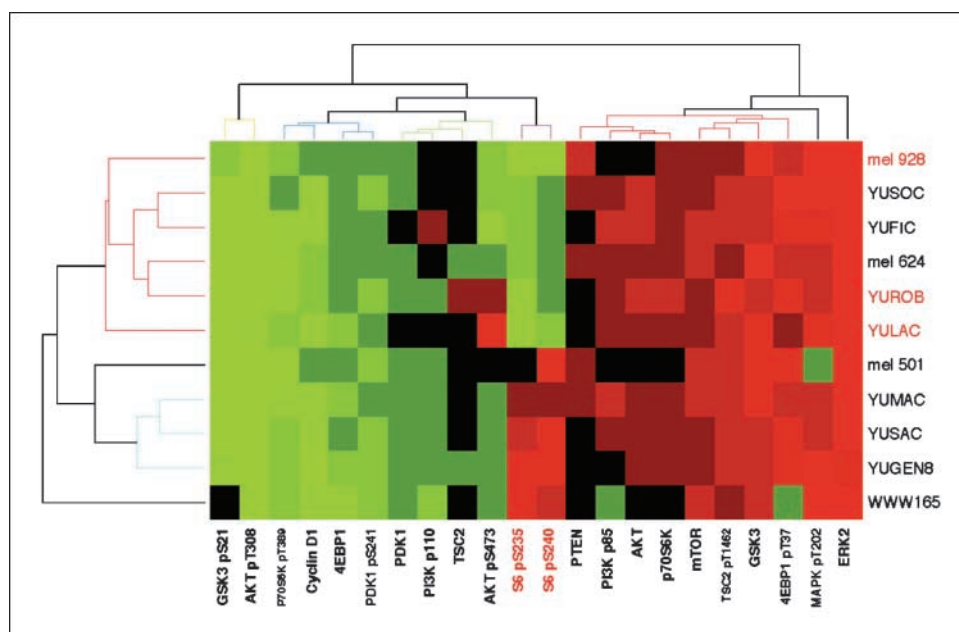


Fig. 4. Unsupervised hierarchical clustering of cell lines (rows) and baseline levels of biomarkers (columns). The cell lines in red are more resistant to LY294002, and those in black are more sensitive. Red, high expression; green, low expression; brightest green, lowest expression. Expression levels represent the average levels of repetitive measurements for each marker, normalized to the loading for the cell line. Levels of pS6 (red) tend to be higher in the more sensitive cell lines than the resistant cell lines.

set of arrays with different cores from tumors of the same patients. Expression of both p85 and p110 α subunits was significantly higher in melanomas than in nevi, and levels of p85 were higher in metastatic than in primary specimens, whereas levels of p110 α were not. Neither subunit was a prognostic marker in either primary or metastatic patients. The p85 subunit was associated with higher Clark level and absence of tumor infiltrating lymphocytes. We found a fair degree of coexpression of the two subunits in the melanoma specimens.

Our findings differ from those of Singh et al. (20), who found that the two PI3K subunits were not expressed in most melanomas. The different results in our study might either be due to the larger cohort used here or to the method of assessment. High expression in our study was measured in a quantitative fashion and was relative to that of nevi. Although levels of PI3K do not necessarily confer activity of PI3K, levels of pAkt are a good surrogate for PI3K activity. Given that we used paraffin-embedded specimens that were not fixed in a uniform fashion and collected over many decades, assessment of levels of phosphorylated proteins might not be accurate. However, activity of this pathway in melanoma is supported by the high levels of pAkt found in the early passage cell lines obtained from tumors excised at our institution used in this study.

The association between PI3K expression and melanoma progression suggests that PI3K might be a valuable drug target in this disease. As with other targeted therapies, there might be an association between target expression or activation and sensitivity to targeted inhibitors. In a panel of cell lines, we found that sensitivity to the PI3K inhibitor, LY294002, was variable, although levels of pAkt (as a surrogate of PI3K activity) declined in all cell lines treated. To assess the association between expression of PI3K and other PI3K pathway members and the degree of sensitivity to LY294002, we used RPPAs. This technology has been validated in previous papers (30). RPPA is a new, sensitive, automated, high-throughput proteomics technology, which provides quantitative analysis of active (phosphorylated) or total protein expression in a large number of lysates prepared either from patient samples or cell lines, all analyzed in a single experiment. RPPA enables the analysis of a large number of proteins (e.g., all known proteins from a signal transduction pathway) from each sample, as tiny amounts of protein are used for each assay. This approach can enable us to develop molecular signature databases. This information can be used for biomarker or protein network assessment. In our study, RPPA results were reproducible, and protein levels correlated well with those obtained from Western blots.

Levels of expression of PI3K pathway members, as well as levels of phosphorylated MAP/ERK kinase (MEK) and extracellular signal-regulated kinase (ERK), were assessed using RPPAs. We initially hypothesized that levels of PI3K and/or pAKT would predict sensitivity to LY294002 treatment. However, no association was seen between levels of pAkt or the p85 and p110 α subunits of PI3K and sensitivity to LY294002. By unpaired *t* tests, we found an association between sensitivity to LY294002 and high levels of phosphorylation of S6 at both the Ser²³⁵ and Ser²⁴⁰ phosphorylation sites. Discordance between Akt activation and S6 activation can occur by activation or inhibition of parallel mediators. For example, cells can have high levels of pAkt and low levels of pS6 if Akt activation inhibits tuberous sclerosis complex, which in turn inhibits mTOR. Conversely, S6 can be

activated in an Akt-independent fashion through activation of S6K by PDK-1, as shown schematically in Fig. 1. The fact that downstream activation of S6 is seen in the sensitive cell lines suggests that full pathway activation is associated with a higher degree of sensitivity to the PI3K inhibitor.

To the best of our knowledge, our study is the first to report an association between levels of pS6 and response to PI3K inhibition. However, we did find one study that mentions an association between high levels of S6K and sensitivity to mTOR inhibition in breast cancer cells (31).

With some targeted therapies, such as Herceptin in breast cancer, expression levels of the target (Her2/neu) are more predictive of response to the drug than the degree of target activation (levels of phosphorylated Her2/neu; ref. 32). Although the results described in this work are preclinical, our data suggest that in the case of PI3K-targeting drugs, activation of the target, and critical downstream mediators might be more predictive of sensitivity than target levels; expression of the p85 and p110 α PI3K subunits were not associated with sensitivity *in vitro*, whereas pS6 was associated.

Our findings have important implications for the therapeutic application of PI3K inhibitors in melanoma. An impressive number of PI3K inhibitors have entered the clinic with variable specificity for the different PI3K family members. SF1126 is a pan-PI3K inhibitor developed by Semafore Pharmaceuticals. It is a conjugate of LY294002 designed for increased solubility and binding to specific integrins within the tumor compartment. It is currently in a phase I clinical trial for solid tumors, and is well-tolerated (33). GDC-0941 (Genentech, Inc.) is an inhibitor of the p110 α subunit, which is also in early phase clinical trials (34). Novartis Pharmaceuticals is developing a number of PI3K inhibitors, including dual inhibitors of PI3K p110 α and mTOR, NVP BEZ235, and NVP BGT226 (35) that have entered clinical trials.⁵ Exelixis has two PI3K inhibitors that are in phase I clinical trials, XL-147 and XL-765.⁶ Early results regarding the latter drug show that it is generally well-tolerated and has resulted in prolonged progression free survival in patients with solid tumors (36). Calistoga Pharmaceuticals is developing a specific inhibitor for the p110 δ subunit (expressed predominantly in hematopoietic cells), which is being studied for hematologic malignancies.⁷ Examples of other PI3K inhibiting drugs in preclinical and clinical stages include compounds developed by Aeterna Zentaris, PX-866, an inhibitor of p110 α , γ , and δ isoforms that results in inhibition of downstream signaling proteins including p-AKT, p-mTOR, and p-S6 (Oncothyreon), GSK1059615 (GlaxoSmithKline), and other pharmaceutical companies are developing drugs that target additional members of the PI3K pathway.^{8,9,10} Although LY294002 is useful for conducting preclinical studies of PI3K inhibition, its poor solubility and narrow therapeutic index preclude its use in humans. Studies using some of the compounds that are in clinical trials are ongoing in our laboratory, with the goal of identifying pretreatment predictors

⁵ <http://www.Novartis.com>

⁶ <http://www.Exelixis.com>

⁷ <http://www.Calistogapharma.com>

⁸ <http://www.Aezsinc.com>

⁹ <http://www.Oncothyreon.com>

¹⁰ <http://www.Gsk.com>

of response in melanoma that might be used for future patient selection.

Careful and rational strategies are needed to target PI3K in melanoma. Expression of pS6 and pAkt was variable in our cell lines, many of which were early passage cell lines, which likely reflect tumor biology. Moreover, although most melanomas had high PI3K expression, PI3K levels were not uniformly high in all primary or metastatic specimens, and expression of PI3K is likely necessary for pathway activation. Further work is needed to determine whether there is a threshold PI3K level in clinical specimens, below which there is no PI3K pathway activity rendering PI3K inhibitors ineffective. However, our data suggest that at the very least, expression and phosphorylation of PI3K pathway members should be assessed in specimens from melanoma patients treated with PI3K inhibitors. Response rates might be maximized if these inhibitors are selectively used for patients with melanomas in which pathway members are both expressed *and* activated, and the therapeutic ratio for patients might be optimized. Appropriate selection of patients, characterization of individual tumor biology, and perhaps interven-

tion with additional rationally targeted agents might be necessary to achieve the best results.

In summary, we have shown a strong association between high PI3K expression and melanoma disease progression, suggesting that PI3K might be a useful drug target in melanoma. In our *in vitro* studies, pS6 seems to be the best predictor of response to the PI3K inhibitor LY294002, and not expression of PI3K and pAkt. Studies are ongoing to assess PI3K pathway members in melanoma cells using clinical quality PI3K inhibitors. Future clinical trials using PI3K inhibitors for melanoma should stratify patients based on expression and phosphorylation of PI3K pathway members, particularly S6, with the goal of future patient selection based on these predictive biomarkers.

Disclosure of Potential Conflicts of Interest

D.L. Rimm and R.L. Camp are stockholders, consultants, and scientific cofounders of HistoRx, which has licensed the Aqua technology from Yale University. The other authors disclosed no potential conflicts of interest.

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