Mutations in the Phosphatidylinositol-3-Kinase Pathway Predict for Antitumor Activity of the Inhibitor PX-866 whereas Oncogenic Ras Is a Dominant Predictor for Resistance

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Abstract

The novel phosphatidylinositol-3-kinase (PI3K) inhibitor PX-866 was tested against 13 experimental human tumor xenografts derived from cell lines of various tissue origins. Mutant PI3K (PIK3CA) and loss of PTEN activity were sufficient, but not necessary, as predictors of sensitivity to the antitumor activity of the PI3K inhibitor PX-866 in the presence of wild-type Ras, whereas mutant oncogenic Ras was a dominant determinant of resistance, even in tumors with coexisting mutations in PIK3CA. The level of activation of PI3K signaling measured by tumor phosphorylated Ser^{473}-Akt was insufficient to predict *in vivo* antitumor response to PX-866. Reverse-phase protein array revealed that the Ras-dependent downstream targets c-Myc and cyclin B were elevated in cell lines resistant to PX-866 *in vivo*. Studies using an H-Ras construct to constitutively and preferentially activate the three best-defined downstream targets of Ras, i.e., Raf, RalGDS, and PI3K, showed that mutant Ras mediates resistance through its ability to use multiple pathways for tumorigenesis. The identification of Ras and downstream signaling pathways driving resistance to PI3K inhibition might serve as an important guide for patient selection as inhibitors enter clinical trials and for the development of rational combinations with other molecularly targeted agents. [Cancer Res 2009;69(1):143–50]

Introduction

An important cell survival mechanism for many cancers is mediated by the phosphatidylinositol-3-kinase (PI3K)/Akt (protein kinase B) signaling pathway (1). Class I PI3Ks phosphorylate membrane phosphatidylinositols to give $PI(3,4,5)P_3$, which then binds and recruits the serine/threonine kinase Akt through its NH_2-terminal pleckstrin homology domain in a process reversed by PTEN phosphatase. The membrane-associated Akt is activated by Thr^{308} phosphorylation by membrane-associated phosphoinositide-dependent kinase-1 (2) and Ser^{473} phosphorylation, most likely through the TORC2 complex (3). Activated Akt detaches from the plasma membrane and moves to the cytoplasm and the nucleus where it phosphorylates a battery of targets, leading to changes in cellular functions (4). PI3K activity has been found to be aberrantly activated in many human cancers, including breast, glioma, prostate, non–small cell lung, ovarian, head and neck, urinary tract, colon, and cervical (5–7). Activation of the PI3K/Akt pathway can occur due to upstream inputs, including deregulated growth factor signaling (8), activating mutations in the proto-oncogene Ras (9), point mutations or overexpression of the PI3K-α catalytic subunit (PIK3CA; refs. 7, 10), mutation or loss of PTEN (11), and activating mutations in the pleckstrin homology domain of Akt (12).

The PI3K/Akt signaling pathway offers several targets for therapeutic intervention and a number of agents are now in or entering clinical trials (13). Much early work on the anticancer potential of PI3K inhibitors was conducted with LY294002, a chromene of low potency and selectivity, that inhibits multiple kinases both related and unrelated to PI3K (14). The other frequently used PI3K inhibitor is the fungal metabolite wortmannin that shows a much higher specificity for the class 1 PI3Ks compared with other related family members. However, wortmannin has proved to be too biologically unstable and toxic for use as an antitumor agent (15). In our studies, we used PX-866, a semisynthetic viridin that has been found to be a specific, irreversible inhibitor of the class 1 PI3Ks with a mechanism similar to wortmannin. Due to its chemically modified tetracyclic core, PX-866 possesses a greatly increased metabolic stability, decreased toxicity, and an increased specificity relative to other PI3K family members (16) and has entered clinical trials as an anticancer agent. PX-866 has been characterized against the class 1 isoforms and was found to selectively inhibit the α, δ, and γ isoforms with IC_{50} values of 5, 9, and 2 nmol/L, respectively (17). PX-866 is a selective inhibitor of PI3K and against a panel of 235 unrelated kinases, PX-866 at 1 μmol/L inhibited only 2 kinases by <30%, Lck by 32%, and LOK by 40%. PX-866 has antitumor efficacy in experimental cancer models as a single agent and in combination with both conventional chemotherapy and targeted agents (17, 18). As PX-866 and other specific inhibitors of PI3K/Akt signaling move through clinical development, the identification of positive and negative predictors of response to identify individuals most likely to receive the maximum therapeutic benefit is critical. Predictors for PI3K/Akt inhibitor sensitivity thus far reported include PI3K pathway–specific mutations (7, 10–12), increased insulin-like growth factor-binding protein-2 in glioma (19), stathmin in breast cancer (20), and acidic ribosomal phosphoprotein P2 (21).

In the present study, we tested PX-866 against a panel of experimental human tumor xenografts derived from cell lines of various tissue origins showing that mutant PIK3CA and loss of PTEN activity were sufficient but not necessary predictors of sensitivity to PX-866 in the presence of wild-type Ras, whereas mutant oncogenic Ras predicted resistance, even in tumors with

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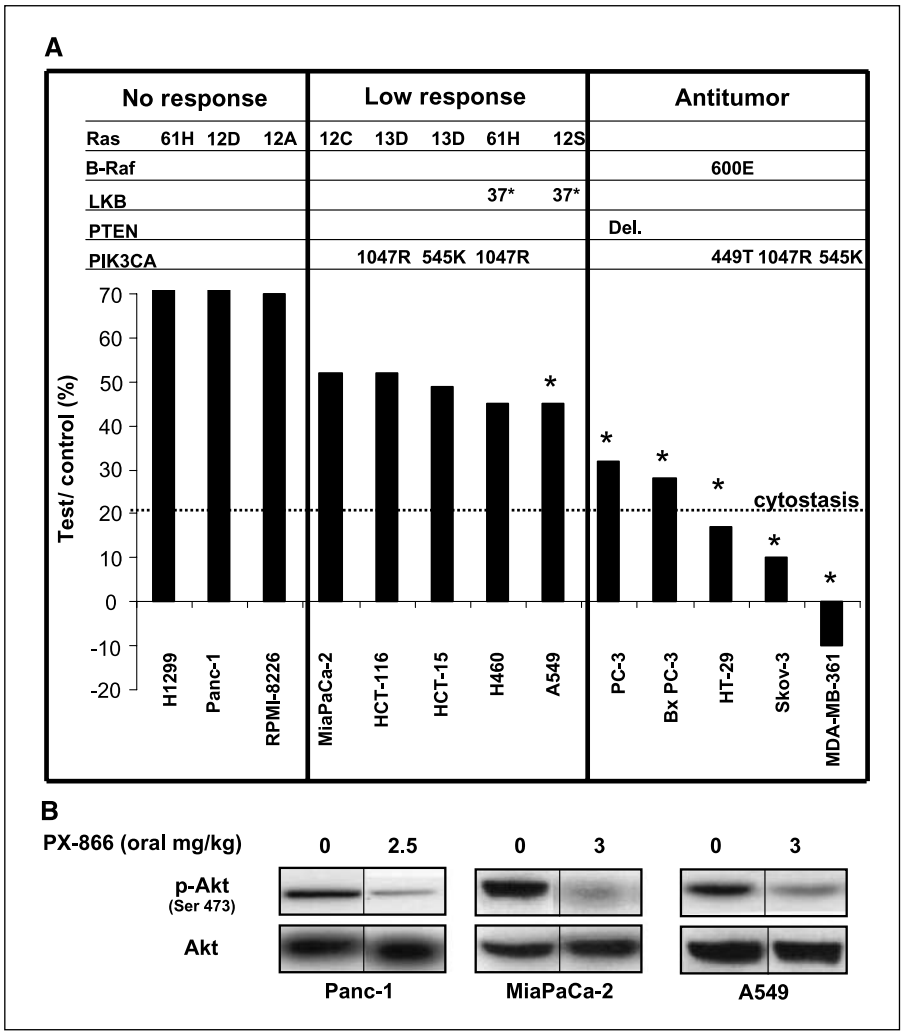


Figure 1. Effect of PX-866 on cell line–derived xenografts. *A,* cell line–derived xenografts were grown subcutaneously in female SCID mice. Upon reaching 200 mm^3, the mice were treated with PX-866, 2.5 to 3.0 mg/kg every other day administered p.o. At the end of treatment, the tumor volume was expressed as a percentage of the increase in the vehicle alone–treated tumor volume (T/C%). *, $P < 0.05$. Tumor responses were characterized as No response (T/C >70%), Low response (T/C 35–69%), or Antitumor (T/C <35%). The Ras, Raf, PIK3A, LKB1, and PTEN mutation status of the tumors is shown. *B,* phosphorylated Ser^{473}-Akt levels measured in representative tumors removed from mice treated with PX-866 (2.5-3 mg/kg p.o.) at the end of treatment. Images were taken from different fields on the same film.

coexisting mutations in PIK3CA. Thus, mutant oncogenic Ras is a primary determinant of resistance to the antitumor activity of PX-866. Further studies used an H-Ras construct to constitutively and preferentially activate the three best-defined downstream targets of Ras: Raf, RalGDS, and PI3K. These constructs revealed that activated Ras mediates resistance through its ability to use these pathways concurrently. The identification of signaling pathways driving resistance to PI3K inhibitors will aid patient selection and reveals a need for the development of rational combinations with other molecularly targeted agents.

Materials and Methods

Cells. A549, H460, and H1299 human non–small cell lung cancer; HT-29, HCT-15, and HCT-116 human colon cancer; MDA-MB-361 human breast cancer; Panc-1, BxPC-3, and MiaPaCa-2 pancreatic cancer; PC-3 prostate cancer; Skov-3 ovarian cancer; and RPMA-8226 multiple myeloma cancer cells were obtained from the American Type Culture Collection. The cell lines were grown in humidified 95% air, 5% CO_2 at 37°C in their American Type Culture Collection recommended medium with 10% fetal bovine serum. All cell lines were tested to be *Mycoplasma*-free using a PCR ELISA assay (Roche Diagnostics, Inc.). HCT-116 K-Ras–deleted cells generated by homologous deletion of the mutant *K-Ras* allele (20, 21) were transfected by electroporation at 600 V for 60 ms using a Multiporator Eppendorf with G418 selectable plasmids expressing mutant active H-Ras (H-Ras V12), and the selective effectors H-Ras V12S35, H-Ras V12G37, or H-Ras V12C40, which preferentially activate Raf, RalGDS, and PI3K enzymes, respectively, and individual colonies were isolated. The plasmids were generously provided by Dr. M. Wigler (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). These Ras mutations have been previously characterized in a number of models (22–26).

Antitumor studies. Approximately 10^7 cells in log cell growth were injected s.c. in 0.1 mL sterile 0.9% NaCl into the flanks of severe combined immunodeficient (SCID) mice. Cell lines with H-Ras constructs were removed from G418 one passage before injection. When the tumors reached 200 mm^2, the mice were stratified into groups of 8 to 10 animals having approximately equal mean tumor volumes and p.o. administration of 2.5 to 3 mg/kg PX-866 begun every other day for 1 to 3 wk. For p.o. administration to mice, PX-866 (4*S*,4a*R*,5*R*,6a*S*,9a*R*,*E*)-1-[(diallylamino)methylene]-11-hydroxy-4-(methoxymethyl)-4a,6a-dimethyl-2,7,10-trioxo-1,2,4,4a,5,6,6a,7,8,9,9a,10-dodecahydroindeno(4,5-*h*)isochromen-5-yl acetate was dissolved at 0.3 to 0.5 mg/mL in 5% ethanol in water and dosed by p.o. gavage. At the end of the study, antitumor activity was expressed as a percentage of test/controls (T/C%) determined by dividing the increase in volume of the PX-866–treated tumors by the increase in volume of the control tumors from the start of treatment. Information on mutations in the cell lines was obtained from the Sanger Institute database[5]

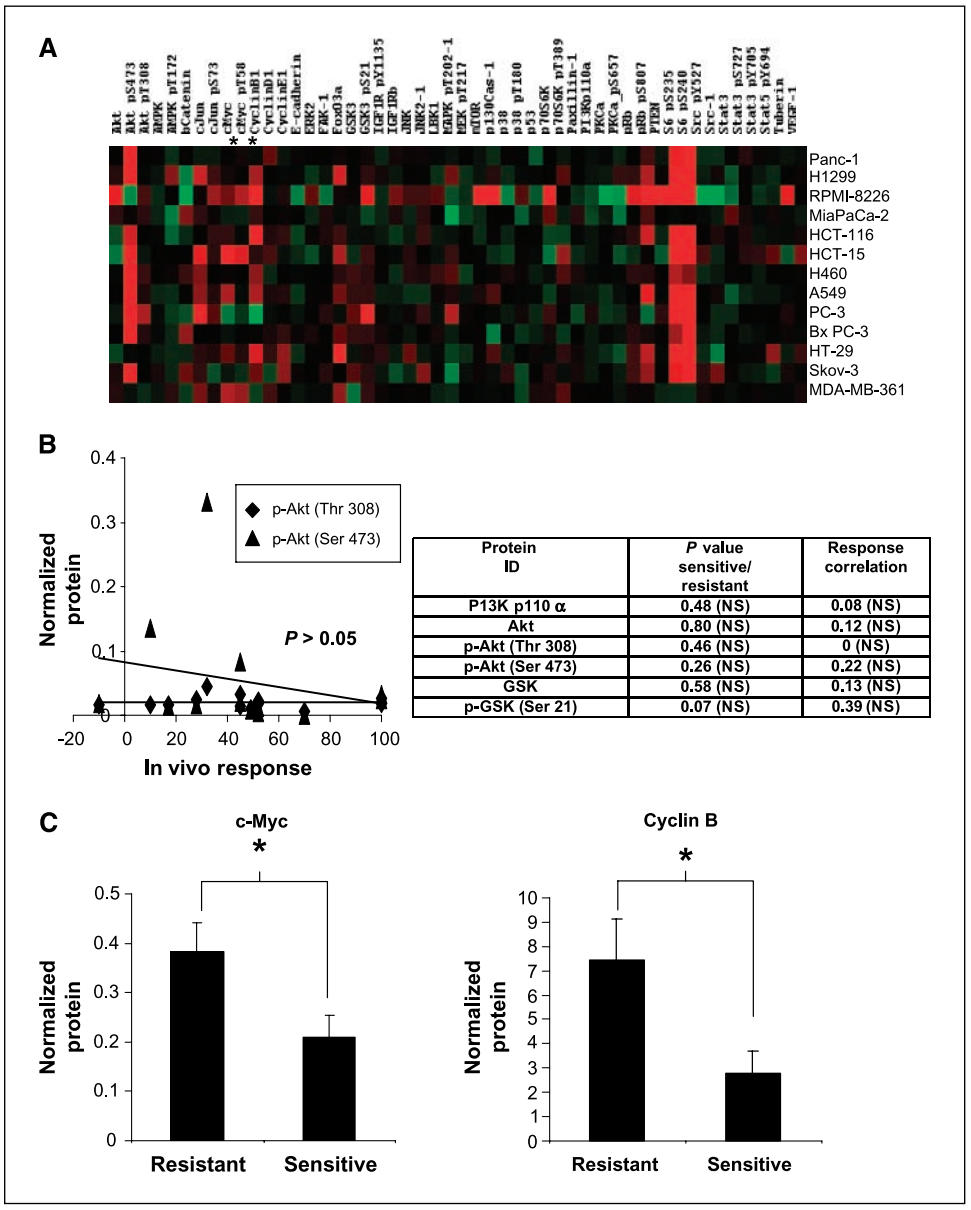
[5] http://www.sanger.ac.uk

and confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometric sequencing.

Tumor PI3K activity. Mice were killed 24 h after the last PX-866 treatment; the tumors were then excised and immediately frozen in liquid nitrogen. For the assay, the tumors were homogenized in lysis buffer containing 50 mmol/L HEPES (pH 7.5), 50 mmol/L NaCl, 0.2 mmol/L NaF, 0.2 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 20 μg/mL aprotinin, 20 μg/mL leupeptin, 1% NP40, and 0.25% sodium deoxycholate. Protein concentration was determined by bicinchoninic acid assay (Pierce Biotechnology) and 50 μg of cell lysate protein were boiled for 5 min with denaturing buffer containing 0.25 mol/L Tris (pH 6.8), 35% glycerol, 8% SDS, and 10% 2-mercaptoethanol, loaded on a 10% acrylamide/bisacrylamide gel, and separated by electrophoresis at 150 V for 40 min. Proteins were electrophoretically transferred to a polyvinylidene fluoride membrane; preincubated with a blocking buffer of 137 mmol/L NaCl, 2.7 mmol/L KCl, 897 mmol/L $CaCl_2$, 491 mmol/L $MgCl_2$, 3.4 mmol/L Na_2HPO_4, 593 mmol/L KH_2PO_4, and 5% bovine serum albumin; and incubated overnight with anti–phosphorylated Ser^{473}-Akt, anti-Akt, anti–phosphorylated Ser^{338}-Raf, anti-Raf, anti–cyclin B, anti–c-Myc polyclonal antibodies (Cell Signaling) or anti–β-actin (Santa Cruz Biotechnology). Donkey anti-rabbit IgG peroxidase-coupled secondary antibody (GE Healthcare) was used for detection. Band density was measured using the Renaissance chemiluminescence system on Kodak X-Omat Blue ML films (Eastman Kodak).

Reverse-phase protein array. Cells were lysed with buffer containing 150 mmol/L NaCl, 50 mmol/L HEPES (pH 7.4), 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 100 mmol/L NaF, 10 mmol/L sodium pyrophosphate, 10% glycerol, 1% Triton X-100 supplemented with Complete protease inhibitor cocktail tablets (Roche Applied Science), and cleared by centrifugation at 15,000 rpm for 10 min at 4°C. Samples were denatured by the addition of 1 part denaturing buffer to 3 parts cell lysate and boiling for 5 min. Sample concentrations were adjusted to 1 mg/mL with dilution buffer (1 part denaturing buffer and 3 parts cell lysis buffer) and printed as serial dilutions on glass slides, and specific proteins were quantified using 52 validated antibodies as previously described (27, 28).

Apoptosis measurement. Cells were treated with 0.5 μmol/L PX-866 for 48 h and harvested following 10 min of exposure to trypsin/EDTA at 37°C. Apoptotic cells that detached from the culture surface were collected by



Protein ID	*P* value sensitive/ resistant	Response correlation
P13K p110 α	0.48 (NS)	0.08 (NS)
Akt	0.80 (NS)	0.12 (NS)
p-Akt (Thr 308)	0.46 (NS)	0 (NS)
p-Akt (Ser 473)	0.26 (NS)	0.22 (NS)
GSK	0.58 (NS)	0.13 (NS)
p-GSK (Ser 21)	0.07 (NS)	0.39 (NS)

Figure 2. Protein analysis of sensitive and resistant cell lines. *A,* cell lines were analyzed by 52 validated antibodies in RPPA. Protein levels were quantified and arranged in a heat map: high expression (*red*), median expression (*black*), and low expression (*green*). *B,* analysis of expression of components of the PI3K/Akt pathway and correlation with *in vivo* antitumor response. *Bottom,* a plot of phosphorylated Akt levels against *in vivo* antitumor response. *C,* levels of c-Myc and cyclin B proteins showing differences between sensitive and resistant cell lines. *, $P < 0.05$.

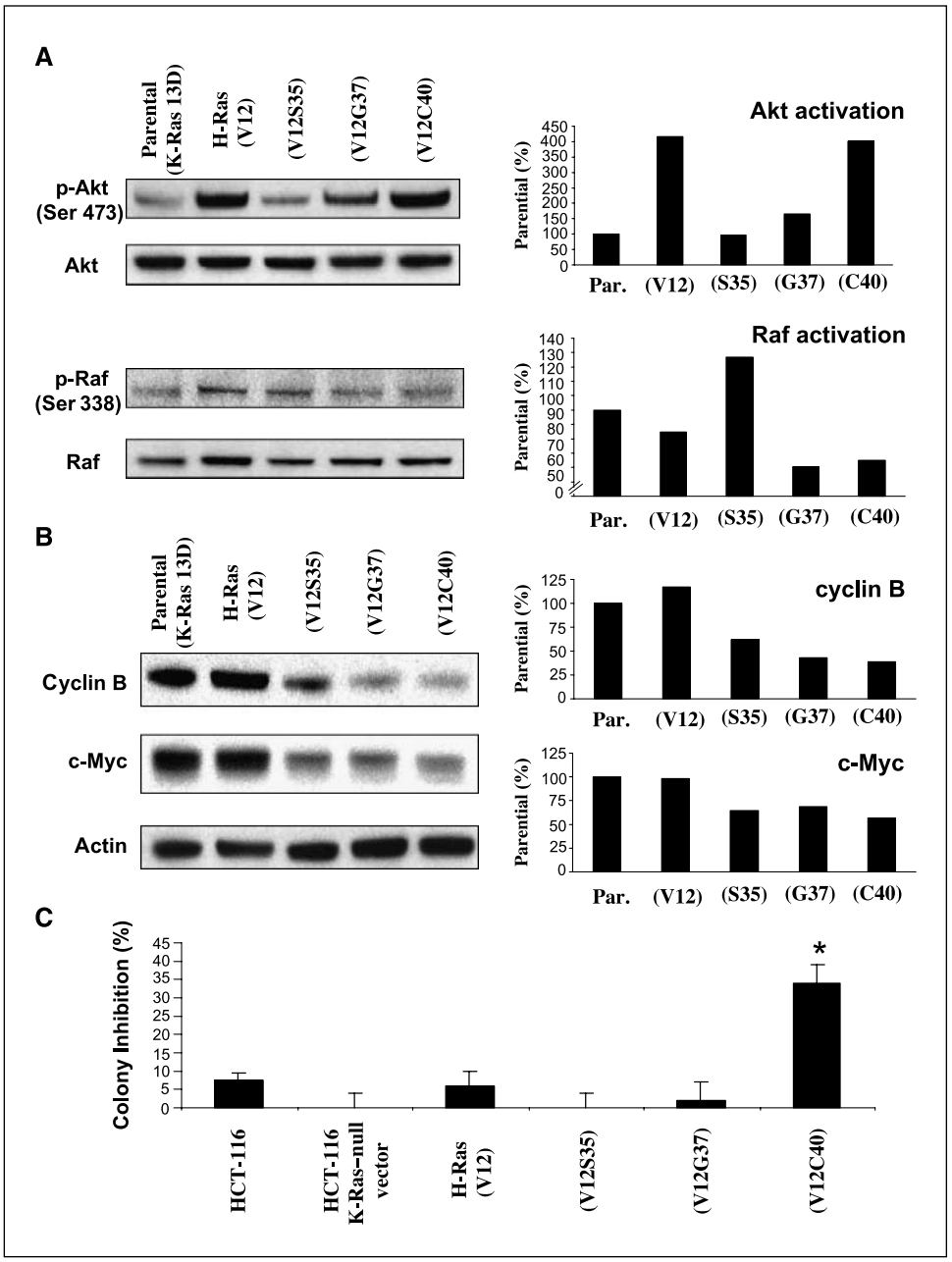


Figure 3. Signaling and clonogenic potential of HCT-116 H-Ras construct cells. *A,* measurement of cellular PI3K activity and Raf activation by Western blot analysis using phosphorylated Ser^{473}-Akt and phosphorylated Ser^{338}-Raf (*left*). Densitometric data from the blots (*right*). *B,* Western blot analysis for cellular cyclin B and c-Myc normalized against actin (*left*). Densitometric data from the blots (*right*). *C,* colony formation assay performed on H-Ras construct HCT-116 cells treated with 0.5 μmol/L PX-866. *, $P < 0.05$ compared with wild-type cells.

centrifugation of the medium at 1,500 rpm for 5 min. The pooled cell pellets were resuspended and mixed with trypan blue dye. Dye incorporation into nonviable cells was measured by counting 500 cells from randomly chosen fields with a light microscope and a hemocytometer and expressed as a percentage of the total number of cells counted. For confirmatory purposes, the extent of apoptosis was evaluated by assessing Hoechst- and TUNEL-stained cytospin slides under fluorescent light microscopy and scoring the number of cells exhibiting the classic morphologic features of apoptosis and necrosis. For each condition, 10 randomly selected fields per slide were evaluated, encompassing at least 1,500 cells. Alternatively, the Annexin V/propidium iodide assay was carried out to determine cell viability according to the instructions of the manufacturer (BD PharMingen) using a Becton Dickinson FACScan flow cytometer.

Colony formation. For studies of the effects of PX-866 on cell survival, 250 to 2,000 cells were plated in a 60-mm dish and 12 h later treated with 0.5 μmol/L PX-866 for 4 h. The medium was changed and the cells were grown for 10 to 14 d. After fixation and staining with crystal violet, colonies of more than 50 cells were counted using a ColCount colony counter (Oxford Optronics). Individual assays were performed at multiple dilutions with a total of six plates per data point.

Data analysis. Comparison of the effects of treatments and comparison of protein levels *in vitro* used a two-tailed *t* test. Differences with a $P < 0.05$ value were considered statistically significant. A two-tailed *t* test and a Pearson correlation was performed between normalized data obtained from the reverse-phase protein array (RPPA) analysis. For studies of the *in vivo* antitumor activity of PX-866, a Whitney Mann *U* test was performed using SPSS software (SPSS, Inc.).

Results

***In vivo* activity of PX-866.** The antitumor activity of PX-866 was measured in 13 human tumor cell line–derived xenografts in SCID

mice. These tumors were then classified into three groups: resistant tumors that showed minimal or no response (No response, T/C >70%), tumors that displayed a slowed but continued growth through treatment (Low response, T/C 35–69%), and sensitive tumors that displayed a antitumor response to PX-866 (Antitumor, T/C <35%), which included two that showed a cytostatic response and one that showed a regression. The mutation status of the cell lines was obtained from the Sanger Institute database and confirmed by mass spectral sequencing (Fig. 1*A*).

PC-3 prostate, BxPC3 pancreatic, HT-29 colon, Skov-3 ovarian, and MDA-MB-361 breast cancers were all sensitive to PX-866. PC-3 prostate cancer is PTEN-null whereas HT-29 colon, Skov-3 ovarian, and MDA-MB-361 breast cancers all have activating mutations in PIK3CA. HT-29 colon cancer has a coexisting activating mutation in B-Raf, but this was insufficient to cause resistance to PX-866 antitumor activity. Of the sensitive tumors, only BxPC3 has no reported mutation in the PI3K/Akt pathway.

All tumors that have an activating mutation in Ras displayed moderate to marked resistance to the antitumor activity of PX-866. This includes HCT-116, HCT-15, and H460 colon cancer, which have an activating PIK3CA mutation as well as an activating Ras mutation. The resistant lines A549 and H460 non–small cell lung cancers also have a mutation resulting in a dysfunctional LKB1 concurrent with an activating mutation in Ras. LKB1 is a known tumor suppressor that down-regulates mTor-mediated protein translation in the presence of low-energy conditions in the cell (29). How LKB1 contributes to the sensitivity of the tumors to PI3K inhibition has not been determined; however, in the two xenografts studied, the intermediate response to PX-866 observed was similar to tumors with a PIK3CA mutation together with oncogenic Ras. PX-866 was tested for its ability to inhibit tumor PI3K activity measured by the phosphorylation of Akt at Ser^{473} by Western blotting, which was found to be equally inhibited in tumors that showed varying degrees of sensitivity to PX-866 treatment (Fig. 1*B*). Thus, RAS mutation seems to be an indicator of resistance to PX-866, which is dominant over the sensitizing effects of PI3K pathway mutations.

PX-866–resistant cells display characteristics of Ras-transformed cells. We next used RPPA technology to address whether response to PX-886 was dependent on the level of expression or activation of proteins of the PI3K/Akt pathway (Fig. 2*A*). Neither PI3K protein levels nor AKT activation measured by Thr^{308} or Ser^{473} phosphorylation, nor the phosphorylation of

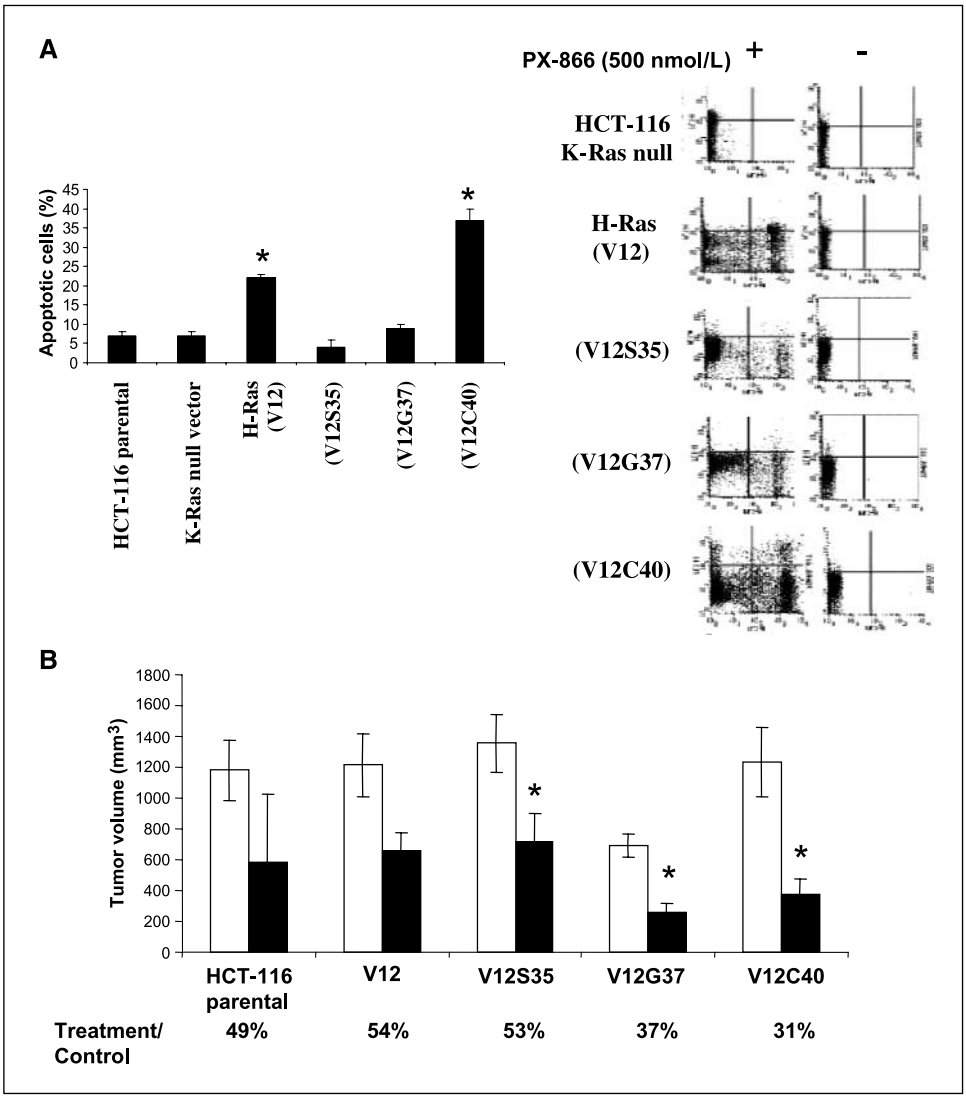


Figure 4. Apoptosis and *in vivo* effects of PX-866 on HCT-116 H-Ras construct cells. *A,* trypan blue and flow cytometry analysis of Annexin-positive cells treated with 0.5 μmol/L PX-866. *, $P < 0.05$ compared with wild-type cells. *B,* comparison of final volumes of vehicle (*white columns*) or PX-866 (*black columns*) treated tumors. *, $P < 0.05$ of treated compared with control cells.

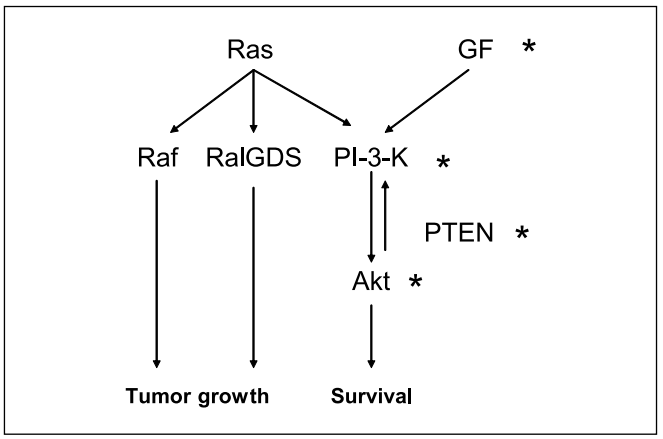


Figure 5. Signaling in resistant and sensitive lines. Diagram showing the interactions of the cell signaling studied in sensitive and resistant lines. Signaling in PX-866–sensitive tumors comes from an increased reliance on the PI3K pathway, arising from aberrant activation through growth factors (*GF*) or mutated components of the pathway itself (*). Tumors with an activated Ras protein show a minimal response to inhibition of the PI3K pathway due to a shared reliance on alternate signaling pathways including the Raf and RalGDS pathways.

the downstream Akt target GSK-3, were significantly altered in sensitive compared with resistant lines, or when correlated with *in vivo* antitumor response (Fig. 2*B*). Two proteins on the array showed a significant difference between sensitive and resistant lines, with levels of c-Myc and cyclin B being significantly higher in lines resistant to PX-866 *in vivo* (Fig. 2*C*). It is noteworthy that an increase in both of these proteins has been reported as a result of ras-induced transformation (30–32).

H-Ras or H-Ras mutants preferentially activating Raf or RalGDS, but not RAS mutants linked to PI3K, are resistant to PX-866 *in vitro* and *in vivo*. Wild-type HCT-116 K-Ras–positive cells; HCT-116 K-Ras–null cells and K-Ras–null cells constitutively expressing an active H-Ras; an H-Ras modified to preferentially activate Raf (H-Ras V12S35), RalGDS (H-Ras V12G37), or PI3K (H-Ras V12C40) were used as a model for the simultaneous and individual activation of proteins effected by Ras signaling as previously described (33). This model was used to determine p-AKT and p-Raf activation (Fig. 3*A*). HCT-116 cells with activated H-Ras, and PI3K-activating H-Ras, showed a robust activation of PI3K signaling measured by phosphorylated Ser^{473}-Akt. H-Ras V12 and H-Ras V12C40 displayed similar levels of Akt activation. H-Ras–activating RalGDS also retained the ability to activate Akt, which has been recently reported (34). Wild-type HCT-116, H-Ras V12, and H-Ras–activating Raf all showed similar levels of Raf activation. H-Ras–activating Ral and H-Ras–activating PI3K showed ~40% decrease in Raf signaling. These cell lines were then probed for cyclin B and c-Myc, which in our RPPA experiment had been shown to be associated with resistance to PI3K inhibition. Both wild-type HCT-116 and the K-Ras–null HCT-116 transfected with activated H-Ras showed a robust expression of cyclin B, as did the H-Ras–activating Raf, whereas the H-Ras linked to RalGDS and PI3K showed lower levels of activation (Fig. 3*B*). Wild-type HCT-116 and the K-Ras–null HCT-116 transfected with activated H-Ras showed high levels of c-Myc protein, whereas the three conditional Ras lines all showed lower levels. Despite slight differences, all the selective H-Ras constructs retained the ability to activate c-Myc or cyclin B to some extent. The lines were also studied for their sensitivity to PX-866 as measured by colony formation (Fig. 3*C*). K-Ras–null cells, H-Ras, Raf, and RalGDS activated cell lines behaved similar to the wild-type HCT-116 (mutant K-Ras, mutant PIK3CA) line when treated with PX-866. In contrast, an H-Ras mutant that preferentially activates PI3K without activating RalGDS or Raf showed significant inhibition of colony formation by PX-866.

Apoptosis was measured in the cell lines both by trypan blue assay and flow cytometry. Cells with active Raf and RalGDS lines showed levels of apoptosis similar to wild-type HCT-116 cells, whereas H-Ras cells showed a moderate but significant increase in apoptosis. In contrast, H-Ras cells with active PI3K, but not Raf or RalGDS activation, showed a large and significant increase in apoptosis (Fig. 4*A*).

Tumors lacking the mutant K-Ras allele have previously been shown to be nontumorigenic (22), indicating that ras is a dominant tumorigenic factor in this cell line. Tumors derived from implanted ras-driven cell lines treated with vehicle or 2.5 mg/kg PX-866 were measured at the final day of treatment (Fig. 4*B*). The T/C percentage for the wild-type HCT-116 cells was 49% for K-Ras–null tumors, 54% for H-Ras tumors, and 53% ($P = 0.05$) for Raf-driven tumors. RalGDS-driven tumors showed a significant decrease in T/C to 37% ($P = 0.01$), and PI3K-driven tumors showed the greatest sensitivity with a T/C value of 31% ($P = 0.02$).

Discussion

The PI3K/Akt signaling pathway is critical for cancer cell growth and survival (1) and a number of inhibitors of PI3K or Akt have been, or will soon be, introduced into clinical trials as antitumor agents (13). Determining which patients respond to these drugs will play a role in how, and at what pace, they move through clinical development (35). Our *in vivo* antitumor studies in a panel of 13 molecularly characterized human tumor cell line–derived xenografts found that tumors with mutant PIK3CA or PTEN-null, but without mutant Ras, were sensitive to PX-866. The three most sensitive lines, which displayed a cytostatic or regression response, had activating mutations in PI3K. It is noteworthy that an activating mutation in Raf in the HT-29–derived xenograft, together with a PIK3CA-activating mutation, was insufficient to reverse the cytostatic effects of PX-866. The BxPC3 pancreatic cancer cell line with no reported mutations in the PI3K/Akt pathway also showed a cytostatic response to PX-866. This cell line has been characterized as having an inactivating mutation in Smad 4, which has recently been shown to allow these cells to down-regulate PTEN and thus activate PI3K signaling (36). This illustrates that whereas individual mutations or deletions in the PI3K/Akt/PTEN signaling pathway may be sufficient to predict response to PX-866, they are not necessary for response as multiple inputs could influence this pathway. Most importantly, we found that mutant oncogenic Ras is a negative predictor of response to the PI3K inhibitor PX-866 in xenografts, even those with concurrent activating mutations in PI3K; thus, PI3K mutation cannot be used as an individual marker for sensitivity.

RPPA technology was used to measure protein levels and activation in the cell lines *in vivo*. Several proteins known to be directly involved in PI3K/Akt signaling were studied, including PIK3CA, Akt, and GSK. Neither total nor phosphoprotein levels were significantly different between sensitive and resistant lines nor was there a significant association with the *in vivo* antitumor response. Thus, the expression level and activation of these PI3K/

Akt pathway proteins *in vitro* under basal conditions does not translate into *in vivo* tumor sensitivity. Although this finding excludes the level of Akt Thr^{308} or Ser^{473} phosphorylation as a predictive biomarker, AKT phosphorylation remains a surrogate end point for measuring the efficacy of target inhibition as phosphorylation of Akt was inhibited by PX-866 *in vivo*, independent of the sensitivity or resistance of the tumor to PX-866 in terms of its growth. Cyclin B and c-Myc were shown to be significantly overexpressed in cell lines forming PX-866–resistant xenografts compared with cell lines showing sensitivity to PX-866. In previous studies, oncogenic Ras has been shown to have the ability to up-regulate total c-Myc levels both through an increase in mRNA levels (31) and increases in protein stability (32). Cyclin B was increased in cells resistant to PX-866 treatment and showed a significant negative association with antitumor response. Cyclin B has been shown to be up-regulated during Ras-induced transformation and is associated with an increased mitotic rate. Whether cyclin B and c-Myc act as factors contributing to the resistance of mutant Ras tumors to PX-866, or only serve as markers of mutant Ras, is not known. Additionally, the data derived from the H-Ras lines possessing the ability to activate specific downstream pathways showed that all three pathways studied are capable of activating cyclin B and c-Myc. Therefore, it is possible that the robust expression of these proteins in the mutant Ras lines in the RPPA may come from a cooperative effect between the pathways.

In colony formation assays, the HCT-116 H-Ras line with specific activation of PI3K was found to be the only line sensitive to the effects of PI3K inhibition, indicating that this line had been made more dependent on PI3K signaling. Cells with the wild-type H-Ras showed greater amounts of apoptosis than cells with mutant K-Ras, which may reflect differences between the two Ras isoforms in their utilization of downstream signaling (37), or the ability of parental K-Ras to use less characterized pathways downstream of Ras. This also suggests that the slowing of growth seen in mutant Ras tumors *in vivo* following PX-866 treatment may be a result of increased apoptosis. The highest apoptosis was seen in the H-Ras cell line selectively activating PI3K, as it lacked the resistance provided by parallel signaling pathways. Of interest, HCT-116 K-Ras–null cells that retain a mutant PI3K were not sensitized to PX-866, suggesting that in cell lines arising from a Ras mutation, in contrast to tumors with exclusive PI3K mutations, the input from Ras may be essential for PI3K signaling to be used in tumorigenic processes. This observation agrees with previous studies which found that despite this PI3K heterozygous mutation, to sensitize HCT-116 to the effects of PI3K inhibitors in colony formation and growth inhibition assays, a homozygous PIK3CA variant needs to be created and the assays performed in low serum conditions. Additionally, HCT-116 cells retaining mutant K-Ras but with only a homozygous wild-type PI3K were able to form tumors (38). In contrast, K-Ras–null HCT-116 cells have been shown to lack the ability to form tumors (22).

When injected into mice, the oncogenic H-Ras and selective H-Ras cells all retained the ability to form tumors. Despite both having increased Akt activity compared with the parental HCT-116 line, the cells with an active H-Ras responded similarly to PX-866 as the parental HCT-116, whereas PI3K-activated H-Ras cells showed a 23% greater response to PX-866 than control cells. H-Ras cells displaying low levels of Akt activity than the other constructs showed activity similar to the parental HCT-116 or HCT-116 H-Ras lines. The H-Ras line specific for RalGDS signaling showed an intermediate response, with a 17% increase in activity of PX-866 against the tumor. Together, these results show that the magnitude of Akt activation does not determine the response to PI3K inhibition but that activation of pathways downstream of oncogenic Ras, parallel or compensatory for active PI3K, can rescue tumors from inhibition.

Mutant active Ras has recently been described to predict resistance to small-molecule inhibitors and antibodies against the epidermal growth factor receptor (39, 40), presumably due to the downstream location of Ras in epidermal growth factor receptor signaling (41). Additionally, Ras-driven cell lines exhibit a modest response to MEK inhibitors, showing a growth delay when grown as xenografts, whereas B-Raf–driven xenografts display a cytostatic response (42). This is similar to our observation with PX-866 in the context of PI3K signaling and derive from the ability of Raf and PI3K to converge on redundant downstream mediators, "funnel factors" of translation, such as eiF4e (43), survival factors such as Bad (44), and cyclin D for cell cycle progression (42). Ultimately, treatment of Ras-driven tumors may lie in the direct inhibition of the active Ras protein itself, or the combination of PI3K inhibitors, including PX-866, with other agents targeting end points of the Ras pathway.

The relationships of the signaling pathways we have studied are shown in Fig. 5. Oncogenic Ras effectively negates the effects of other potential predictors such as mutant PIK3CA, which has been proposed to be a positive marker of response to inhibitors of PI3K/Akt signaling and decreases the reliance of the tumor on PI3K signaling, in favor of other Ras-dependent signaling pathways. Thus, it may be necessary to know the mutational status of K-Ras, PIK3CA, and PTEN as markers for predicting response to PI3K inhibition. This information may have considerable significance in tumor types, such as ovarian, endometrial, and colon tumors in which mutations in PIK3CA or PTEN and Ras have been found to coexist at relevant rates (45, 46).

In summary, we have studied the activity of the PI3K inhibitor PX-866 in a panel of tumor cell line–derived xenografts and have shown mutant oncogenic Ras to be a negative predictor of response to PX-866, both independently and in the presence of positive prognostic indictors such as PI3KCA and loss of PTEN activity. Known effects of Ras-induced transformation such as increased cyclin B and c-Myc were also negatively associated with antitumor response to PX-866 The level of activation of PI3K signaling measured with phosphorylated Akt was not sufficient to predict *in vivo* antitumor response to PX-866. Ras constructs modified to activate specific components of the Ras signaling pathway showed that multiple pathways are used for growth and survival both *in vitro* and *in vivo* in Ras-dependent signaling.

Disclosure of Potential Conflicts of Interest

G. Powis is a founder of, owns stock in, and is a consultant to Oncothyreon, Inc., which is developing PX-866. L. Kirkpatrick is an employee of and owns stock in Oncothyreon, Inc. The other authors declared no potential conflicts of interest.

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References

1. Cantley LC. The phosphoinositide 3-kinase pathway. Science 2002;296:1655–7.
2. Alessi DR, Cohen P. Mechanism of activation and function of protein kinase B. Curr Opin Genet Dev 1998; 8:55–62.
3. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 2005;307:1098–101.
4. Meier R, Alessi DR, Cron P, Andjelkovic M, Hemmings BA. Mitogenic activation, phosphorylation, and nuclear translocation of protein kinase Bβ. J Biol Chem 1997; 272:30491–7.
5. Nicholson KM, Anderson NG. The protein kinase B/Akt signalling pathway in human malignancy. Cell Signal 2002;14:381–95.
6. Moore SM, Rintoul RC, Walker TR, Chilvers ER, Haslett C, Sethi T. The presence of a constitutively active phosphoinositide 3-kinase in small cell lung cancer cells mediates anchorage-independent proliferation via a protein kinase B and p70s6k-dependent pathway. Cancer Res 1998;58:5239–47.
7. Shayesteh L, Lu Y, Kuo WL, et al. PIK3CA is implicated as an oncogene in ovarian cancer. Nat Genet 1999;21: 99–102.
8. Soltoff SP, Rabin SL, Cantley LC, Kaplan DR. Nerve growth factor promotes the activation of phosphatidylinositol 3-kinase and its association with the trk tyrosine kinase. J Biol Chem 1992;267:17472–7.
9. Campbell PM, Groehler AL, Lee KM, Ouellette MM, Khazak V, Der CJ. K-Ras promotes growth transformation and invasion of immortalized human pancreatic cells by Raf and phosphatidylinositol 3-kinase signaling. Cancer Res 2007;67:2098–106.
10. Samuels Y, Ericson K. Oncogenic PI3K and its role in cancer. Curr Opin Oncol 2006;18:77–82.
11. Wu X, Senechal K, Neshat MS, Whang YE, Sawyers CL. The PTEN/MMAC1 tumor suppressor phosphatase functions as a negative regulator of the phosphoinositide 3-kinase/Akt pathway. Proc Natl Acad Sci U S A 1998;95:15587–91.
12. Carpten JD, Faber AL, Horn C, et al. A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. Nature 2007;448:439–44.
13. Cheng JQ, Lindsley CW, Cheng GZ, Yang H, Nicosia SV. The Akt/PKB pathway: molecular target for cancer drug discovery. Oncogene 2005;24:7482–92.
14. Gharbi SI, Zvelebil MJ, Shuttleworth SJ, et al. Exploring the specificity of the PI3K family inhibitor LY294002. Biochem J 2007;404:15–21.
15. Bain J, Plater L, Elliott M, et al. The selectivity of protein kinase inhibitors: a further update. Biochem J 2007;408:297–315.
16. Wipf P, Minion DJ, Halter RJ, et al. Synthesis and biological evaluation of synthetic viridins derived from C(20)-heteroalkylation of the steroidal PI-3-kinase inhibitor wortmannin. Org Biomol Chem 2004;2:1911–20.
17. Ihle NT, Paine-Murrieta G, Berggren MI, et al. The phosphatidylinositol-3-kinase inhibitor PX-866 overcomes resistance to the epidermal growth factor receptor inhibitor gefitinib in A-549 human non-small cell lung cancer xenografts. Mol Cancer Ther 2005;4: 1349–57.
18. Ihle NT, Williams R, Chow S, et al. Molecular pharmacology and antitumor activity of PX-866, a novel inhibitor of phosphoinositide-3-kinase signaling. Mol Cancer Ther 2004;3:763–72.
19. Mehrian-Shai R, Chen CD, Shi T, et al. Insulin growth factor-binding protein 2 is a candidate biomarker for PTEN status and PI3K/Akt pathway activation in glioblastoma and prostate cancer. Proc Natl Acad Sci U S A 2007;104:5563–8.
20. Saal LH, Johansson P, Holm K, et al. Poor prognosis in carcinoma is associated with a gene expression signature of aberrant PTEN tumor suppressor pathway activity. Proc Natl Acad Sci U S A 2007;104:7564–9.
21. Akashi T, Nishimura Y, Wakatabe R, Shiwa M, Yamori T. Proteomics-based identification of biomarkers for predicting sensitivity to a PI3-kinase inhibitor in cancer. Biochem Biophys Res Commun 2007;352:514–21.
22. Shirasawa S, Furuse M, Yokoyama N, Sasazuki T. Altered growth of human colon cancer cell lines disrupted at activated Ki-ras. Science 1993;260:85–8.
23. White MA, Nicolette C, Minden A, et al. Multiple Ras functions can contribute to mammalian cell transformation. Cell 1995;80:533–41.
24. Khosravi-Far R, White MA, Westwick JK, et al. Oncogenic Ras activation of Raf/mitogen-activated protein kinase-independent pathways is sufficient to cause tumorigenic transformation. Mol Cell Biol 1996; 16:3923–33.
25. Hamad NM, Elconin JH, Karnoub AE, et al. Distinct requirements for Ras oncogenesis in human versus mouse cells. Genes Dev 2002;16:2045–57.
26. Lim KH, Counter CM. Reduction in the requirement of oncogenic Ras signaling to activation of PI3K/AKT pathway during tumor maintenance. Cancer Cell 2005;8: 381–92.
27. Nishizuka S, Charboneau L, Young L, et al. Proteomic profiling of the NCI-60 cancer cell lines using new high-density reverse-phase lysate microarrays. Proc Natl Acad Sci U S A 2003;100:14229–34.
28. Hu J, He X, Baggerly KA, Coombes KR, Hennessy BT, Mills GB. Non-parametric quantification of protein lysate arrays. Bioinformatics 2007;23:1986–94.
29. Shaw RJ, Bardeesy N, Manning BD, et al. The LKB1 tumor suppressor negatively regulates mTOR signaling. Cancer Cell 2004;6:91–9.
30. Santana C, Ortega E, Garcia-Carranca A. Oncogenic H-ras induces cyclin B1 expression in a p53-independent manner. Mutat Res 2002;508:49–58.
31. Lloyd AC, Paterson HF, Morris JD, Hall A, Marshall CJ. p21H-ras-induced morphological transformation and increases in c-myc expression are independent of functional protein kinase C. EMBO J 1989;8:1099–104.
32. Sears R, Leone G, DeGregori J, Nevins JR. Ras enhances Myc protein stability. Mol Cell 1999;3:169–79.
33. Martin AP, Miller A, Emdad L, et al. Lapatinib resistance in HCT116 cells is mediated by elevated MCL-1 expression, decreased BAK activation, and not by ERBB receptor mutation. Mol Pharmacol 2008;74:807–22.
34. Hao Y, Wong R, Feig LA. RalGDS couples growth factor signaling to Akt activation. Mol Cell Biol 2008;28: 2851–9.
35. Dietel M. Predictive medicine: incipient reality or fata morgana? J Pathol 2007;212:353–5.
36. Chow JY, Dong H, Quach KT, Van Nguyen PN, Chen K, Carethers JM. TGF-β mediates PTEN suppression and cell motility through calcium-dependent PKC-α activation in pancreatic cancer cells. Am J Physiol Gastrointest Liver Physiol 2008; 294:G899–905.
37. Caron RW, Yacoub A, Li M, et al. Activated forms of H-RAS and K-RAS differentially regulate membrane association of PI3K, PDK-1, and AKT and the effect of therapeutic kinase inhibitors on cell survival. Mol Cancer Ther 2005;4:257–70.
38. Samuels Y, Diaz LA, Jr., Schmidt-Kittler O, et al. Mutant PIK3CA promotes cell growth and invasion of human cancer cells. Cancer Cell 2005;7:561–73.
39. Massarelli E, Varella-Garcia M, Tang X, et al. KRAS mutation is an important predictor of resistance to therapy with epidermal growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancer. Clin Cancer Res 2007;13:2890–6.
40. Khambata-Ford S, Garrett CR, Meropol NJ, et al. Expression of epiregulin and amphiregulin and K-ras mutation status predict disease control in metastatic colorectal cancer patients treated with cetuximab. J Clin Oncol 2007;25:3230–7.
41. Qin B, Ariyama H, Baba E, et al. Activated Src and Ras induce gefitinib resistance by activation of signaling pathways downstream of epidermal growth factor receptor in human gallbladder adenocarcinoma cells. Cancer Chemother Pharmacol 2006;58:577–84.
42. Solit DB, Garraway LA, Pratilas CA, et al. BRAF mutation predicts sensitivity to MEK inhibition. Nature 2006;439:358–62.
43. Armengol G, Rojo F, Castellvi J, et al. 4E-binding protein 1: a key molecular "funnel factor" in human cancer with clinical implications. Cancer Res 2007;67: 7551–5.
44. She QB, Solit DB, Ye Q, O'Reilly KE, Lobo J, Rosen N. The BAD protein integrates survival signaling by EGFR/MAPK and PI3K/Akt kinase pathways in PTEN-deficient tumor cells. Cancer Cell 2005;8:287–97.
45. Thomas RK, Baker AC, Debiasi RM, et al. High-throughput oncogene mutation profiling in human cancer. Nat Genet 2007;39:347–51.
46. Oda K, Stokoe D, Taketani Y, McCormick F. High frequency of coexistent mutations of PIK3CA and PTEN genes in endometrial carcinoma. Cancer Res 2005;65: 10669–73.

Mutations in the Phosphatidylinositol-3-Kinase Pathway Predict for Antitumor Activity of the Inhibitor PX-866 whereas Oncogenic Ras Is a Dominant Predictor for Resistance

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