

Key Role of Phosphoinositide 3-Kinase Class IB in Pancreatic Cancer

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Abstract

Purpose: Phosphoinositide 3-kinase (PI3K) signaling is well established as important in cancer. To date most studies have been focused on the PI3K/p110 α isoform, which has been found to be mutated in several different cancers. The aim of our study was to determine which specific PI3K isoforms are involved in pancreatic ductal adenocarcinoma (PDAC) and investigate the effects of these isoforms on proliferation, survival, and induction of Akt activation in pancreatic cancer cells.

Experimental Design: The expression of all PI3K isoforms and downstream targets was analyzed by immunohistochemistry in human pancreatic cancer tissue and normal counterparts. Isoform selective inhibitors and short interfering RNA (siRNA) were employed to investigate the effects of the different PI3Ks on proliferation, survival, and intracellular signaling in PDAC cell lines.

Results: Immunohistochemical screening revealed high specific expression of the PI3K/p110 γ isoform. Scoring indicated that 72% of the PDAC tissue stained positive for PI3K/p110 γ , whereas no stain was detected in normal pancreatic ducts. Proliferation analyses after selective inhibition and siRNA downregulation of PI3K/p110 γ showed that PI3K/p110 γ , but not other PI3K isoforms, was required for cell proliferation. Overexpression of PI3K/p110 γ indeed increased cell numbers and mediated activation of Akt in PDAC cell lines. Moreover, PI3K/p110 γ was required for Akt activation via lysophosphatidic acid receptors.

Conclusions: These data represent the first identification of a tumor-specific accumulation of the PI3K isoform p110 γ in human cancer. Further, our results signify a critical role for PI3K/p110 γ in pancreatic cancer, and we hypothesize that PI3K/p110 γ overexpression is a key event in the disease progression. *Clin Cancer Res*; 16(20); 4928–37. ©2010 AACR.

Pancreatic cancer has one of the poorest prognoses among all cancers because of its tendency for late discovery and its peculiar resistance to chemotherapy and radiation therapy (1, 2). The 5-year survival rate for pancreatic cancer is the lowest among all cancers (3). Although pancreatic cancer is well characterized at genetic level, the molecular mechanisms linking the genetic changes to the

aggressive nature of this disease remain poorly understood. Studies using pancreatic tumor tissues and pancreatic cancer cell lines have identified a number of common genetic and epigenetic events. Mutations in *K-Ras* oncogene accumulate early in the disease progression and occur in 75% to 90% of pancreatic adenocarcinomas (4, 5). A key downstream target of the Ras family is phosphoinositide 3-kinase (PI3K), the enzyme responsible for generation of 3-phosphorylated phosphoinositides and activation of the protein kinase B/Akt (6–9). Activation of Akt represents a biological indicator of the aggressiveness of pancreatic cancer (10–12), and preclinical studies have shown that generic inhibitors of PI3K, such as wortmannin and LY294002, induce apoptosis and inhibit tumor growth of pancreatic cancer xenografts (13). In addition to activated Akt in general, the Akt2 isoform has been shown to be overexpressed to some extent (10, 14). It is yet not known what specifically activates Akt in pancreatic cancer, but there are no reports suggesting that Akt itself is mutated (15–17).

The PI3K/Akt pathway is involved in inhibition of apoptosis and stimulation of cell proliferation (9, 18). It is now well known that deregulation of intracellular signaling

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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doi: 10.1158/1078-0432.CCR-10-1210

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Translational Relevance

In this study we screened pancreatic ductal adenocarcinomas and normal counterparts for constitutive expression of the different phosphoinositide 3-kinase (PI3K) isoforms, and we here reveal selective tumoral overexpression of the PI3K class IB catalytic subunit p110 γ . Further, we show that PI3K/p110 γ is necessary for pancreatic cancer cell line proliferation, indicating that overexpression of this specific PI3K isoform has a functional role in pancreatic cancer progression. These results suggest PI3K/p110 γ as a rational target for development of novel therapeutic agents.

mediated by the family of PI3K enzymes is associated with cancer development, such that up to 50% of all cancer types exhibit deregulation of this signaling pathway (19). Three classes of PI3Ks have been described (20, 21), with PI3K class I divided into two groups: class IA and class IB. Class IA encompasses three isoforms of the catalytic subunit known as p110 α , p110 β , and p110 δ , whereas class IB has the single p110 γ . Gain of p110 α function by mutation is common in several human cancers (22, 23). The potential contribution to cancer initiation and progression by the other isoforms has not been fully investigated. Although no somatic mutations of the genes encoding p110 β , p110 γ , and p110 δ have been reported so far, an increased expression of the p110 β and p110 δ isoforms has been observed in some colon and bladder tumors, and in glioblastoma (24, 25). The p110 β isoforms has been shown to stimulate cell proliferation and invasive cell growth (26); p110 δ controls proliferation in acute myeloid leukemia (27) and migration of breast cancer cells (28), whereas a role has been suggested for p110 γ in tumor angiogenesis (29) and drug resistance of chronic myeloid leukemia cells (30). Interestingly, it has been reported that overexpression of wild-type p110 α does not seem to have transforming potential, whereas overexpression of the wild-type catalytic subunits p110 β , p110 γ , or p110 δ is sufficient to induce an oncogenic phenotype in cultured cells (31). It has further been suggested that the transforming activity of the p110 γ isoform depends on Ras binding (31). These data suggest that although the oncogenic potential of p110 α is associated with mutations of the gene, elevated levels of wild-type p110 β , p110 γ , or p110 δ might induce cellular transformation on their own.

Materials and Methods

Immunohistochemistry

Informed consent was obtained as appropriate. Paraffin-embedded tissues were used for identification of indicated proteins in human cancer and donor samples. Briefly, deparaffinized and rehydrated sections were incubated in blocking solution (TBS, 3% bovine serum albumin) and

sequentially incubated with primary antibodies [p110 α , p110 β , p110 γ , and p110 δ (Santa Cruz Biotechnology); anti-pSer473-Akt and phosphatase and tensin homolog deleted on chromosome ten (PTEN; Cell Signaling Technology); anti-PI3K-C2 α and anti-PI3K-C2 β (BD Transduction Laboratories); and anti-phosphatidylinositol 3,4,5-trisphosphate (PIP $_3$), kindly provided by Prof Glenn Prestwich (University of Utah)], secondary biotinylated goat anti-rabbit, anti-mouse antibodies, and peroxidase-labeled streptavidin. Positive reactions were visualized by diaminobenzidine/hydrogen peroxide (DAKO) as substrate. The sections were counterstained with hematoxylin (Sigma). Control samples exposed to secondary antibodies alone did not show nonspecific staining.

Scoring of immunohistochemistry

The degree of staining (0, none; 1, weak; 2, moderate; 3, strong) and the number of positive ducts (0, 0-10%; 1, 10-25%; 2, 25-50%; 3, 50-75%; 4, 75-100%) were manually scored in samples stained for p110 γ (12 control and 29 cancer samples) and p110 α and p110 β (11 control and 22 cancer samples). The combined score for each sample translated to the sum of the scores for degree of staining and number of positive ducts.

Cell lines

Cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin-streptomycin-L glutamine, and gentamicin, all from Invitrogen. The cell lines ASPC1, PANC1, BXPC3, Capan1, HPAF, and PT45 had been short tandem repeat (STR) genotyped and confirmed identical to the published DNA profiles of the American Type Culture Collection. The PT45 was identical to an original vial from Dr. Kalthoff who established the cell line. The cell lines Dec-hTert and HPDE had been characterized previously and their authenticity was confirmed by Dr. Kocher. The cell lines GER, PaCa44, PSN1, MCC1, and CFPac were kindly provided by Prof. Aldo Scarpa (University of Verona, Italy) and had been characterized by his lab (32).

Real-time PCR analysis

Real-time PCR analyses were done on an Applied Biosystems 7500. Total RNA was isolated using RNeasy minikit (Qiagen). Superscript cDNA synthesis kit (Invitrogen) was used for cDNA preparation with random hexamers. The cDNA was amplified using Maxima SYBR (Fermentas) using primers for indicated genes (Supplementary Table S1). Results are presented as the fold expression compared with control as indicated in the figures, calculated with the 2(-ddCt) method (33) using Glutamyl-tRNA synthetase (Qars) as reference gene.

Transfection of short interfering RNA and plasmid DNA

Cells were transfected with human p110 γ plasmid, kindly provided by Prof. B. Vanhaesebroeck (Queen Mary University London, United Kingdom), and pcDNA3 empty vector using Lipofectamine 2000 and Smartpool siRNA targeting p110 α , p110 β , p110 δ , or p110 γ (Thermo

Fischer Scientific) using Oligofectamine according to Invitrogen's respective protocols (Invitrogen). Transfection efficiency of siRNA was verified with real-time PCR analysis and Western blotting (Supplementary Fig. S3A-C and Fig. 6).

Cell counting and MTT assay

Cells were treated as indicated in the figures with inhibitors AS252424, TGX-221, IC87114 (kind gift from Prof. P.R. Shepherd, University of Auckland, New Zealand), and LY294002 (Calbiochem, Merck) or cells transfected with the indicated siRNA/DNA. The inhibitor concentrations used were chosen so as to be specific according to published IC₅₀ values (34). Cells were counted on a CASY cell counter at indicated time points. MTT (Thiazolyl Blue Tetrazolium Bromide, M2128, Sigma) was diluted in experiment-specific culture media (0.5 mg/mL), incubated 2 hours, and dissolved in DMSO before the absorbance was read at 570 nm.

Propidium iodide incorporation and Annexin V staining

For the flow cytometry analysis cells were pretreated as described in the figures. Subsequently, cells were incubated for 30 minutes in propidium iodide (PI) solution [20 mmol/L Tris-HCL (pH 7.5), 100 mmol/L NaCl, 50 mg/mL PI, 0.1% Nonidet P40, and 20 µg/mL RNase] or stained with Annexin V and PI according to kit protocol (BD biosciences) before analysis on a Becton Dickinson LSR II analyzer.

Caspase3 assay

Cells were treated as described and the caspase-3 activity was assayed according to manufacturer's protocol using the EnzChek Caspase-3 Assay Kit 2 (Invitrogen).

Western blotting

Cell pellets were prepared from the indicated cell lines cultured in normal growth media and lysed in buffer [150 mmol/L NaCl, 0.5% Triton X-100, 10 mmol/L Tris (pH 7.6), 5 mmol/L EDTA (pH 8.0), 30 mmol/L sodium pyrophosphate, 1 mmol/L sodium orthovanadate, 50 mmol/L sodium fluoride, 10% glycerol, 10 µg/mL aprotinin, 10 µg/mL leupeptin, and 100 mmol/L phenylmethylsulfonyl fluoride) or in β-mercaptoethanol sample buffer. Complete lysates were separated on SDS-PAGE. For stimulation experiments cells were transfected with siRNA 72 hours prior to overnight starvation and then stimulated for 15 minutes. Alternatively, cells were starved overnight prior to a 30-minute pretreatment with inhibitors and a 15-minute stimulation with 25 µmol/L lysophosphatidic acid (LPA; Sigma) or 20 ng/mL epidermal growth factor (EGF; PeproTech). The gels were transferred to nitrocellulose membranes (Whatman) and blocked in 5% milk-PBS-Tween and incubated with antibodies: tubulin (Sigma), p110α, p110β, p110γ, PTEN, pThr308-Akt, pSer473-Akt (Cell Signaling Technology), and Akt (Santa Cruz Biotechnology) according to the manufacturers'

protocol. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Sigma), washed with PBS-Tween, and developed using ECLplus (GE healthcare).

Statistics

Error bars indicate SE. The *P* values were calculated with χ^2 test, Mann-Whitney *U*-test, or Student's *t*-test as indicated in the figure legends. The χ^2 tests were calculated with one degree of freedom. Student's *t*-test was calculated with the assumption of two-sample equal variance and two-tailed distribution or otherwise indicated. *P* values < 0.05 were considered significant.

Results

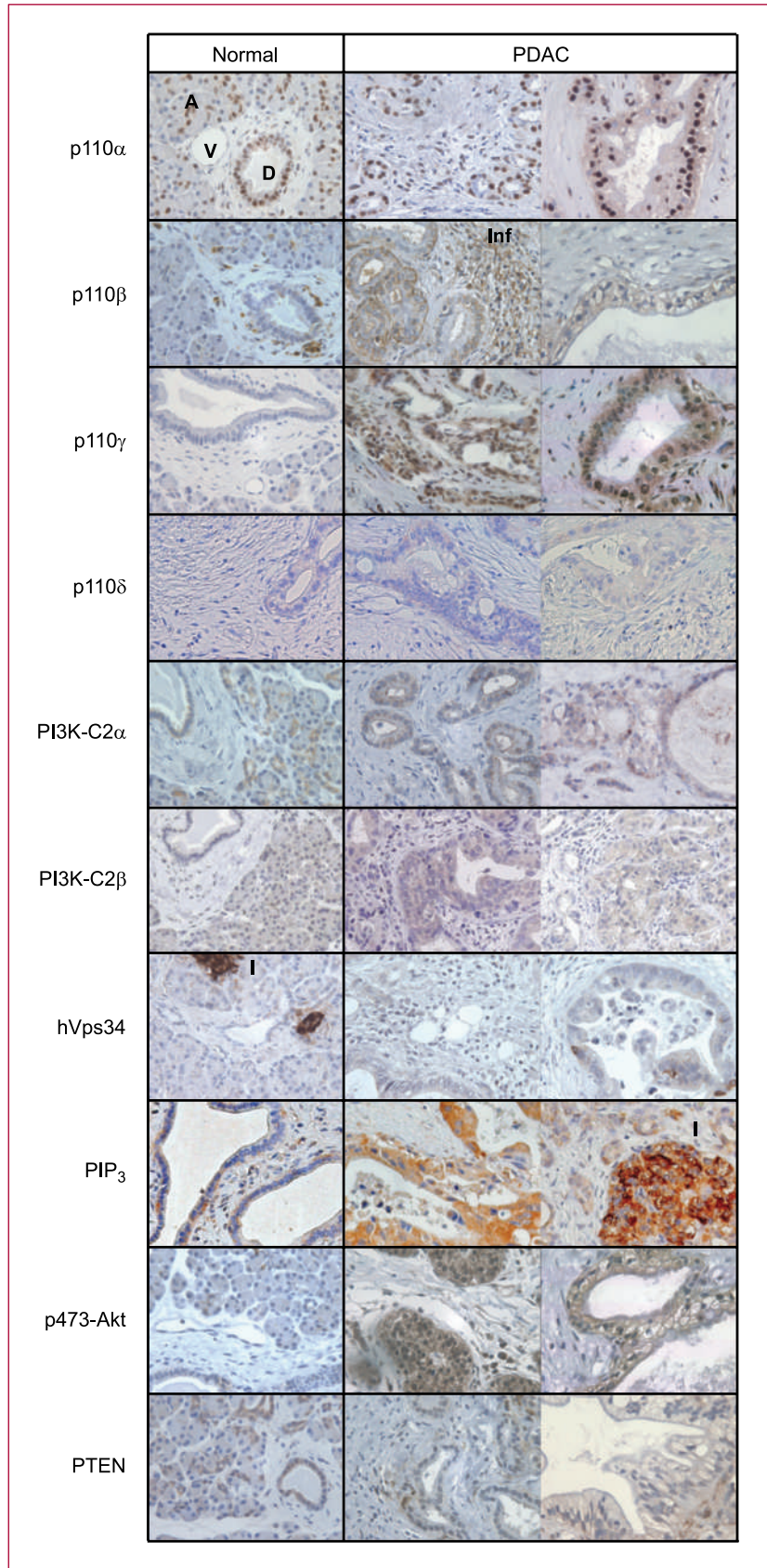
PI3K Class IB isoform p110γ is overexpressed in human pancreatic cancer tissues and cell lines

To investigate the molecular events associated with PI3K in pancreatic cancer we decided to do an immunohistochemical screening of the molecules involved in PI3K signaling in human pancreatic normal (healthy donors and nonneoplastic controls from areas surrounding tumors) and pancreatic ductal adenocarcinoma (PDAC) tissue. In the screening we analyzed the expression and distribution of the different isoforms of PI3K, as well as the lipid product PIP₃. Activation of the downstream target Akt was determined by monitoring phosphorylation at residue Serine473. In addition, expression levels of PTEN, which specifically dephosphorylates PIP₃, were determined.

Analysis of the PI3K class IA isoform p110α showed it to be expressed in both pancreatic normal and cancer tissue samples with similar nuclear distribution in both groups (Fig. 1). In normal samples the protein was present in ducts and in acini with different intensity grade. In PDAC tissues, p110α exhibited a strong staining in dysplastic and distorted ducts cells and in atrophic acini. Staining for the PI3K class IA isoforms p110β and p110δ was found to be weak or absent in most normal and cancer samples. Where detectable, p110β expression was found in acinar cells, smaller pancreatic ducts, intrapancreatic nerve fibers, and around blood vessels in the normal tissue. In the PDAC tissue p110β was strongly expressed in few of the neoplastic ducts and in inflammatory cells. The protein was localized in the cytoplasm in both groups. The p110δ isoform showed similar weak staining in normal and cancerous parts of the pancreas. Low expression levels were detected in ductal cells whereas stronger expression levels were observed in the islets of Langerhans. The images in Fig. 1 represent samples where positive stain was seen.

Interestingly, our analysis showed that the PI3K class IB isoform p110γ was not detectable at all in normal duct epithelium and acinar cells, whereas it showed a cytoplasmic localization in islet cells and nerve fibers. In contrast, high levels of the protein were observed in pancreatic cancer tissue. In particular p110γ was highly expressed in pancreatic intraepithelial neoplasia and ductal carcinoma, and

Fig. 1. Immunohistochemical analyses reveal overexpression of PI3K/p110 γ in human pancreatic cancer. Immunohistochemical screening of PI3K classes I to III, PIP₃, phosphorylated Akt, and PTEN in PDAC samples and normal controls. The expression of p110 γ , phosphorylated Akt (Ser473), and PIP₃ are visibly overexpressed in the PDAC samples. The pictures are of positively stained samples, one for normal and two distinct images for cancer. All stains of normal tissue depict pancreatic ducts. PDAC samples show atrophic acinar cells and dysplastic/aberrant ducts typical for pancreatic cancer. Examples of stained islets are displayed. Key features identified by morphology are indicated (on first occasions only): A, acinar cells; D, duct; V, blood vessel; I, islet; Inf, inflammatory cells/stroma. Magnification for all pictures, $\times 20$.



it seemed to be localized both in the nuclear compartment and in the cytoplasm of these cells (Fig. 1).

The PI3K class II isoform PI3K-C2 α was expressed in a subset of the samples. Positive stain was found in acini and ducts in cancer and donor tissue, but with higher intensity staining in acini with high cellular atypia and in dysplastic ducts (cytoplasmic). The PI3K-C2 β isoform was only visibly stained in few of the samples. In the positive samples the protein was expressed in ductal and acinar structures in both normal and cancer (cytoplasmic).

The PI3K class III enzyme human vacuolar protein sorting 34 (hVps34) was exclusively and strongly localized in subsets of Langerhans islets in both normal and cancer tissues (Fig. 1). Furthermore, the screening indicated that pancreatic cancer is also characterized by high levels of phosphorylated Ser473-Akt and PIP₃. Phosphorylated Akt was visible with different intensity in the cytoplasm of neoplastic ductal cells and in atrophic acini, whereas it was only detectable in islets in the normal tissues, corroborating previous reports (10–12). PIP₃ showed faint staining in acinar cells and ducts of the normal pancreas, but in the PDAC samples PIP₃ was abundant in aberrant ducts and in atrophic acinar cells. Staining of PIP₃ was detected in the cytoplasmic compartment. Finally, the phosphatase PTEN was expressed at similar levels mainly in acinar cells in both normal and PDAC tissue (Fig. 1).

To quantitate the immunohistochemical analysis of p110 γ , the degree of staining (none, weak, moderate, or strong) and the number of positive ducts (0–100%) were scored manually in 12 control samples and 29 cancer samples. The total scores show that 72% of the cancers were positive for p110 γ whereas none of the control samples showed p110 γ staining in the ducts (0%; Fig. 2A). Examples of scoring for p110 γ are shown in Supplementary Fig. S1. Scoring of other PI3K class I isoforms showed that p110 α was expressed in 82% of the normal tissue samples and 78% of PDAC samples, confirming non-tumor-specific staining (Fig. 2B). Scoring of p110 β stain indicated that, although few samples strongly expressed p110 β , most samples did not express detectable levels (11% positive in normal pancreatic tissue and 22% positive in PDAC tissue; Fig. 2C). The overexpression of p110 γ protein in the tumor tissues was confirmed at mRNA level by real-time PCR. Tissues from 10 pancreatic cancer patients were compared with 9 control samples establishing a significant general increase in p110 γ mRNA level in pancreatic tumor tissues (Fig. 2D).

Taken together the immunohistochemical screening clearly indicates that the PI3K pathway is highly activated in PDAC and specifically that there is a selective overexpression of p110 γ together with increased levels of PIP₃ and phosphorylated Akt. To our knowledge, this is the first report of a tumor-specific accumulation of the PI3K class IB isoform in a human cancer type.

We then examined the protein levels of the p110 α , p110 β , and p110 γ isoforms and phosphorylated Akt in 11 pancreatic cancer cell lines and 1 immortalized non-cancerous cell line (Fig. 3). The protein levels of p110 γ

were similar across the different cancer cell lines, with the exception of CFPac, ASPC1, and PANC1, which showed fairly low levels. The “normal” transformed pancreatic ductal cells HPDE showed the lowest p110 γ protein expression. Analyses of the level of p110 α and p110 β isoforms, PTEN, and activated Akt indicate expression at varying degrees in all tested cell lines (Fig. 3).

p110 γ blockade inhibits cell proliferation in pancreatic cancer cells

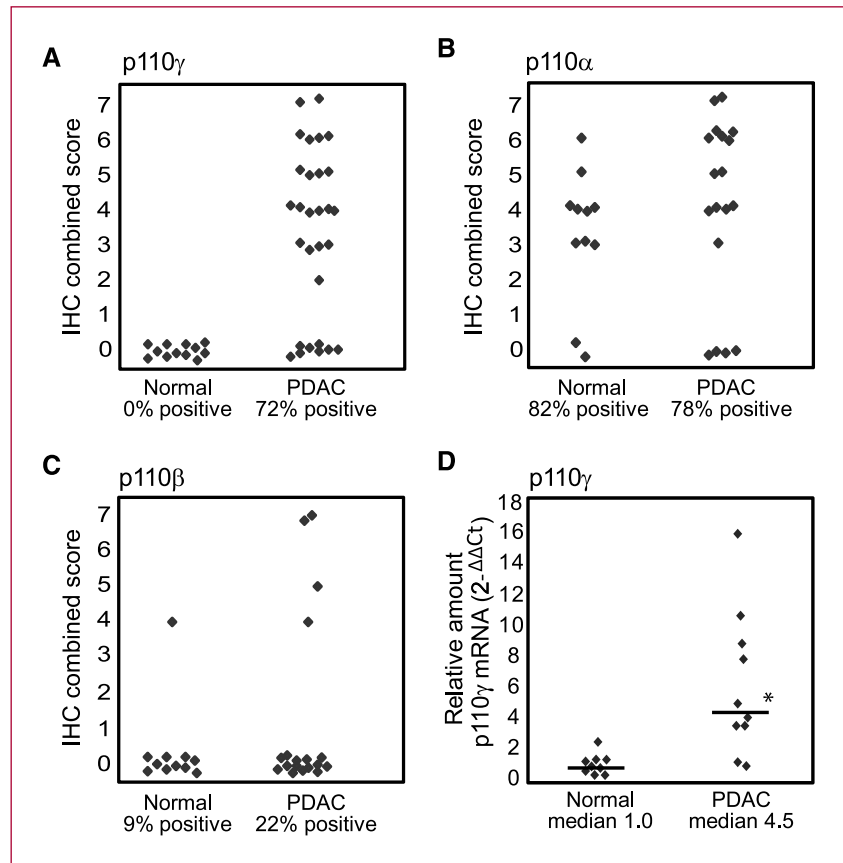
In an effort to define the specific role of p110 γ in pancreatic cancer cells, we analyzed the effect of inhibition of class I PI3K isoforms using chemical inhibitors or siRNA. Different PI3K-dependent functions such as cell proliferation, cell cycle progression, apoptosis, and activation of PI3K-dependent signaling were investigated. Potency and specificity of all inhibitors used have been previously reported (34).

As shown in Fig. 4, chemical inhibition of p110 γ with AS252424 specifically blocked proliferation in the pancreatic cancer cell lines HPAF and Capan1, as assessed by cell counting (Fig. 4A). The p110 γ effect on proliferation was confirmed in ASPC1, HPAF, and PT45 by MTT assay (Supplementary Fig. S2A). No decrease of proliferation was observed upon specific inhibition of the p110 β (TGX-221) and p110 δ (IC87114) isoforms (Fig. 4A). On the contrary, selective inhibition of p110 β slightly increased proliferation of Capan1 cells. As a control we used the PI3K generic inhibitor LY294002, which showed that complete inhibition of PI3K class I isoforms severely blocked proliferation, suggesting the existence of an alternative target. Downregulation of the PI3K class I isoforms using specific siRNAs in PT45 cells show that only the p110 γ isoform had a significant effect on proliferation analyzed by cell counting, consistent with data obtained using isoform-selective inhibitors (Fig. 4B). The effect of p110 γ downregulation was confirmed in HPAF cells assessed by cell counting and MTT (Fig. 4B and Supplementary Fig. S2B). Furthermore, we found that overexpression of exogenous p110 γ in ASPC1 cells increased the proliferative activity assessed by MTT (Fig. 4C). Specificity and efficiency for siRNA downregulation was validated at mRNA as well as protein level (Supplementary Fig. S3A and B and Fig. 6A). The overexpression of p110 γ was further validated at mRNA level (Supplementary Fig. S3C).

Examination of the cell cycle state upon p110 γ inhibition displayed accumulation of cells in G₂-M phase (Fig. 4D). General inhibition of PI3K isoforms with LY294002 resulted in G₁ arrest, whereas no effect on the cell cycle was observed in cells treated with the p110 β - and p110 δ -specific inhibitors alone (Fig. 4D).

To investigate if inhibition of p110 γ activity induced apoptosis we analyzed caspase-3 activity (Fig. 5A) and Annexin V binding (Fig. 5B) following treatment with the p110 γ -specific inhibitor AS252424. Caspase-3 activity, which is a relatively early indicator of apoptosis, was measured after 24 hours. The results indicated that p110 γ inhibition did not induce apoptosis at the concentration

Fig. 2. Scoring of immunohistochemistry data and mRNA analyses confirm upregulation of PI3K/p110 γ . A to C, combined scores for p110 γ (A), p110 α (B), and p110 β (C). A, $\chi^2 = 13.7$, corresponding to $P < 0.001$. B, $\chi^2 = 0.32$, $P > 0.5$. C, $\chi^2 = 0.83$, $P > 0.3$. IHC, immunohistochemistry. D, real-time PCR analysis of p110 γ mRNA level in human pancreatic normal tissue (9 samples) compared with cancer (10 samples). Two-tailed Mann-Whitney's U -test, $P = 0.0015$.



(1 $\mu\text{mol/L}$) sufficient to block proliferation. However, treatment with 10 times higher concentration (10 $\mu\text{mol/L}$) did induce apoptosis, possibly because of off-target effects of the inhibitor. The general PI3K inhibitor LY294002 was

able to induce apoptosis in the Capan1 cell line (Fig. 5A). Annexin V staining was used to measure apoptosis at a later time point. The cells were treated for 3 days with AS252424 or with LY294002. Consistent with the caspase-3 activity assay, p110 γ inhibition did not induce apoptosis when used at the lower concentration. At higher concentration (10 $\mu\text{mol/L}$) almost all cells died after 3 days of incubation. Generic inhibition of PI3Ks increased the number of apoptotic cells although not reaching statistical significance (Fig. 5B).

p110 γ blockade inhibits LPA-induced Akt activation in pancreatic cancer cells

To further analyze the PI3K pathway we determined the effect of blockade of the different PI3K isoforms on Akt activation in HPAF and PT45 cell lines. The phosphorylation status (p-S473 and p-T308) of Akt was assessed by Western blotting after downregulation with siRNA or pretreatment with the isoform-selective inhibitors and stimulation with either LPA or EGF (Fig. 6). The PI3K class IB isoform is believed to be mainly activated via G-protein coupled receptors and not via tyrosine kinase receptors (21). Consistently, experiments using selective siRNAs revealed that p110 γ was indeed required for full activation of Akt upon LPA, but not EGF, stimulation. Further, we found that

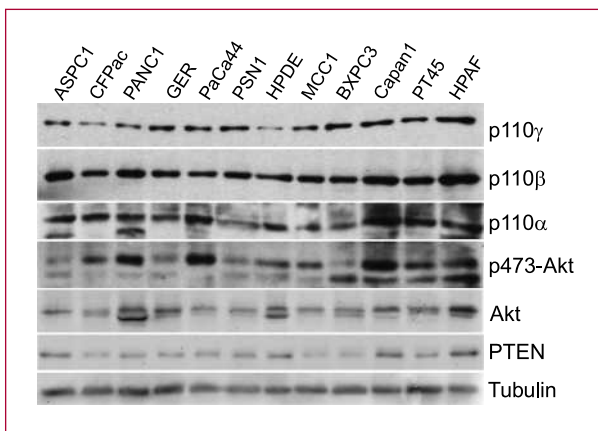


Fig. 3. Analyses of protein levels of PI3K isoforms in pancreatic cancer cell lines. Protein levels for p110 γ , p110 β , p110 α , phosphorylated Akt (S473), total Akt, and PTEN were analyzed by Western blot. Tubulin was used as loading control. HPDE is an immortalized nontumor pancreatic cell line.

p110 β was required for activation of Akt following both LPA and EGF stimulation, consistent with data suggesting that p110 β can be activated both via tyrosine kinase receptors and G-protein coupled receptors (21). Noticeably, neither p110 α nor p110 δ seemed to play a role in the Akt activation in this cell system (Fig. 6A). More-

over, pretreatment with inhibitors show that p110 γ is required for Akt activation in the PT45 and HPAF cell lines. Surprisingly, p110 β inhibition had effect in the PT45 cell line but not in the HPAF cell line. The general PI3K inhibitor LY294002 blocked Akt phosphorylation in both cell lines as expected (Fig. 6B). Additionally,

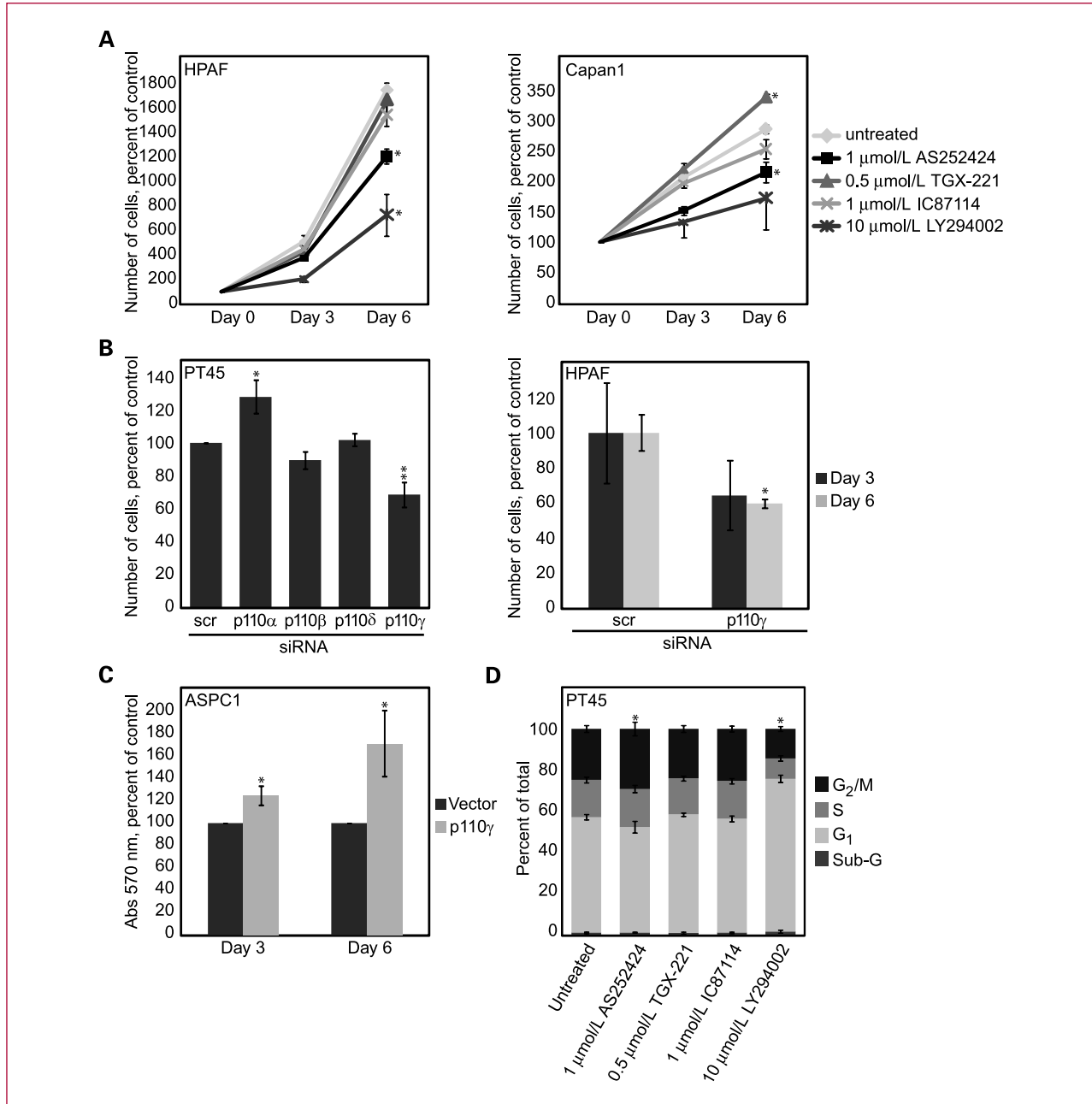


Fig. 4. PI3K/p110 γ regulates proliferation in pancreatic cancer cell lines. A, cell counting of HPAF and Capan1 cells following 0, 3, and 6 days of treatment with inhibitors targeting p110 γ (AS252424), p110 β (TGX-221), p110 δ (IC87114), and generic PI3K (LY294002). Cells were cultured in low-serum condition and counted in duplicate on a CASY cell counter. B, PT45 and HPAF transfected with 120 nmol/L siRNA targeting PI3K class I isoforms or scrambled siRNA were kept for 6 days in complete media before cell counting. Abs, absorbance. C, ASPC1 cells overexpressing p110 γ or vector control assayed by MTT following 3 and 6 days of incubation in serum-free media ($n \geq 3$ for each experiment). *, $P < 0.05$; **, $P < 0.01$, with Student's t -test. D, PI incorporation of PT45 cells following 72-hour inhibitor treatment as indicated in the figure. Cells were cultured in low-serum media. Error bars, SE. *, $P < 0.05$ with paired Student's t -test compared with untreated control.

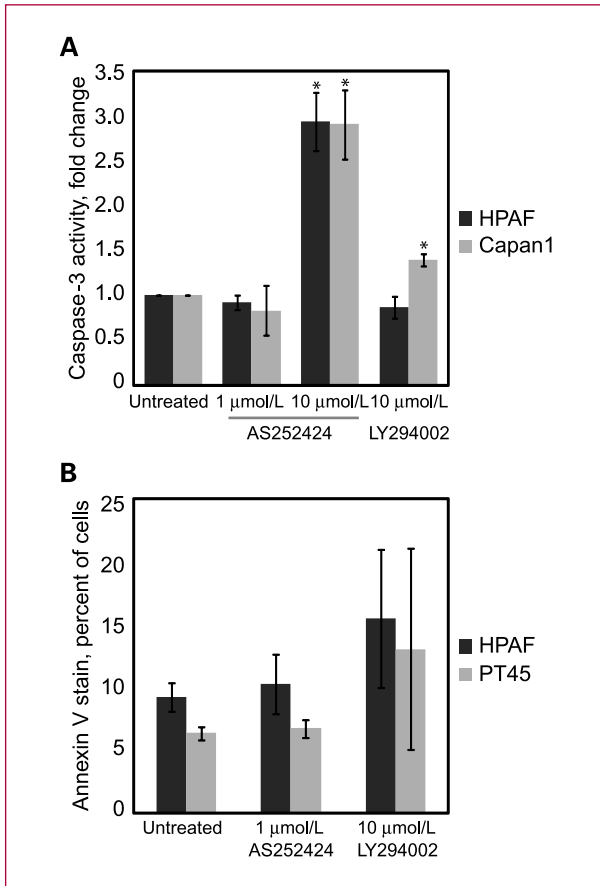


Fig. 5. Inhibition of PI3K/p110 γ does not induce apoptosis in pancreatic cancer cell lines. A, induction of apoptosis in HPAF and Capan1 cells analyzed by caspase-3 activity assay following treatment with inhibitors for 24 hours. Induced apoptosis is displayed as fold-change to untreated cells. B, Annexin V staining of HPAF and PT45 cells analyzed by flow cytometry following 3-day treatment as indicated. Percentage of apoptotic cells is displayed ($n \geq 3$ for each experiment). *, $P < 0.05$ with Student's t -test.

Western blot analysis of ASPC1 and Dec-hTert cells transiently transfected with wild-type p110 γ shows increased phosphorylation of Akt in correlation with overexpressed p110 γ (Fig. 6C). These results, in combination with the immunohistochemistry screening, indicate that Akt activation detected in pancreatic cancer might be specifically driven by overexpression of the PI3K isoform p110 γ .

Discussion

Several studies have established that many growth factors and their receptors are overexpressed in human pancreatic cancer (1). These alterations in signal transduction pathways confer an enormous survival and growth advantage to pancreatic cancer cells. Thus, there is intense interest in the molecular mechanisms of pancreatic cancer to understand its particular aggressiveness and resistance to drug treatments. Several lines of evidence indicate a crucial

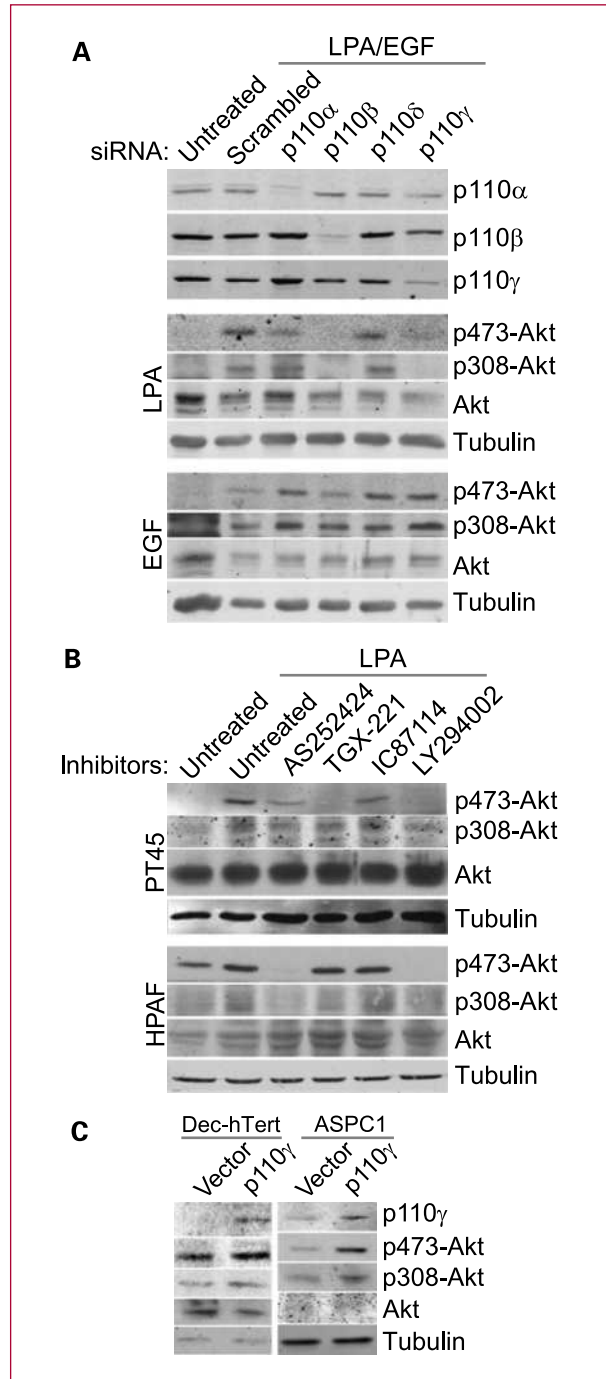


Fig. 6. PI3K/p110 γ is required for LPA activation of Akt in pancreatic cancer cell lines. A, PT45 cells were transfected with indicated siRNA (120 nmol/L) and incubated 48 hours prior to starvation overnight. Starved cells were then stimulated with either 25 μ mol/L LPA or 20 ng/mL EGF for 15 minutes. Western blots display protein levels of class I PI3K isoforms, phosphorylated Akt (S473 and T308), and total Akt. Tubulin was used as loading control. B, HPAF and PT45 cells were starved overnight and treated with indicated inhibitors for 30 minutes prior to stimulation with LPA for 15 minutes. The blots are representative of at least three separate experiments. C, Western blot analysis of protein levels of p110 γ , phosphorylated Akt (S473 and T308), and total Akt in Dec-hTert and ASPC1 cells overexpressing p110 γ compared with empty vector.

role of the PI3K enzymes in human cancer biology, and deregulation of the PI3K-Akt pathway is also implicated in pancreatic cancer (10, 18). So far, however, the majority of studies have been focused on the PI3K isoform p110 α . Therefore, the question was whether the “non α ” PI3K isoforms could also play a role in cancer biology.

This study represents the first detailed analysis of PI3K pathway expression in human pancreatic cancer. We identified the p110 γ isoform as being selectively overexpressed in pancreatic cancer, and we show that p110 γ is necessary for pancreatic cancer cell proliferation. Cell cycle analysis indicates that p110 γ participates in driving the cell through the G₂-M phase. Hence, lack of p110 γ slows down proliferation, whereas inhibition of other PI3Ks seems to result in G₁ arrest in combination with induction of apoptosis, leading to decreased proliferation and eventually cell death. More detailed investigations of all the known players in the cell cycle machinery are required before conclusions regarding the mechanism behind this can be drawn. Interestingly, we find that the low concentration of inhibitor, sufficient to block proliferation, does not induce apoptosis, although ten times higher concentration does. We believe this is most likely due to off-target effects of the inhibitor. This is further supported by our data indicating that the LY294002 compound is able to induce apoptosis and to completely inhibit Akt activation in pancreatic cancer cell lines in which p110 γ inhibition has only a partial effect. This is an intriguing hypothesis, and therefore the identification of this additional target, i.e., another kinase in the PI3K pathway, may provide us the opportunity to completely block pancreatic cell growth by hitting two different targets. Furthermore, in this study we show that p110 γ and p110 β , but not p110 α and p110 δ , are required for activation of Akt. Because the p110 β isoform is not expressed in most of the examined tissue samples, this indicates that the overexpression of the p110 γ isoform is responsible for the detected activation of Akt in the cancer tissue. This is supported by our data showing that overexpression of p110 γ itself indeed can increase the level of phosphorylated Akt in the ASPC1 and Dec-hTert cell lines.

Chronic inflammatory disorders are often associated with an increased risk of developing cancer. A classic example of the connection between inflammation and cancer is the increased risk of pancreatic cancer in patients with chronic pancreatitis (35). Furthermore, chronic pancreatitis accelerates cancer progression in genetically engineered mouse models and is essential for induction of

PDAC by *K-Ras* oncogenes in adult mice (36). Genetic targeting of p110 γ and p110 δ in mice has underlined a central role of these PI3K isoforms in inflammation (37). Therefore, it is important to emphasize that p110 γ expression was also observed in patients with inflammation of the pancreas (Supplementary Fig. S4). These data are in agreement with a recent finding suggesting a role for p110 γ in the development of pancreatitis (38). We hypothesize that p110 γ expression and activation could be an early event that links chronic pancreatitis and pancreatic adenocarcinoma, but further studies are needed to show this link. The potential role of p110 γ in inflammatory cells and in pancreatic cancer cells gives us the appealing opportunity to target both tumor cell as well as inflammation.

In summary our findings represent the first identification of a cancer-specific expression of class IB PI3K and the first clear showing of a link among p110 γ expression, Akt activation, and pancreatic cancer progression. Furthermore, our data firmly support the emerging hypothesis that deregulation of PI3Ks in cancer may not be only restricted to mutations of the p110 α but also include overexpression of other PI3K isoforms. These data strongly signify the PI3K isoform p110 γ as a potential therapeutic target in the prevention and treatment of pancreatic cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

C.E. Edling was supported by the European Commission FP6 program Apotherapy (#037344, grant to M. Falasca) and by the Pancreatic Cancer Research Fund (PCRF, grant to M. Falasca and C.E. Edling). R. Buus was supported by PCRF (grant to M. Falasca). We thank Prof. Lewis Cantley (Harvard Medical School) and Prof. Bart Vanhaesebroeck (Queen Mary University London, United Kingdom) for critically reading the manuscript, Prof. Mauro Piantelli (University of Chieti, Italy) for support with immunohistochemical analysis, Prof. Peter Shepherd (University of Auckland, New Zealand) for the PI3K inhibitors, Prof. Aldo Scarpa (University of Verona, Italy) for the pancreatic cancer cell lines, and Dr. Paul Nielsen (Echelon, USA) and Prof Glenn Prestwich (University of Utah) for the anti-phosphoinositide antibodies.

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Received 05/07/2010; revised 08/16/2010; accepted 08/20/2010; published OnlineFirst 09/28/2010.

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