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Frequent Activation of AKT2 Kinase in Human Pancreatic Carcinomas

Deborah A. Altomare,[1] Satoshi Tanno,[1] Assunta De Rienzo,[1] Andres J. Klein-Szanto,[2] Sachie Tanno,[1] Kristine L. Skele,[1] John P. Hoffman,[3] and Joseph R. Testa[1,2]*

[1]Human Genetics Program, Fox Chase Cancer Center, Philadelphia, Pennsylvania
[2]Tumor Cell Biology Program, Fox Chase Cancer Center, Philadelphia, Pennsylvania
[3]Department of Surgical Oncology, Fox Chase Cancer Center, Philadelphia, Pennsylvania

Abstract Activation of AKT/protein kinase B promotes a variety of biological activities important in tumorigenesis, such as cell survival and cell cycle progression. We previously demonstrated amplification and overexpression of the *AKT2* gene in a subset of human pancreatic carcinomas. In this investigation, we assessed AKT2 catalytic activity in 50 frozen pancreatic tissues (37 carcinomas, four benign tumors, and nine normal pancreata) by in vitro kinase assay. Twelve of 37 (32%) pancreatic carcinomas showed markedly elevated levels of AKT2 activity compared to normal pancreata and benign pancreatic tumors. To delineate mechanisms contributing to AKT2 activation in malignant pancreatic tumors, we examined the status of upstream components of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway. Western blot analysis revealed loss of PTEN protein expression in two of the 12 pancreatic carcinomas with activated AKT2. In vitro PI3K assays demonstrated high levels of PI3K activity in seven carcinoma specimens that showed AKT2 activation. Immunohistochemical staining confirmed high levels of phosphorylated (active) AKT in malignant pancreatic tumors compared to normal pancreata. Overall, these data suggest that upstream perturbations of the PI3K/AKT pathway contribute to frequent activation of AKT2 in pancreatic cancer, which may contribute to the pathogenesis of this highly aggressive form of human malignancy. J. Cell. Biochem. 87: 470–476, 2002.

Key words: AKT2; serine–threonine kinase; oncogene; kinase activity; PTEN; phosphatidylinositol 3-kinase; pancreatic cancer

The AKT/protein kinase B family consists of several closely related serine/threonine kinases, designated AKT1, AKT2, and AKT3 in man [reviewed in Testa and Bellacosa, 1997]. The AKT kinases are major downstream targets of growth factor receptor tyrosine kinases that signal via phosphatidylinositol 3-kinase (PI3K) [reviewed in Alessi and Cohen, 1998; Coffer et al., 1998; Downward, 1998; Kandel and Hay, 1999; Cantley, 2002]. AKT is recruited to sites of PI3K activation by binding of its pleckstrin homology domain to the PI3K product, phosphatidylinositol-3,4,5-trisphosphate($PI(3,4,5)P_3$). Phospholipid binding at the plasma membrane facilitates phosphorylation of AKT by phosphoinositide-dependent kinase 1, which also associates with $PI(3,4,5)P_3$ at the membrane. This phosphorylation promotes catalytic activation of AKT. Upon its release from the membrane, activated AKT phosphorylates numerous downstream targets involved in essential processes such as cell growth and survival.

AKT has emerged as a central player in a signaling pathway of which many components have been linked to tumorigenesis. *AKT1* is the cellular homologue of a viral oncogene [Staal, 1987; Bellacosa et al., 1991]. In addition, avian sarcoma virus 16 contains a potent transforming gene derived from the cellular gene for the p110α catalytic subunit of PI3K [Chang et al., 1997]. Its human homologue, *PIK3CA*, has been implicated as an oncogene in ovarian cancer

Deborah A. Altomare and Satoshi Tanno contributed equally to this work.

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*Correspondence to: Joseph R. Testa, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111. E-mail: JR_Testa@fccc.edu

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[Shayesteh et al., 1999]. Furthermore, the negative regulator of this pathway, PTEN, is a tumor suppressor. This phosphatase inhibits AKT activation by dephosphorylating the 3′ position of $PI(3,4,5)P_3$ to produce $PI(4,5)P_2$, thus suppressing tumor formation by inhibiting PI3K/AKT signaling [Cantley and Neel, 1999; Di Cristofano and Pandolfi, 2000]. Loss or inactivation of PTEN causes an increased concentration of $PI(3,4,5)P_3$, leading to hyperactivation of AKT. PTEN loss has been documented in many human cancers and, depending on the tissue type, appears to be involved in tumor initiation or progression [Ali et al., 1999].

Investigations conducted over the last few years have revealed a burgeoning list of AKT substrates implicated in oncogenesis [reviewed in Testa and Bellacosa, 2001]. Among its pleiotropic effects, activated AKT has been shown to promote cell survival, cell cycle progression, angiogenesis, telomerase activation, and tumor cell invasiveness. In addition, AKT activation antagonizes $p21^{WAF1}$-mediated cell cycle arrest [Zhou et al., 2001] and acts in concert with Mdm2 to reduce both p53 levels and transactivation [Mayo and Donner, 2001].

Pancreatic cancer is the fifth most common cause of cancer deaths in the United States, with an annual incidence of nearly 30,000 cases and a 5-year survival rate of only 4% [Hawes et al., 2000]. In pancreatic cancer, tumorigenesis is associated with increasing histologic atypia and with the accumulation of genetic and epigenetic alterations in specific cancer-related genes [Hruban et al., 2000]. In the progression from intraductal proliferation to invasive ductal carcinoma, activation of the *KRAS* oncogene and overexpression of HER2/neu occur relatively early; inactivation of the $p16^{INK4a}$ tumor suppressor gene is at an intermediate stage; and inactivation of *TP53*, *DPC4*/*SMAD4*, and *BRCA2* occurs late. We and others previously reported amplification and overexpression of *AKT2* in $\sim 20\%$ of human pancreatic carcinomas [Cheng et al., 1996; Miwa et al., 1996; Ruggeri et al., 1998]. Furthermore, *AKT2* antisense RNA has been shown to inhibit the tumorigenicity and in vivo invasive potential of human pancreatic carcinoma cell lines exhibiting amplification of *AKT2* [Cheng et al., 1996]. However, to date the activation status of the AKT2 kinase in pancreatic cancer has not been reported.

As a follow-up to our previous investigations of AKT2 in pancreatic cancer, we now report that AKT2 is frequently activated in human pancreatic carcinomas. We show that AKT2 activation is often associated with perturbations of upstream regulators of AKT2 catalytic activity, including hyperactivation of PI3K and/or loss of PTEN expression. Immunohistochemical staining of a subset of matched paraffin-embedded pancreatic tumors revealed that AKT activation is significantly increased in malignant pancreatic tumor cells compared to normal pancreata. Overall, the data presented here indicate that AKT2 activation is a common occurrence in the progression of human pancreatic cancer and suggest that the PI3K/PTEN/AKT pathway is a potentially important target for therapeutic intervention in this malignancy.

MATERIALS AND METHODS

Tissue Samples

A total of 37 primary pancreatic carcinoma tissues and four benign pancreatic lesions were surgically excised from 41 patients with pancreatic tumors. Histologically, the 37 malignant tumors consisted of 23 ductal adenocarcinomas, five islet cell carcinomas, three mucinous cystadenocarcinomas, three neuroendocrine adenocarcinomas, one mucinous adenocarcinoma, one acinar cell carcinoma, and one small cell carcinoma. The benign tumors consisted of two mucinous cystadenomas, one serous cystadenoma, and one intraductal papillary-mucinous tumor. Six normal pancreatic samples were obtained from patients with gastric cancer or other non-pancreatic diseases, and three samples were obtained from normal pancreatic tissue adjacent to tumor specimens. Tissue samples were snap frozen in liquid nitrogen and stored at -80°C prior to examination.

Immunoprecipitation and Western Blot Analysis

For detection of AKT2 and PTEN expression, 200 μg of protein from tissue samples was immunoprecipitated with 2 μg of anti-AKT2 (Upstate Biotechnology, Lake Placid, NY) or anti-PTEN (N-19, Santa Cruz Biotechnology, Santa Cruz, CA) antibodies, following incubation with protein A:protein G agarose beads. Immunoprecipitates were subjected to 7.5% SDS–PAGE followed by immunoblotting. Actin protein expression was used as a control to assess for protein extract quality. For detection of actin expression, 30 μg of protein was subjected

to Western blot analysis. Membranes were blocked and incubated with primary antibodies against AKT2 (D-17), PTEN (A2B1), and actin (I-19) (all from Santa Cruz Biotechnology) for 1 h in Tris-buffered saline containing 1% nonfat dry milk/0.1% Tween 20. Detection of antigen-bound antibody was carried out with the Renaissance Chemiluminescence Reagent Plus system (NEN Life Science, Boston, MA).

In Vitro AKT2 Kinase Assays

Tissue samples were homogenized in lysis buffer [50 mM Tris-HCl (pH 7.5), 137 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.2% Triton X-100, 10% glycerol, 0.1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, and 2 μg/ml aprotinin]. Insoluble material was removed by centrifugation at 4°C for 15 min at 18,400*g*. Protein concentration was determined with a BioRad Protein Assay Kit (BioRad Laboratories, Hercules, CA). Protein extract (200 μg) was incubated with 2 μg of anti-AKT2 antibody (Upstate Biotechnology), and the immunocomplex was precipitated with protein A:protein G (1:1) agarose beads (Life Technologies, Gaithersburg, MD) at 4°C overnight. Immunoprecipitates were incubated with 5 μM [γ-^{32}P]-ATP in kinase buffer [20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$] at 30°C for 25 min using histone H2B as substrate. The reactions were terminated by the addition of 2 × Laemmli sample loading buffer and then subjected to 15% SDS–PAGE. Phosphorylation of histone H2B was visualized by autoradiography and quantitated with a BAS-1000 phosphorimager (Fuji Medical Systems, Stamford, CT). Relative activity was determined by comparison to that of normal tissues given a value of 1.0. AKT2 was considered to have elevated activity when the level of phosphorylated AKT2 was at least threefold greater than the average value of several normal pancreata, which were loaded on every gel.

In Vitro PI3K Assay

Tissue lysates containing 200 μg of protein were immunoprecipitated with anti-p85 (Upstate Biotechnology) and protein A:protein G agarose beads. Phosphoinositides (Sigma, St. Louis, MO) were sonicated in 30 mM HEPES (pH 7.5), mixed with 5 μM [γ-^{32}P]-ATP, and then incubated at 30°C for 30 min. Reactions were stopped by addition of HCl and then extracted with chloroform–methanol (1:1). Lipids were separated by thin layer chromatography, and the chromatography plates were visualized by autoradiography.

Immunohistochemical Analysis

A subset of formalin-fixed, paraffin-embedded sections matching frozen tumors and pancreata used for the in vitro kinase and Western blot analyses were cut into 4 μ sections and placed onto charged slides. The sections were deparaffinized and hydrated in water, followed by antigen retrieval in 10 mM citrate buffer, pH 6.0. Preparations were incubated in 3% H_2O_2 for 20 min, washed with H_2O or PBS, and blocked with 10% serum for 30 min. Primary antibodies used for immunohistochemistry included anti-phospho-AKT (Ser473, Cell Signaling Technology, Beverly, MA) and anti-AKT2 (D-17, Santa Cruz Biotechnology). Primary antibodies were detected with biotinylated secondary antibodies (BioGenex, San Ramon, CA). Negative controls were incubated with primary antibody pre-absorbed with blocking peptide (Cell Signaling Technology). Tissue sections were stained with DAB chromagen and counterstained with hematoxylin.

RESULTS

Among the 37 pancreatic carcinomas, 12 (32%) exhibited > threefold higher levels of AKT2 kinase activity than in normal pancreata as determined by in vitro kinase assay. Of these 12 cases, five had > fivefold higher levels of AKT2 activity than in normal pancreata. Of the 25 remaining cases, four others exhibited levels of AKT2 activity that were considered borderline elevated (i.e., 2.5 to 3.0-fold higher than in normal tissues). The levels of AKT2 kinase activity in benign pancreatic tumors were similar to normal pancreatic specimens. Examples of AKT2 kinase levels in pancreatic specimens are depicted in Figure 1.

Because AKT2 kinase activity is positively regulated by PI3K and negatively regulated by PTEN, we examined PI3K activity and PTEN expression in the 12 pancreatic carcinomas that showed activation of AKT2. Immunoblot analysis revealed loss of PTEN protein expression in two specimens (cases 11 and 35) (Fig. 2A), both of which showed markedly elevated (> fivefold) levels of AKT2 activity (Fig. 1).

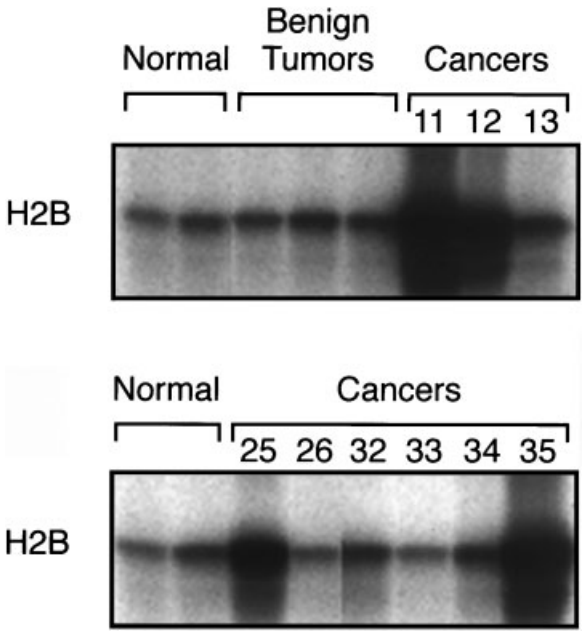

Fig. 1. Increased AKT2 kinase activity in human pancreatic carcinomas compared to normal pancreata and benign pancreatic tumors. Note AKT2 activation in several pancreatic carcinomas. Phosphorylation of histone H2B was visualized by autoradiography.

Since AKT2 is a downstream target of PI3K, we next assayed PI3K activity by in vitro kinase assay. We evaluated in vitro PI3K activity in 12 specimens (nine available pancreatic carcinomas with activated AKT2 and three normal pancreata). Compared to normal pancreatic tissue, PI3K activity was elevated in seven of the nine carcinomas examined, several of which are shown in Figure 2B. One carcinoma (case 11) with elevated PI3K activity also showed loss of PTEN expression (Fig. 2A).

To confirm high levels of phosphorylated (active) AKT in a subset of pancreatic carcinomas, we next performed immunohistochemical analyses on five paraffin-embedded specimens for which increased AKT2 activity had been detected by in vitro kinase assay. Immunohistochemistry with antibodies against AKT2 and phospho-specific pan-AKT (a phospho-specific AKT2 antibody is currently unavailable) revealed positive staining in all five malignant tumor specimens tested. Staining was increased in malignant cells compared to surrounding stromal tissue (Fig. 3). Immunostaining with phospho-AKT or AKT2 antibodies was negative in the ductal tissue of each of two normal pancreata examined, although weak staining was observed with either antibody in glandular components.

DISCUSSION

Amplification and overexpression of the *AKT2* gene has been previously reported in about 20% of human pancreatic carcinomas [Cheng et al., 1996; Miwa et al., 1996; Ruggeri et al., 1998]. *AKT2* antisense RNA inhibits the tumorigenicity and in vivo invasive potential of human pancreatic carcinoma cell lines exhibiting amplification of *AKT2* [Cheng et al., 1996], suggesting that aberrant expression of AKT2 contributes to the malignant phenotype of some pancreatic cancers. The data presented here indicate that AKT2 activation is a frequent occurrence in pancreatic cancer, occurring in about 30–40% of cases depending on the threshold value used to define elevated activity. The threshold used here (> threefold increase)

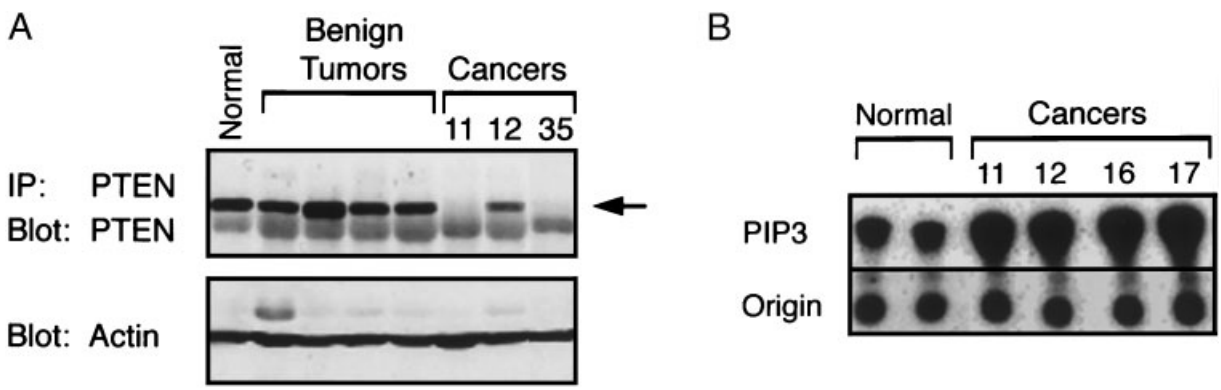

Fig. 2. **(A)** Immunoprecipitation followed by Western blotting to assess PTEN expression in pancreatic carcinomas compared to normal pancreata and benign pancreatic tumors. Note absence of detectable PTEN protein in pancreatic carcinoma cases 11 and 35. **(B)** In vitro PI3K activity in two normal pancreata and four pancreatic carcinomas previously shown to have elevated AKT2 kinase activity. Chromatography plates were visualized by autoradiography. The position of PI(3,4,5)P_3 is shown.

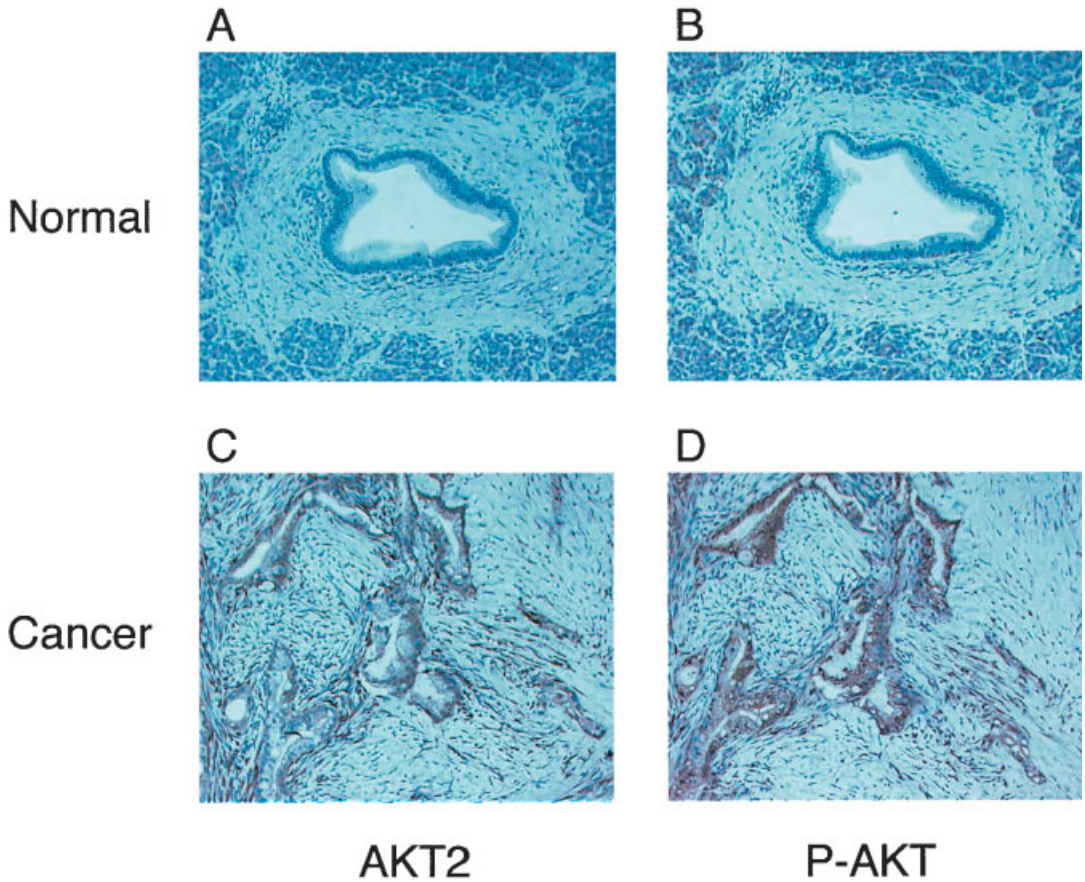

Fig. 3. Immunohistochemical staining of a representative normal pancreata and a pancreatic carcinoma with AKT2 and phospho-specific (Ser473) pan-AKT antibodies. **(A,B)** Consecutive sections of pancreata exhibiting negative staining for AKT2 (A) and phosphorylated (active) AKT (B). **(C,D)** Consecutive sections of adenocarcinoma staining positive for both AKT2 (C) and phospho-AKT (D). Note that this tumor also exhibited elevated AKT2 activity by in vitro kinase assay. Hematoxylin plus immunostain.

is consistent with that used by others to characterize AKT activation in human tumor samples [Yuan et al., 2000; Sun et al., 2001]. In any case, the data presented here suggest that perturbations of AKT2 may be involved in a higher percentage of pancreatic carcinomas than estimated previously based on Southern, Northern, or immunohistochemical analyses [Cheng et al., 1996; Miwa et al., 1996; Ruggeri et al., 1998]. Consistent with this notion, four of our 12 carcinomas with elevated AKT2 kinase activity did not express abundant levels of AKT2 protein based on Immunoblot analysis (data not shown)

Since all AKT isoforms are downstream targets of PI3K, pancreatic tumors with activation of AKT2 would also be expected to show increased AKT1 and AKT3 activity. In fact, an AKT1 kinase assay performed in parallel with the AKT2 kinase assay on 19 pancreatic carcinomas revealed very similar patterns of activation for both AKT isoforms (data not shown).

A growing body of evidence has suggested that AKT activation plays a central role in human cancer [reviewed in Testa and Bellacosa, 2001]. Activation of the AKT2 kinase has been detected in approximately 40% of ovarian cancers [Yuan et al., 2000]. Moreover, activation of AKT1 is frequently observed in breast and prostate cancers [Sun et al., 2001], and increased AKT3 activity has been reported in estrogen receptor-deficient breast cancer and androgen-insensitive prostate cancer cell lines [Nakatani et al., 1999]. Furthermore, constitutive activation of AKT is a frequent event in lung cancer cell lines and promotes cell survival and resistance to chemotherapy and radiation [Brognard et al., 2001].

To delineate mechanisms contributing to AKT2 activation in pancreatic tumors, we

examined the status of upstream components of the AKT pathway. We show that most pancreatic carcinomas with activated AKT2 have either PI3K activation or PTEN down-regulation. PI3K activity was elevated in seven of nine carcinomas examined that exhibited activated AKT2. Of the remaining two cases, one had loss of PTEN expression. Thus, activation of AKT2 was associated with perturbations of PI3K and/or PTEN in eight of these nine cases. In previous studies of other tumor types, activation of PI3K was frequently observed in carcinomas of the breast and ovary, but not prostate, whereas loss of PTEN protein expression was observed in most prostate cancers and a small number of ovarian carcinomas [Sun et al., 2001].

PI3K/AKT signaling is now known to play a prominent role in several processes considered to be hallmarks of cancer [Testa and Bellacosa, 2001]. AKT activation inhibits programmed cell death and promotes cell cycle progression, angiogenesis, telomerase activation, and tumor cell invasiveness. AKT2 is overexpressed in some pancreatic carcinomas [Ruggeri et al., 1998], which may permit a tumor cell to become overly responsive to ambient levels of growth factors that normally would not provoke cell proliferation. Moreover, insulin-like growth factor-1 (IGF-1) receptor is often overexpressed in pancreatic cancers [Bergmann et al., 1995], and we have shown that activation of AKT2 or AKT1 up-regulates IGF-1 receptor expression and promotes invasiveness [Tanno et al., 2001]. Co-transfection experiments also demonstrated that IGF-1 receptor expression induced by wild type or constitutively active AKT2 or AKT1 is inhibited by expression of PTEN.

AKT is a major mediator of survival signals that protect cells from undergoing apoptosis and, thus, is a potentially important therapeutic target. The fact that inhibition of AKT can down-regulate IGF-1 receptor expression suggests that AKT could represent an important therapeutic target in human pancreatic cancer. Recent in vitro studies have demonstrated that treatment of human tumor cells with the specific PI3K inhibitor LY294002 promotes chemotherapy- and radiation-induced apoptosis in cells with high AKT levels, but does not significantly increase therapy-induced apoptosis in cells with low AKT levels [Brognard et al., 2001]. Thus, pharmacological agents that inhibit AKT signaling might preferentially kill tumor cells that have a dependence on AKT activity for survival not shared by normal cells [Brognard et al., 2001]. Collectively, these data provide strong rationale for identifying inhibitors or modulators of the PI3K/PTEN/AKT pathway, with the intention of developing novel chemopreventive or chemotherapeutic strategies directed at tumors exhibiting AKT activation.

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