

Differential Mechanisms of Constitutive Akt/PKB Activation and Its Influence on Gene Expression in Pancreatic Cancer Cells

Joe Matsumoto,^{1,2,3} Masako Kaneda,^{1,2,3} Mitsuhiro Tada,^{1,4} Jun-ichi Hamada,¹ Shunichi Okushiba,² Satoshi Kondo,² Hiroyuki Katoh² and Tetsuya Moriuchi¹

¹Division of Cancer-related Genes, Institute for Genetic Medicine, Hokkaido University and ²Department of Surgical Oncology, Hokkaido University Graduate School of Medicine, N15 W7, Kita-ku, Sapporo 060-0815

Activated Akt/protein kinase B transmits oncogenic signals leading to inhibition of apoptosis, cellular proliferation, and tolerance to hypoxia. Presently, mutational inactivation of *PTEN* and activation of Ras are considered to be the major causes of Akt activation. Here we report differential mechanisms of constitutive Akt activation in 4 human pancreatic cancer cell lines (KMP-3, KMP-4, PCI-66, and PCI-68). These 4 cell lines displayed phosphorylation and functional activation of Akt both in the presence and absence of serum, while three control cell lines (PCI-79, KMP-8, and PSN-1) did so only in the presence of serum in culture. All the 7 cell lines harbored K-Ras activated by mutations at codon 12 resulting in MAP kinase kinase (MEK1/2) phosphorylation, and all except one (KMP-8) had p53 mutations, indicating that these mutations are not sufficient for constitutive Akt activation. KMP-3 and KMP-4 had lost *PTEN* function owing to loss of expression or a mutation, but PCI-66 and PCI-68 retained wild-type *PTEN*. Phosphorylation of Akt was inhibited by the phosphatidylinositol-3-kinase (PI3K) inhibitor LY294002 and the tyrosine kinase inhibitor genistein in KMP-3 and KMP-4 cells, indicating that upstream signals are required for Akt activation in these two cell lines. In contrast, neither LY294002 nor genistein inhibited Akt activation in PCI-66 and PCI-68 cells, indicating the involvement of another unknown mechanism of Akt activation independent of PI3K-mediated signaling to Akt. Irrespective of the differential mechanisms, the 4 cell lines showed similar mRNA expression patterns of 49 genes assessed by cDNA array as compared to the 3 cell lines without Akt activation, suggesting that the mechanisms have the same consequences on the downstream signaling of the constitutive Akt activation.

Key words: Pancreatic cancer — Akt/protein kinase B — Constitutive activation — K-ras — *PTEN*

Akt/protein kinase B regulates important cellular events including apoptosis, cellular proliferation, and responses to hypoxic stress. Activated Akt inhibits apoptosis through phosphorylating Bad,¹ I κ B kinase (IKK),² Forkhead transcriptional factor,³ and caspase-9.⁴ Akt is implicated in cell cycle progression.^{5,6} Also, activated Akt enhances expression of hypoxia-inducible factor (HIF)-1 and thereby confers capacity to tolerate hypoxia on cells.⁷

Activation of Akt depends on the level of phosphatidylinositol-3,4,5-triphosphate (PIP-3), which is determined by the balance between production of PIP-3 by phosphatidylinositol-3-kinase (PI3K) and dephosphorylation of PIP-3 into PIP-2 by *PTEN* phosphatase.⁸ Activation of PI3K occurs by binding of the regulatory p85 subunit to tyrosine-phosphorylated protein (receptor tyrosine kinase),⁹ and by binding of the p110 catalytic subunit to activated Ras.¹⁰ Loss of *PTEN* function causes Akt activation through accumulation of PIP-3.^{8,11}

Abnormal activation of Akt due to the imbalance in favor of PIP-3 is thought to play an important role in many human cancers including brain tumors,^{12,13} endometrial cancers,¹⁴ malignant melanomas,¹⁵ thyroid cancers,¹⁶ and pancreatic cancers.¹⁷ It remains unclear, however, whether a single event among enhanced growth factor signaling, Ras activation, and *PTEN* mutation/deletion is sufficient or whether their combination is necessary for Akt activation in each type of cancer. The situation is complicated by the facts that 1) PI3K can act upstream of Ras,¹⁸ 2) small GTPases such as R-Ras, Cdc42, and Rac bind to and activate PI3K,^{19,20} and 3) another tumor suppressor p53 acts upstream of Ras.^{21,22}

Here, we present our study analyzing the mechanisms of Akt activation in human pancreatic cancer cell lines. While all 7 cell lines examined showed activated Akt in the presence of serum, 4 of the 7 showed constitutively activated Akt in the absence of serum. We demonstrate that *PTEN* dysfunction together with tyrosine kinase-Akt and Ras-Akt signaling through PI3K resulted in Akt activation in two of the cell lines, whereas Akt activation in two other cell lines was independent of tyrosine kinase,

³ These two authors contributed equally to this study.

⁴ To whom correspondence should be addressed.

E-mail: m_tada@med.hokudai.ac.jp

PI3K activity or PTEN loss, strongly suggesting the presence of an undefined pathway leading to Akt activation. We also show that, in spite of the differential mechanisms of constitutive Akt activation, a common consequent mRNA expression profile was present in the 4 cell lines as compared to the 3 cell lines without Akt activation.

MATERIALS AND METHODS

Cell lines Human pancreatic cancer cell lines, PCI-66, -68, and -79 were established and provided by Dr. H. Ishikura (the First Department of Pathology, Hokkaido University); KMP-3, -4, and -8 by Dr. Y. Shimada (Department of Surgery and Surgical Basic Science, Kyoto University); and PSN-1 by Dr. T. Yoshida (Genetics Division, National Cancer Center, Japan). The cells were maintained in Dulbecco's modified essential medium (DMEM, Gibco-BRL, Tokyo) supplemented with 0.1 mM L-glutamine, 0.1% NaHCO₃, 100 U/ml penicillin-G, and 10% fetal calf serum (FCS, Gibco-BRL) at 37°C in a humidified 5% CO₂ atmosphere.

Immunoblot analyses for constitutive MEK, Akt, and EGFR phosphorylation Confluent cells in 6-cm dishes were serum-starved with DMEM with 0.5% FCS for 24 h and DMEM without FCS for an additional 24 h. Protein was extracted from the cells with Cell Lysis Buffer (New England Biolabs, Beverly, MA), applied at 10 µg per lane on 7.5–12.5% SDS-polyacrylamide gel, separated, and electro-transferred to a poly-vinylidene difluoride membrane (Millipore, Bedford, MA). The blots were probed with anti-Akt, anti-phospho-Akt (Ser473), anti-MEK1/2 (MAP kinase kinase 1/2), and anti-phospho-MEK1/2 (Ser217/Ser221) antibodies from New England Biolabs, and anti-EGFR (epidermal growth factor receptor) and anti-phospho-EGFR (Tyr1173) antibodies from Upstate Biotechnology (Lake Placid, NY). They were visualized with the ECL kit (Amersham, Little Chalfont, UK). The antibodies against Akt, phospho-Akt, MEK1/2, and phospho-MEK1/2 cross-react to the corresponding human and mouse molecules. For immunoblot analyses using these antibodies, protein extracts from NIH3T3 cells with or without PDGF stimulation (provided by the antibody supplier) were used as negative and positive controls, respectively.

Akt kinase assay Protein extracted from cells serum-starved as described above was incubated overnight with Immobilized Akt1G1 monoclonal antibody (New England Biolabs). After extensive washing, the kinase reaction was performed in the presence of ATP and GSK-3 as the substrate for Akt kinase. Phosphorylation of GSK-3 was assayed by a western blot using anti-phospho-GSK-3α/β (GSK-3α, Ser21; GSK-3β, Ser9) antibody (New England Biolabs).

Sequence analysis of *K-Ras* and *H-Ras* genes Exon 1

spanning codons 11 and 12 of the *K-Ras* gene, and exon 1 spanning codons 11 and 12, and exon 2 spanning codon 61 of the *H-Ras* gene were PCR-amplified from genomic DNA of each cell line with the use of Pfu polymerase (Stratagene, La Jolla, CA) on a Thermal Cycler Model 2400 (Perkin-Elmer, Chiba). The primers used were 5'-CTG GTG GAG TAT TTG ATA GTG T-3' and 5'-CTT TAT CTG TAT CAA AGA ATG GT-3' for *K-Ras* exon 1; 5'-CCC CTG AGG AGC GAT GAC G-3' and 5'-TCC TGG GGT GCT GAG ACG AG-3' for *H-Ras* exon 1; and 5'-ATG GGG AGA CGT GCC TGT TG-3' and 5'-CCT CAC GGG GTT CAC CTG TA-3' for *H-Ras* exon 2. The fragments were cloned into pCR2.1 vector (Invitrogen, Groningen, The Netherlands) and at least 6 clones each were sequence-analyzed in an ABI 377 automated sequencer (Applied Biosystems, Chiba).

Yeast p53 functional assay Yeast p53 functional assay and subsequent sequence analysis were performed to examine p53 status as described elsewhere.^{23,24)}

PTEN stop codon assay and sequence analysis PTEN stop codon assay was performed to screen PTEN mutations as described elsewhere.¹³⁾ For detection of missense mutations, chain-terminating mutations of *PTEN*, and *ψPTEN* (pseudogene) expression, plasmids recovered from at least 4 independent clones each of the white, red, and pink colonies were sequence-analyzed.

LY294002 and genistein treatments Confluent cells serum-starved for 48 h were treated with 40 µM LY294002 or 100 µM genistein for 8 h. Protein was extracted as described above and subjected to western blot analyses for Akt and phosphorylated Akt.

Sequence analysis of *Akt1* and *Akt2* cDNA fragments of *Akt1* (codons 1–480), and *Akt2* (codons 1–349, 345–481) were PCR-amplified from genomic DNA of PCI-66 and PCI-68. The primers used were 5'-AGC CTG GGT CAA AGA AGT CAA A-3' and 5'-AAA TGC ACC CGA GAA ATA AAA A-3' for *Akt1*; 5'-CAT GTC CTG CTG CCC TGA G-3' and 5'-CTC GTG GTC CTG GTT GTA GAA G-3' for the upper portion of *Akt2*; and 5'-CCG CCT GCC CTT CTA CAA C-3' and 5'-AAA CCA CCC AGC GGT GAT G-3' for the lower portion of *Akt2*. The fragments were cloned into PCR2.1 vector, and at least 6 clones each were sequence-analyzed.

cDNA array analysis PolyA⁺ RNA was extracted from the total RNA (ca. 50 µg) with the use of a MagExtractor kit (Toyobo, Tokyo) according to the manufacturer's instructions. Contaminating DNA was removed with DNase I (Toyobo) in the final step of polyA⁺ RNA extraction. The extracted polyA⁺ RNA (ca. 1 µg) was reverse-transcribed for 50 min at 42°C in a 20 µl reaction mixture containing 1× RT buffer, 0.25 mM each of dNTPs, 0.5 u/µl RNase inhibitor (Toyobo), and 5 u/µl ReverTra Ace reverse transcriptase (Toyobo). The cDNA was ethanol-precipitated and subjected to polyC⁺ tailing for 10 min at

37°C in a 10 μ l reaction mixture containing 0.2 mM dCTP, 1 \times TdT buffer, and 1.25 u/ μ l terminal deoxynucleotidyl transferase (Toyobo). The polyC⁺ cDNA (2.5 μ l) was then amplified by PCR for biotin-16-dUTP labeling with a Biotin cDNA amplification system (Toyobo); the PCR cycle consisted of 3 min at 98°C for initial denaturation, and 25 cycles of 20 s at 98°C, 10 s at 55°C, and 6 min at 74°C. The labeled cDNA was ethanol-precipitated, denatured at 68°C, and used as a hybridization probe in 10 ml of PerfectHyb solution (Toyobo). Hybridization was done overnight at 68°C on a GeneticLab (Sapporo) in-house cDNA array of 1281 genes plus 11 house keeping genes. The membrane was washed successively with 2 \times SSC+0.1% SDS and 0.1 \times SSC+0.1% SDS, each three times, at 68°C. Detection was performed by means of a streptavidin-biotinylated alkaline phosphatase system (Imaging High-Chemilumi-Gene Navigator, Toyobo) using CDP-Star as a luminogen. Acquisition of the signals was done with Fluoro-S MultiImager (BioRad Laboratories, Hercules, CA).

Analysis of expression data cDNA array data values of each cell line were normalized with the mean expression value of the 1281 genes in order to cancel out size shift due to independent hybridization. Expression profile analysis and cluster analysis were done by using programs in MATLAB 6.1 (MathWorks, Natick, MA).

RESULTS

Constitutive Akt activation in pancreatic cancer cell lines We first tested Akt status in 7 pancreatic cancer cell lines: PCI-66, PCI-68, PCI-79, KMP-4, KMP-8, PSN-1,

and KMP-3. We evaluated levels of whole Akt expression and phosphorylated Akt at Ser473 in serum-deprived cells by immunoblot analyses, and assessed Akt kinase activity towards GSK-3 by an Akt kinase assay. Although the *Akt2* gene is known to be amplified in some pancreatic cancer cell lines such as ASPC-1 and PANC-1²⁵⁾ and in cancers,¹⁷⁾ no such overexpression of Akt was found in the present cell lines. In the presence of serum (10%), all the cell lines showed phosphorylation at Ser473 of Akt (data not shown), whereas Akt phosphorylation was observed in 4 cell lines, PCI-66, PCI-68, KMP-4, and KMP-3, in the absence of serum stimulation (Fig. 1). Akt kinase activity

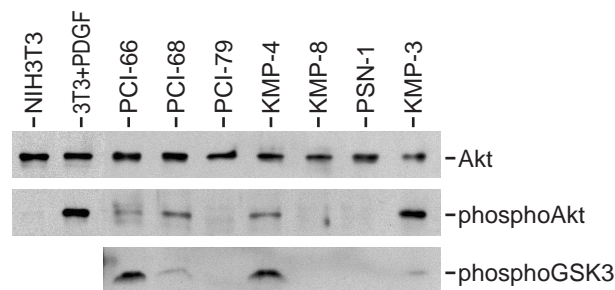


Fig. 1. Constitutive Akt activation in pancreatic cancer cells. Cells were deprived of serum for 48 h. Levels of total Akt (–Akt) and phosphorylated Akt at Ser437 (–phosphoAkt) were determined by immunoblots using specific antibodies. Phosphorylation of GSK-3 α and - β (–phosphoGSK3) at Ser21 and Ser9, respectively, by the Akt purified from the cells was measured as described in “Materials and Methods.”

Table I. Molecular Status of the Seven Pancreatic Cancer Cell Lines

	Akt activation			<i>K-Ras</i> mutation ^{b)}	<i>H-Ras</i> mutation	<i>p53</i> mutation ^{b)}	<i>PTEN</i> mutation ^{b)}	<i>Akt1</i> , <i>Akt2</i> mutation
	Constitutive	Dependence to PI3K	Dependence to TK ^{a)}					
PCI-66	positive	no	no	Gly 12 Asp (GGT→GAT)	none	Pro 250 Ser (CCC→TCC)	wild-type	none
PCI-68	positive	no	no	Gly 12 Asp (GGT→GAT)	none	His 179 Tyr (CAT→TAT)	wild-type	none
PCI-79	—			Gly 12 Val (GGT→GTT)	none	Arg 248 Gln (CGG→CAG)	wild-type	none
KMP-4	positive	yes	yes	Gly 12 Arg (GGT→CGT)	none	codons 212–215 inframe 12 b deletion	237 AAG→AAAG (frameshift)	none
KMP-8	—			Gly 12 Asp (GGT→GAT)	none	wild-type	wild-type	none
PSN-1	—			Gly 12 Arg (GGT→CGT)	none	Lys 132 Gln (AAG→CAG)	wild-type	none
KMP-3	positive	yes	yes	Gly 12 Val (GGT→GTT)	none	212–215 12b (4 codons) deletion	<i>PTEN</i> no expression ψ <i>PTEN</i> expression	none

a) TK, tyrosine kinases; b) numbers indicate mutant codons.

was also observed in the same 4 cell lines in the absence of serum (Fig. 1), while there was no apparent correlation between the amounts of phosphorylated Akt and the degrees of phosphorylation of GSK-3 in the 4 cell lines.

K-Ras, H-Ras, p53, and PTEN mutations in pancreatic cancer cell lines Since Ras activated by mutation is known to activate the p110 catalytic subunit of PI3K, resulting in Akt activation,¹⁰⁾ we sequenced *K-Ras* and *H-Ras* in the 7 cell lines. Three types of missense mutations (Gly→Asp, Val, or Arg) were found at codon 12 of *K-Ras*, showing no apparent correlation to constitutive Akt activation (Table I). None of the cell lines contained *H-Ras* mutations, however. As p53 is known to activate Ras upstream,^{21, 26)} we determined *p53* status in the cell lines by means of a yeast *p53* functional assay.^{23, 24)} All the cell lines, except KMP-8 which retained wild-type *p53*, showed complete loss of *p53* function due to distinct mutations (Table I). We then determined *PTEN* status by means of *PTEN* stop codon assay followed by sequence analyses. We identified a frameshift mutation (1 base insertion at codon 237) in KMP-4 cells as 100% red colonies (Fig. 2, b and e). KMP-3 showed loss of wild-type *PTEN* expression (no white colonies), but expression of the pseudogene ψ *PTEN* was identified as pink colonies,

and this was confirmed by sequence analysis (Fig. 2, a, c and d). Other cell lines showed white colonies, and sequence analysis of the plasmids recovered from white colonies ruled out the presence of *PTEN* missense mutations.

Constitutive MEK1/2 phosphorylation in pancreatic cancer cell lines The major Ras-signaling other than PI3K-Akt is activation of Raf1, which results in the acti-

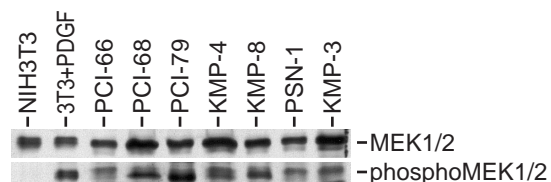


Fig. 3. Constitutive activation of MEK in pancreatic cancer cells. Cellular extracts from the cells deprived of serum for 48 h were electrophoresed together with extracts from serum-deprived NIH3T3 cells (negative control) and NIH3T3 cells cultured with serum (-3T3+PDGF, positive control), which were provided by the manufacturer (New England Biolabs), and probed with antibodies to MEK1/MEK2 and phosphorylated MEK1/MEK2 (Ser217/Ser221).

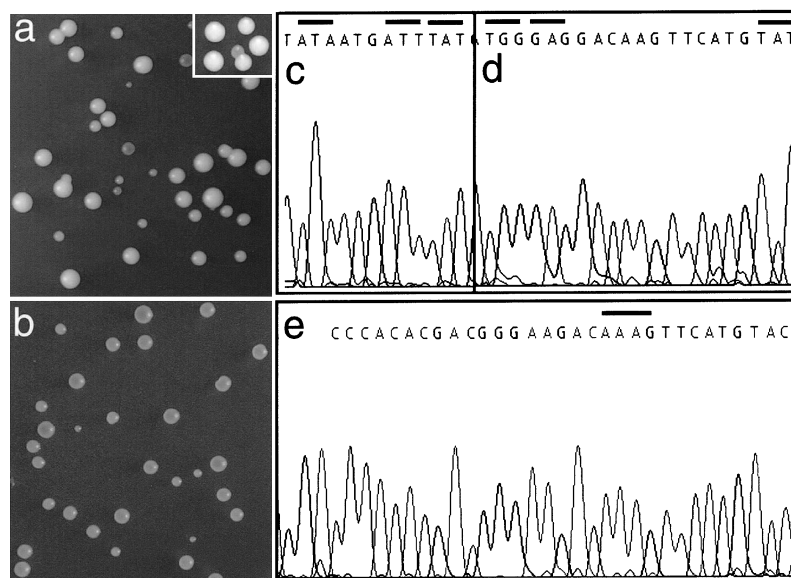


Fig. 2. ψ *PTEN* expression and *PTEN* mutation in KMP-3 and KMP-4. **a.** Pink colonies observed in the *PTEN* stop codon assay for KMP-3. Note the color differences from red colonies of the assay background and white colonies in PCI-66 (right upper corner inset). **b.** Red colonies observed in the *PTEN* stop codon assay for KMP-4. **c.** Sequence chromatogram of ψ *PTEN* cDNA detected in KMP-3 cells, showing codons 99–102 corresponding to *PTEN* codons 133–136, where three base-replacements in ψ *PTEN* from *PTEN* are noted (bars): codon 133 *PTEN* GTA (Val)→99 ψ *PTEN* ATA (Ile); codon 135 *PTEN* ATA (Ile)→101 ψ *PTEN* ATT (Ile); codon 136 *PTEN* TGT (Cys)→102 ψ *PTEN* TAT (Tyr). **d.** Codons 200–206 of ψ *PTEN* cDNA in KMP-3, corresponding to *PTEN* codons 234–240, depicting three base replacements (bars): codon 234 *PTEN* CGG (Arg)→codon 200 ψ *PTEN* TGG (Trp); codon 235 *PTEN* GAA (Glu)→codon 201 ψ *PTEN* GAG (Glu); codon 240 *PTEN* TAC (Tyr)→codon 206 ψ *PTEN* TAT (Tyr). **e.** One base insertion at codon 237 (AAG→AAAG, frameshift, bar) detected in KMP-4.

vation of extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase/ERK kinase (MEK).²⁷⁾ To confirm the augmented signaling consequent to Ras activation, we then examined MEK phosphorylation at Ser217 and Ser221 by an immunoblot analysis. All 7 cell lines showed MEK phosphorylation in the absence of serum stimulation (Fig. 3), confirming the equal effect of Ras activation in all the cell lines.

Inhibition of Akt activation by PI3K inhibitor LY294002 Since PCI-66 and PCI-68 cells showed constitutive activation of Akt without loss of *PTEN*, we questioned whether excessive production of PIP-3 by activated PI3K might be responsible for the Akt activation in these cell lines. To confirm this, we treated PCI-66, PCI-68, KMP-3, and KMP-4 with the specific PI3K inhibitor LY294002²⁸⁾ and evaluated Akt phosphorylation status. LY294002 inhibited Akt phosphorylation completely in KMP-3 and partially in KMP-4, indicating that Akt activation was dependent on PI3K in KMP-3 and at least partially so in KMP-4 cells. In PCI-66 or PCI-68, such inhibition was not observed, indicating that Akt activation in PCI-66 and PCI-68 cells was independent of PIP-3 over-production (Fig. 4).

Participation of receptor tyrosine kinases An autocrine mechanism via coexpression of EGFR and transforming growth factor (TGF)- α is known in pancreatic cancers, and may also lead to activation of PI3K.^{29, 30)} As a possible cause of the PI3K activation bypassing Ras,⁹⁾ we questioned whether there might be EGFR autophosphorylation

at Tyr1173 in the cells. An immunoblot analysis showed the absence of autophosphorylation in all the cells deprived of serum (Fig. 5a), whereas EGFR stimulation induced phosphorylation of EGFR (Fig. 5b). We then

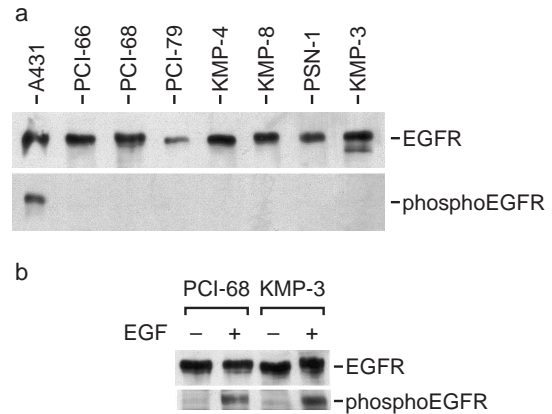


Fig. 5. Absence of autophosphorylation of EGFR in pancreatic cancer cells. **a.** Cellular extracts from pancreatic cancer cells serum-deprived for 48 h and A431 epidermoid cancer cells (positive control, Transduction Laboratories, Lexington, KY)⁶¹⁾ were subjected to immunoblot analyses for EGFR and phosphorylated EGFR. **b.** PCI-68 and KMP-3 cells were treated with none or 100 ng/ml EGF for 20 min. Levels of EGFR and phosphorylated EGFR were determined by immunoblotting.

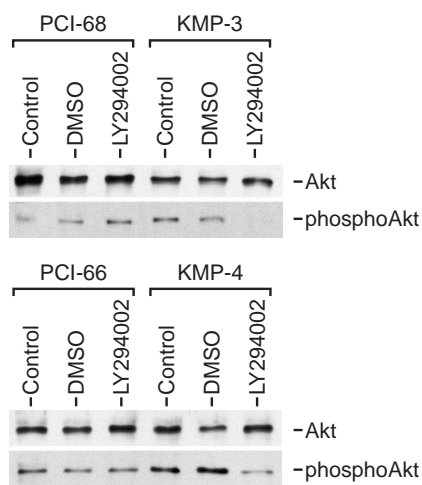


Fig. 4. PI3K-dependent and -independent constitutive Akt activation. PCI-68, KMP-3, PCI-66, and KMP-4 cells were deprived of serum for 48 h and treated with none (-Control), 0.2% DMSO (solvent, -DMSO), or 40 μ M LY294002 (PI3K inhibitor) for an additional 8 h. Levels of Akt and phosphorylated Akt were assessed by immunoblots with specific antibodies.

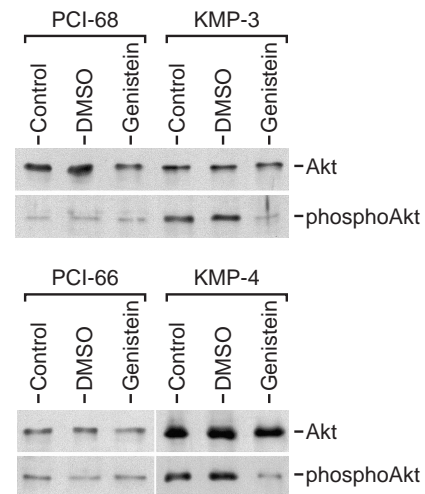


Fig. 6. Tyrosine kinase-dependent and -independent constitutive Akt activation. PCI-68, KMP-3, PCI-66, and KMP-4 cells were deprived of serum for 48 h and treated with none (-Control), 0.1% DMSO (solvent, -DMSO), or 100 μ M genistein (tyrosine kinase inhibitor) for an additional 8 h. Levels of Akt and phosphorylated Akt were assessed by immunoblots with specific antibodies.

treated cells with genistein, a broad-spectrum tyrosine kinase inhibitor,³¹⁾ and evaluated Akt phosphorylation. As seen in the case of LY294002 treatment, genistein failed to inhibit Akt phosphorylation in PCI-66 and PCI-68 cells, whereas it inhibited Akt phosphorylation in KMP-3 and KMP-4 cells (Fig. 6).

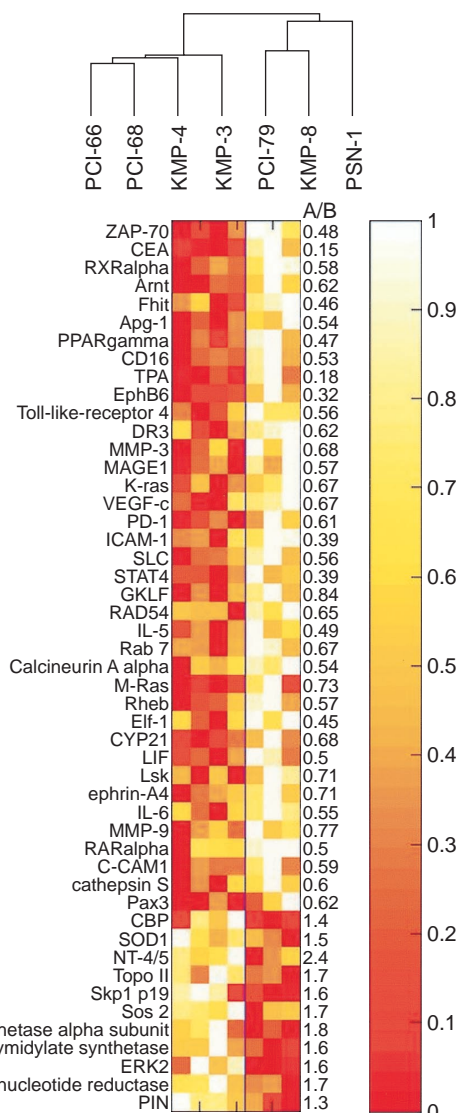


Fig. 7. Differential expression of 49 genes in the pancreatic cell lines. RNAs from the 7 pancreatic cell lines were analyzed by cDNA array. Genes that showed significant differences in expression ($P < 0.05$) between group A (PCI-66, PCI-68, KMP-4, and KMP-3) and group B (PCI-79, KMP-8, and PSN-1) were extracted by using a computer program. The cell lines were clustered according to the expression profiles of the 49 genes (top of the figure). Expression of each gene is shown in a relative scale of color.

Mutation of Akt1 and Akt2 in PCI-66 and PCI-68 cells
 Because PIP-3 is the only direct effector so far identified in Akt activation, we inferred that the cause of activation of Akt in PCI-66 and PCI-68 cells lies in Akt itself—possibly autonomous phosphorylation owing to some mutation in Akt protein. Thus, we sequenced the entire open reading frames of *Akt1* and *Akt2* in the cells. However, we found no mutation in *Akt1* or *Akt2*.

mRNA expression profiles in the pancreatic cancer cell lines
 To establish the influence of constitutive Akt activation on mRNA expression profiles in the pancreatic cancer cell lines, we performed a cDNA array analysis. By computer analysis, we identified a total of 49 genes which were expressed at significantly higher (11 genes) or lower (38 genes) levels ($P < 0.05$) in KMP-3, KMP-4, PCI-66 and PCI-68 cells as compared to PCI-79, KMP-8, and PSN-1 cells. A cluster analysis of the expression profiles successfully classified the cell lines according to Akt status (Fig. 7). Expression analysis, however, did not disclose a notable difference in gene expression between PCI-66/PCI-68 cells and KMP-3/KMP-4 cells.

DISCUSSION

In the present study, we demonstrated Akt phosphorylation at Ser473 and consequent Akt activation assessed by GSK-3 in 4 pancreatic cancer cell lines (PCI-66, PCI-68, KMP-3, KMP-4) in the absence of serum, while all 7 tested cell lines showed Akt phosphorylation in the presence of serum (data not shown). Hence, we call the Akt activation in the 4 cell lines “constitutive,” in the sense that it is independent of growth factor stimulation. Although phospho-GSK-3 bands are positive exclusively in the 4 cell lines, the band intensities did not apparently correlate with the amounts of phosphorylated Akt. One possible explanation for this is that there might be some imbalance in phosphorylation between the two phosphorylation sites (Thr308 and Ser473) in Akt, and that both are required for full activation of Akt, whereas Thr308 phosphorylation itself can partially activate Akt.^{32, 33)} In the 3 cell lines that did not show constitutive Akt phosphorylation at Ser473, Akt activity was completely negative, suggesting that Thr308 phosphorylation without Ser473 phosphorylation is unlikely to be the sole determinant of constitutive Akt activation.

We demonstrated that at least two different mechanisms were responsible for the constitutive Akt activation observed in the 4 cell lines. One is what we have seen in KMP-3 and KMP-4, which involved loss of PTEN function by loss of expression of *PTEN* and by a frameshift mutation at codon 237, respectively. Since we demonstrated the activating-*K-Ras* mutations and the consequent MEK phosphorylation in all 7 cell lines, *K-Ras* activation itself did not appear to be a sufficient cause of the consti-

tutive activation of Akt. It has already been reported that there was no direct correlation of *K-Ras* mutational status and “constitutive” activation or the expression levels of activated MEK in pancreatic cancer cell lines.³⁴⁾ However, we recognized suppression of Akt phosphorylation in KMP-3 and KMP-4 using LY294002, which inhibits PI3K downstream of K-Ras²⁸⁾ and genistein, which inhibits tyrosine kinase upstream of K-Ras. This finding indicated that the Ras-PI3K-mediated signal at least is required for Akt activation in the absence of PTEN function in the two cell lines. This may be mediated via MEK or an MEK-independent pathway. These observations are consistent with the previous findings of oncogenic roles of Ras and Akt: 1) combined activation of Ras and Akt is necessary and sufficient for tumor formation (glioblastoma) in mice,³⁵⁾ and 2) transforming activity of oncogenic H-Ras can be reverted by wild-type PTEN.³⁶⁾ This raises a question about the mutational selection of K-Ras, especially at codon 12, in pancreatic cancers rather than H-Ras, which is known to be more potent in activating PI3K.³⁷⁾ None of the cell lines used in this study harbored *H-Ras* mutations. Specific selection of Ras isoforms is also observed in other types of cancers. For example, *K-Ras* mutations occur in 50% of colon cancers, whereas *N-* and *H-Ras* mutations are extremely uncommon.³⁸⁾ Conversely, *H-Ras* and *N-Ras* mutations are found in 80% of bladder cancers, but *K-Ras* mutations are a rare event.³⁹⁾ These mutational selections of Ras necessarily suggest the importance of another Ras-signaling pathway, as indicated by previous observations that a balanced and parallel activation of MEK and Akt pathways by Ras occurs in cellular transformation by oncogenic tyrosine kinases.^{40,41)} The balance between the Ras-Raf1-MEK pathway and the Ras-PI3K-Akt pathway corresponding to a cancer cell type may be associated with specific roles of oncogenic components (tyrosine kinases, Ras, and PTEN). Although Akt is known to be able to inhibit the Raf1-MEK pathway,^{42,43)} this is unlikely in pancreatic cancer cells, because no apparent reciprocity was observed between the activities of MEK and Akt in the present study.

Another mechanism of the constitutive Akt activation which we consider to be implicated in PCI-66 and PCI-68 cells has not previously been reported. PTEN sequence was wild-type in these cell lines. Both the PI3K inhibitor

LY294002 and the tyrosine kinase inhibitor genistein failed to inhibit the Akt activation in these cells, showing that the mechanism was completely independent of the tyrosine kinase receptors/K-Ras/PI3K/PTEN pathway. Mutational analysis of *Akt1* and *Akt2* in these cells disclosed no mutation in the entire coding sequences. There might be an unknown supplier of phosphatidylinositol-3,4,5-triphosphate (PIP-3) that is resistant to LY294002, including a possible mutation in PIK3. Alternatively, it is possible that a mediator (PDK) or a coactivator of Akt phosphorylation is over-active even at low levels of PIP-3 in the phosphorylation of Akt. For instance, it is plausible that a change in an unknown serine kinase (putatively called PDK2) that acts on Akt at Ser473, or in its upstream regulators, is responsible for the constitutive Akt activation in PCI-66 and PCI-68 cells. An example is a mutant PDK1 (A280V) whose phosphorylation of Ser308 of Akt is resistant to PI3K inhibitors.⁴⁴⁾

Irrespective of the mechanisms of Akt activation, we identified 49 common genes expressed in the 4 cell lines at significantly different levels compared to the 3 control cell lines without constitutive Akt activation. Among the 49 genes, many are known to be involved in Ras- or Akt-signaling, i.e., PPAR γ ,⁴⁵⁾ Ephrin B6,⁴⁶⁾ Rheb,⁴⁷⁾ CBP,⁴⁸⁾ NT-4/5,⁴⁹⁾ SOS2,⁵⁰⁾ and ERK2²⁷⁾; some are known to be regulated by Ras-signals, i.e., tissue plasminogen activator (TPA),⁵¹⁾ MMP-3,⁵²⁾ and MMP-9,⁵³⁾ ribonucleotide reductase⁵⁴⁾; and others are aberrantly expressed in pancreatic cancer cells, i.e., CEA,⁵⁵⁾ Fhit,⁵⁶⁾ VEGF-c,⁵⁷⁾ ICAM-1,⁵⁸⁾ and LIF.⁵⁹⁾ Low expressions of M-Ras⁶⁰⁾ and H-Ras in cells with activated Akt is of note, because they suggest a possible direct or indirect negative feedback loop from Akt-signaling. It remains unknown, however, whether these 49 genes are actually regulated negatively or positively by Akt-signaling. Further analyses seem warranted to uncover the regulatory mechanisms of these genes.

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