

Prognostic Significance of Activated Akt Expression in Pancreatic Ductal Adenocarcinoma

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ABSTRACT

Purpose: Akt is a serine/threonine kinase that plays a central role in tumorigenesis. Among the members of Akt family, Akt2 is associated with the development of human cancers. The present study was designed to clarify the prognostic significance of Akt2 and activated Akt expression in pancreatic ductal adenocarcinoma (PDAC). In addition, activated extracellular signal-regulated kinase 1 and 2 (ERK1/2) and the proliferation activity of tumor cells detected by Ki-67 immunohistochemistry were examined.

Experimental Design: Immunohistochemical analysis was performed on paraffin-embedded specimens from 65 patients with PDAC; 36 males and 29 females with ages ranging from 48 to 79 years (median, 66 years) of age. Expression levels of Akt2, phosphorylated Akt (*p*-Akt), and phosphorylated ERK 1/2 (*p*-ERK 1/2) were categorized as either weaker (low intensity) or equal to stronger (high intensity) compared with those in the endothelial cells of the same specimens. For Ki-67 immunohistochemistry, cases were divided into two groups: level 1, Ki-67 labeling index (LI), <20%; level 2, Ki-67 LI, ≥20%.

Results: Twenty-six (42.6%), 28 (45.9%), 39 (63.9%), and 46 (75.4%) of the tumors showed high intensity of Akt2, *p*-Akt, and *p*-ERK 1/2 expression, and Ki-67 LI level 2, respectively. A significant positive correlation was observed between Akt2 and *p*-Akt expression ($P < 0.01$). Multivariate analysis revealed that *p*-Akt expression, Ki-67 LI, and histological differentiation are independent prognosticators for PDAC.

Conclusions: *p*-Akt expression is a significant prognostic indicator for PDAC. Inhibition of Akt is a possible molecular approach for treatment of PDAC.

INTRODUCTION

Three variations of the serine/threonine kinase Akt are present in the human genome, thus designated as *Akt1*, *Akt2*, and *Akt3* (1, 2). Akt is activated by various growth factors and plays a role in tumorigenesis by inhibiting apoptosis and mediating cell proliferation (1–3). Phosphorylation of Thr³⁰⁸ is necessary for Akt activation, and phosphorylation of Ser⁴⁷³ results in maximum activity (4).

Pancreatic ductal adenocarcinoma (PDAC) usually has a poor prognosis (5–7). The altered expression of genes such as the *K-ras* oncogene frequently occurs during the development of PDAC (8). However, specific patterns of gene alternations responsible for PDAC have not been identified (9).

The detection of Akt activation is a useful method for predicting the progression and prognosis of PDAC because it represents the signaling of various growth factors that playing key roles in tumorigenesis (1). Among the members of the Akt family, Akt2 is associated with the development of human cancers (10). Overexpression of the *Akt2* gene is observed in ~20% of all cases of PDAC (11). Transfection of antisense *Akt2* RNA into PDAC cell lines inhibits its tumorigenicity (11). Therefore, an association between the activation or overexpression of Akt, and the clinicopathological behavior of PDAC is postulated.

In the present study, the expression of Akt was immunohistochemically examined in 65 patients with PDAC, and its relationship with clinicopathological factors and the patient survival rate was evaluated. In addition, the activation status of extracellular signal-regulated kinase 1 and 2 (ERK1/2), another serine/threonine kinase that plays a role in cell proliferation and differentiation (12, 13), and the proliferation activity of tumor cells seen by using Ki-67 immunohistochemistry (14) was examined.

MATERIALS AND METHODS

Patients. Sixty-five patients who underwent curative resection of primary PDAC were selected for the study. They underwent surgery at the Gastroenterological Surgery Division, Osaka University Hospital and Division of Surgery, Osaka Medical Center for Cancer and Cardiovascular Diseases from June 1982 to August 2001. The patients consisted of 36 males and 29 females ranging from 48 to 79 years (median, 66 years) of age. The stage of the disease was classified according to the pathological Tumor-Node-Metastasis staging system (15).

Surgically resected specimens were macroscopically examined to determine the location and size of the tumors. Then, tissue samples were fixed in 10% formalin and routinely processed for paraffin embedding. Histological sections cut with a

Received 11/23/02; revised 11/17/03; accepted 12/10/03.

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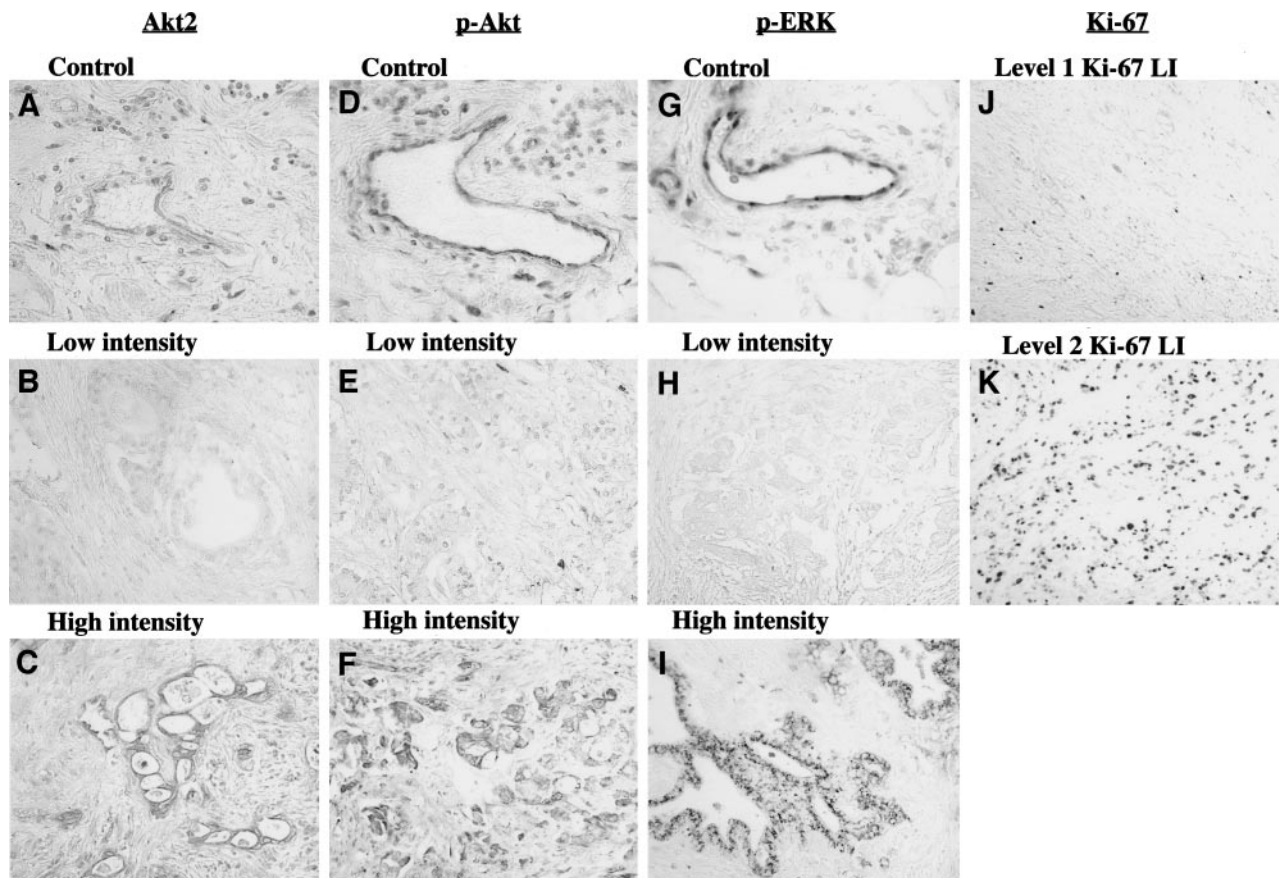


Fig. 1 Akt2 (A–C), phosphorylated-Akt (*p*-Akt; D–F), phosphorylated extracellular signal regulated kinase 1 and 2 (*p*-ERK 1/2; G–I) immunostaining. A, D, and G, internal controls for Akt2, *p*-Akt, and *p*-ERK 1/2 staining. The cytoplasm of endothelial cells is positively stained (magnification, $\times 50$). B, E, and H, low intensity staining of Akt2, *p*-Akt, and *p*-ERK 1/2. Tumor cells are weakly stained compared with that of endothelial cells (magnification, $\times 25$). C, F, and I, high intensity staining of Akt2, *p*-Akt, and *p*-ERK 1/2. Tumor cells are strongly stained similar to that of endothelial cells (magnification, $\times 25$). J, Ki-67 labeling index (LI) level 1. Less than 20% of the tumor cells show nuclear staining (magnification, $\times 25$). K, Ki-67 LI level 2. More than 20% of the tumor cells show nuclear staining (magnification, $\times 25$).

4- μ m thickness were stained with H&E and reviewed by one of the authors (Y. Hoshida) to determine histological differentiation and the existence of metastasis in the lymph nodes. All tumors were adenocarcinomas with well- (31 cases), moderately (24 cases), and poorly differentiated (10 cases) morphologies.

After surgery, serum CA 19-9 level was measured, and ultrasonography and computed tomography scan were performed at 3-month intervals. The patients were followed-up until March 31, 2002: the follow-up periods for survivors ranged from 3.4 to 105.5 months (median, 23.6 months) after surgery.

Immunohistochemical Analysis. The specificity of antiphosphorylated Akt (*p*-Akt; Thr³⁰⁸) polyclonal antibody (Cell Signaling Technology, Beverly, MA) was examined by Western blot analysis using extracts from Jurkat cells, which showed a positive band after platelet-derived growth factor treatment. Antibody preincubated with antigen peptide was used for a negative control test and gave a uniformly negative result.

The immunoperoxidase procedure (avidin-biotin-complex method) was performed on the paraffin-embedded sections. Antigen retrieval was carried out by heating the sections in 10 mM citrate buffer for 5 min. Anti-Akt2 monoclonal antibody

(Santa Cruz Biotechnology, Santa Cruz, CA), anti-*p*-Akt polyclonal antibody, anti-*p*-ERK1/2 (Thr²⁰²/Tyr²⁰⁴) monoclonal antibody (Cell Signaling Technology), and anti-Ki-67 (clone MIB 1) monoclonal antibody (Dako Cytomation A/S, Copenhagen, Denmark) were used as primary antibodies at dilutions of 1:400, 1:100, 1:100, and 1:50, respectively. Sections were counterstained lightly with methyl green. For negative controls, non-immunized mouse or rabbit IgG serum (Vector Laboratories, Burlingame, CA) was used as primary antibodies. Positive intracytoplasmic staining of endothelial cells in the noncancerous areas in each specimen was used as an internal positive control. Stained sections were blindly evaluated without prior knowledge of the clinicopathological parameters. The staining intensity in the cytoplasm of the tumor cells was categorized as follows: weaker (low intensity) or equal to stronger (high intensity) than that of the endothelial cells. Cases with negative endothelial cell staining were regarded as having underwent poor antigen preservation and excluded from additional analyses. For Ki-67 immunohistochemistry, cells showing intranuclear staining were judged as Ki-67 positive. The Ki-67-positive cells among 200 tumor cells were counted, and the percentage was

Table 1 Correlation between Akt2, *p*-Akt^a, *p*-ERK 1/2^a, and Ki-67 expression and clinicopathological factors in 61 patients with pancreatic ductal adenocarcinoma

Factors	Category	Total no. of patients	Patients with high-intensity Akt2 (n = 26)	<i>P</i>	Patients with high-intensity <i>p</i> -Akt (n = 28)	<i>P</i>	Patients with high-intensity <i>p</i> -ERK 1/2 (n = 39)	<i>P</i>	Patients with level 2 Ki-67 LI (n = 46)	<i>P</i>
Age (years)	1: ≤60	15	6 (40.0%)	N.S.	7 (46.7%)	N.S.	9 (60.0%)	N.S.	10 (66.7%)	N.S.
	2: >60	46	20 (43.5%)		21 (45.7%)		30 (65.2%)		36 (78.3%)	
Gender	1: Male	34	13 (38.2%)	N.S.	12 (35.3%)	N.S.	20 (58.8%)	N.S.	28 (82.4%)	N.S.
	2: Female	27	13 (48.1%)		16 (59.3%)		19 (70.4%)		18 (66.7%)	
Location of tumor	1: Head	50	23 (46.0%)	N.S.	24 (48.0%)	N.S.	31 (62.0%)	N.S.	36 (72.0%)	N.S.
	2: Body/Tail	11	3 (27.3%)		4 (36.4%)		8 (72.7%)		10 (90.9%)	
T (pTNM)	1: T ₁	4	1 (25.0%)	N.S.	1 (25.0%)	N.S.	2 (50.0%)	N.S.	3 (75.0%)	N.S.
	2: T ₂	7	3 (42.9%)		3 (42.9%)		5 (71.4%)		3 (42.9%)	
	3: T ₃	31	13 (41.9%)		15 (48.4%)		20 (64.5%)		23 (74.2%)	
	4: T ₄	19	9 (52.6%)		9 (52.6%)		12 (63.2%)		17 (89.5%)	
Histological differentiation	1: Well	29	13 (44.8%)	N.S.	14 (48.3%)	N.S.	19 (65.5%)	N.S.	25 (86.2%)	N.S.
	2: Moderately	23	8 (34.8%)		11 (47.8%)		14 (60.9%)		14 (60.9%)	
	3: Poorly	9	5 (55.6%)		3 (33.3%)		6 (66.7%)		7 (77.8%)	
Lymph node metastasis	1: Present	36	15 (41.7%)	N.S.	17 (47.2%)	N.S.	24 (66.7%)	N.S.	28 (77.8%)	N.S.
	2: Absent	25	11 (44.0%)		11 (44.0%)		15 (60.0%)		18 (72.0%)	
Stage (pTNM)	1: I	4	1 (25.0%)	N.S.	1 (25.0%)	N.S.	2 (50.0%)	N.S.	2 (50.0%)	N.S.
	2: II	38	16 (42.1%)		18 (47.4%)		25 (65.8%)		27 (71.1%)	
	3: III	19	9 (47.4%)		9 (47.4%)		12 (63.2%)		17 (89.5%)	
Akt2 expression	1: Low intensity	35			11 (31.4%)	<0.01	21 (60.0%)	N.S.	25 (71.4%)	N.S.
	2: High intensity	26			17 (65.4%)		18 (69.2%)		21 (80.8%)	
<i>p</i> -Akt expression	1: Low intensity	33	9 (27.3%)	<0.01			20 (60.6%)	N.S.	26 (78.8%)	N.S.
	2: High intensity	28	17 (60.7%)				19 (67.9%)		20 (71.4%)	
<i>p</i> -ERK 1/2 expression	1: Low intensity	22	8 (36.4%)	N.S.	9 (40.9%)	N.S.			16 (34.8%)	N.S.
	2: High intensity	39	18 (46.2%)		19 (48.7%)				30 (76.9%)	
Ki-67 LI	1: Level 1 (<20%)	15	5 (33.3%)	N.S.	8 (53.3%)	N.S.	9 (60.0%)	N.S.		
	2: Level 2 (≥20%)	46	21 (45.7%)		20 (43.5%)		30 (65.2%)			

^a *p*-Akt, phosphorylated Akt; *p*-ERK 1/2, phosphorylated extracellular signal-regulated kinase 1 and 2; Ki-67 LI, Ki-67 labeling index; N.S., nonsignificant; pTNM, pathological Tumor-Node-Metastasis.

used as the Ki-67 labeling index (LI). Cases were divided into two groups: level 1, Ki-67 LI, <20%; level 2, Ki-67 LI, ≥20%.

Statistics. Statistical analysis was performed using JMP software (SAS Institute, Inc., Cary, NC). χ^2 test and Fisher's exact probability test were used to analyze the correlation between Akt overexpression or activation and clinicopathological features. The Kaplan-Meier method was used to calculate the overall patient survival rate, and the difference in survival curves was evaluated using a log-rank test (16). Cox's proportional hazards regression model with stepwise manner was used to analyze the independent prognostic factors (17). $P < 0.05$ was considered to be statistically significant.

RESULTS

Akt2, *p*-Akt, and *p*-ERK Expression and Ki-67 LI in PDACs. Four (6.1%) of the 65 cases that did not show endothelial staining for Akt2, *p*-Akt, or *p*-ERK were regarded as having undergone poor antigen preservation and were excluded from additional analyses. Twenty-six, 28, and 39 cases showed high intensity staining for Akt2, *p*-Akt, and *p*-ERK, respectively, and 46 cases showed Ki-67 LI level 2 (Fig. 1).

Univariate and Multivariate Analyses for Prognostic Factors for PDAC. A significant positive correlation was observed between Akt2 and *p*-Akt staining patterns ($P < 0.01$; Table 1). Correlation between other combinations was not observed.

The 5-year overall survival rate for the 61 patients was

32.3%. Patients with low intensity of *p*-Akt expression showed a significantly better 5-year survival rate (57.0%) than those with high-intensity expression (14.1%; $P < 0.05$, Fig. 2). Akt2 and *p*-ERK expression were not significant prognosticators. A univariate analysis revealed that *p*-Akt expression, Ki-67 LI, histological differentiation, and T factor of the pathological Tumor-Node-Metastasis staging system were significant factors of the overall survival rate (Table 2). A subsequent multivariate analysis revealed that *p*-Akt expression, Ki-67 LI, and histological differentiation were independent prognosticators (Table 3).

DISCUSSION

To establish proper therapeutic modalities for PDAC, an accurate assessment of the factors affecting tumor progression and patient prognosis is critical. Although the conventional Tumor-Node-Metastasis staging system, which is defined by tumor size, tumor progression, lymph node involvement, and distant metastasis (15), is useful for PDAC classification, the outcome is poor for patients even in the low-stage (I and II) groups (5). Therefore, the prognostic use of several molecular markers for PDAC classification have been investigated (8), although none proved useful for predicting patient prognosis (9). We undertook the present study to determine whether Akt2, *p*-Akt, and *p*-ERK expression and the proliferation activity seen by Ki-67 LI are valid biological indicators of the aggressiveness of PDAC.

The constitutive activation of Akt in endothelial cells is

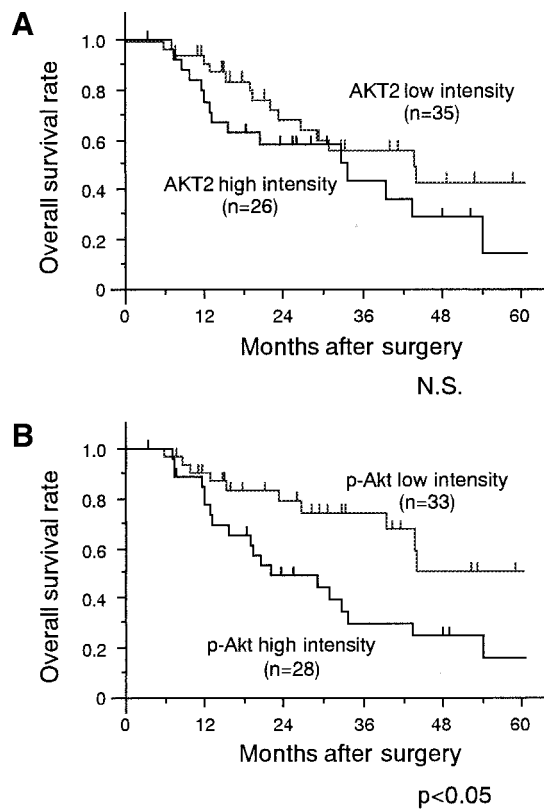


Fig. 2 Overall survival rate of pancreatic ductal adenocarcinoma patients with high and low intensity Akt2 and *p*-Akt expression. A significant difference was observed between the two groups for *p*-Akt expression (B; $P < 0.05$) but not for Akt2 expression [A; nonsignificant (N.S.)].

essential for the maintenance of endothelial cell-extracellular matrix attachment (18). In the present study, Akt2 and *p*-Akt expressions in endothelial cells were used as an internal positive control for each specimen, which showed a consistent moderate staining intensity. However, the staining intensity of nontumorous pancreatic tissues varied among the samples (data not shown). This is because of the different conditions of pancreatic tissue frequently affected by varying degrees of inflammation and fibrosis.

The patients characteristics such as gender, age, and 5-year survival rates were similar to those in the previous studies from Japan (5, 6) and Western countries (7). The present uni- and multivariate analyses confirmed the prognostic significance of tumor differentiation and T-factor for the pathological Tumor-Node-Metastasis classification of PDAC, as previously reported (5, 7). Therefore, the results of the present cases are generally applicable to PDAC.

The present study showed a significant correlation between Akt2 and *p*-Akt for PDAC ($P < 0.01$), which implies a close association between increased Akt activity and overexpression. Additional investigation is necessary to clarify the mechanism that controls Akt expression and activation.

Uni- and multivariate analyses showed that activated Akt expression is an independent prognosticator for PDAC patients together with histological differentiation and Ki-67 LI. The cause for poor prognosis of PDAC is local recurrence (5–7). Pleiotropic Akt activities such as antiapoptosis (3), proliferation (3), invasiveness (19), and angiogenesis (20) provide PDAC a microinvasiveness at the resected margin and promotes the recurrence of cancer. These findings show that activated Akt expression is a sign of poor prognosis for PDAC.

The phosphatase and tensin homologue deleted from chromosome 10 (*PTEN*) is a tumor suppressor gene that negatively

Table 2 Univariate analysis of clinicopathological factors for the overall survival rate of 61 patients with pancreatic ductal adenocarcinoma

Factors	Category	No. of patients	5-year overall survival rate (%)	<i>P</i>
Age (years)	1: ≤60	15	71.1	N.S.
	2: >60	46	25.2	
Gender	1: Male	34	34.9	N.S.
	2: Female	27	30.5	
Location of tumor	1: Head	50	37.9	N.S.
	2: Body/Tail	11	0	
T(pTNM)	1: T ₁	4	66.7	<0.05 ^b
	2: T ₂	7	55.6	
	3: T ₃	31	32.2	
	4: T ₄	19	18.4	
Histological differentiation	1: Well/Moderately	52	37.6	<0.01
	2: Poorly	9	0	
Lymph node metastasis	1: Present	36	19.6	N.S.
	2: Absent	25	44.9	
Akt2 expression	1: Low intensity	35	43.6	N.S.
	2: High intensity	26	14.6	
<i>p</i> -Akt expression	1: Low intensity	33	50.6	<0.05
	2: High intensity	28	16.4	
<i>p</i> -ERK 1/2 expression	1: Low intensity	22	45.1	N.S.
	2: High intensity	39	28.4	
Ki-67 LI	1: Level 1 (<20%)	15	66.3	<0.05
	2: Level 2 (≥20%)	46	18.8	

^a N.S., nonsignificant; pTNM, pathological Tumor-Node-Metastasis; *p*-Akt, phosphorylated Akt; *p*-ERK 1/2, phosphorylated extracellular signal-regulated kinase 1 and 2; Ki-67 LI, Ki-67 labeling index.

^b 1 and 2 versus 3 and 4.

Table 3 Multivariate analysis of clinicopathological factors for overall survival rate of 61 patients with pancreatic ductal adenocarcinoma

Factors	Category	Relative risk	95% confidence interval	χ^2 value	P
Histological differentiation	1: Well/Moderately 0: Poorly	3.69	1.19–2.94	6.67	0.0098
p-Akt ^a expression	1: Low intensity 0: High intensity	3.44	1.28–2.80	10.04	0.0015
Ki-67 LI	1: Level 1 (< 20%) 0: Level 2 (\geq 20%)	3.92	1.20–3.72	7.70	0.0055

^a p-Akt, phosphorylated Akt; Ki-67 LI, Ki-67 labeling index.

regulates the Akt pathway, and its mutations are often associated with an aggressive tumor phenotype (21). Somatic deletions or mutations of this gene have been identified in a large proportion of tumors, including glioblastomas, and endometrial and prostate cancers, thus placing *PTEN* among the most commonly mutated genes in human cancers (21). The decreased expression of *PTEN* for PDAC occurs at the mRNA and protein levels, whereas deletions or mutations are not frequently observed (22).

Constitutive activation of the Akt pathway occurs in many human PDAC cell lines (23). Inhibition of the phosphatidylinositol 3'-kinase/Akt pathway by inhibitors of phosphatidylinositol 3'-kinase (LY294002) induces apoptosis in tumor cells *in vitro* and inhibits the growth of tumor xenografts *in vivo* without excessive toxicity in nude mice (23). *In vitro* studies showed that Akt activation inhibits gemcitabine-induced apoptosis, and the addition of Akt inhibitors diversely enhances apoptosis (24). The Akt activation pathway could be a target for the treatment of PDAC.

In conclusion, activated Akt expression determined by immunohistochemistry is a new prognosticator for PDAC and is a new way to explore effective modalities for the treatment of PDAC. The inhibition of Akt might be a possible molecular target for the treatment of PDAC.

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Clinical Cancer Research

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Clin Cancer Res 2004;10:2846-2850.

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