Original Article

Phosph-Akt1 Expression is Associated with a Favourable Prognosis in Pancreatic Cancer

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Abstract

Introduction: Akt, a serine/threonine protein kinase, mediates growth factor-associated cell survival. In several human cancers, including pancreatic cancer, constitutive activation of Akt (phosphorylated Akt, p-Akt) has been observed and may be associated with chemotherapy and radiotherapy resistance. However, there are contradictory viewpoints in p-Akt in pancreatic cancer on prognosis, and the clinical relevance of p-Akt in pancreatic cancer is not well understood. This study aims to investigate the expressions and relevance of Akt and p-Akt1 in pancreatic cancer tissues and their clinical significance. Materials and Methods: The expressions of Akt and p-Akt in 74 surgically resected paraffin-embedded pancreatic ductal adenocarcinoma samples and 10 normal pancreatic tissue samples were examined by immunohistochemistry. The associations of their expression with clinicopathological and survival data were analysed. Results: The positive expression rate of Akt and p-Akt1 were 87.8% and 83.8%, respectively, which were remarkably higher then those in normal pancreatic tissue (P <0.05). There was a positive correlation between the expression of Akt and p-Akt1. High p-Akt1 expression correlated with lower T stage (P = 0.004), while Akt was not associated with any clinicopathologic variables. Kaplan-Meier survival analysis revealed that higher expression of Akt, p-Akt1 were respectively correlated with favourable prognosis (16.0[4.7-27.3] vs 9.3[9.0-9.6] months, P = 0.007, and 23.0[12.2-33.8] vs 11.1[7.5-14.7] months, P = 0.004, respectively). Multivariate analysis identified p-Akt1 as a significant independent favourable prognostic factor (HR=0.421, P = 0.010). Conclusions: These results suggest that high p-Akt1 expression may be a favourable prognostic factor in pancreatic cancer.

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Key words: Clinicopathological variable, Immunohistochemistry, Survival time

Introduction

Pancreatic cancer is the fourth leading cause of cancer-related mortality worldwide with an estimated 34,290 deaths recorded in the United States in 2008. Of these, pancreatic ductal adenocarcinoma (PDAC) accounts for ~90% of all cases.[1] Despite advances in surgery, chemotherapy and other therapies, the lack of significant improvement in 5-year survival rates for PDAC has driven the search for new strategies aimed at improving pancreatic cancer management. Understanding the biology of pancreatic cancer may lead to the identification of novel targets for the treatment and chemoprevention of this disease.

Akt (also called protein kinase B) is a serine/threonine protein kinase activated by a variety of growth factors, including insulin, insulin-like growth factor-I and epidermal growth factor, via the phosphatidylinositol 3-kinase (PI3K) pathway,[2] and plays a role in tumorigenesis by inhibiting apoptosis and mediating cell proliferation.[3] Full activity of Akt is achieved by phosphorylation at Thr^{308} and Ser^{473}.[4] Phospho-Akt (p-Akt) is a powerful promoter of cell survival as it antagonises and inactivates various components of the apoptotic cascade, such as proapoptotic Bad,[4] caspase-9,[5] and members of the forkhead transcription factor family.[6] Furthermore, Akt has been implicated in regulating metastasis,[7-8] which is an important process in cancer development.

It has been well-documented that Akt is overexpressed in a variety of human cancer types, including gliomas and pancreatic cancer,[9-10] and its expression also seems to correlate with stage and tumour grade in prostate cancer.[11]

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Because it plays an important role in the signalling pathways of various growth factors important for tumorigenesis,[12] the detection of Akt activation may be a useful tool for predicting the progression and prognosis of PDAC. However, the usefulness of p-Akt as a prognostic indicator in PDAC is a matter of debate.[13-14] Akt has three isoforms, Akt1, Akt2 and Akt3. These three isoforms play different, or even opposing, roles in the growth and progression of mammary tumours.[15-16] Akt1 controls the expression of the SKP2 gene, an oncogene up-regulated in PDAC, by regulating the binding of E2F1 to the proximal SKP2 gene promoter in PDAC cells.[17] Akt1 knockdown inhibits both SKP2 transcription and the G1 phase progression of PDAC cells. However, the activation of Akt-1 has also been shown to suppress tumour invasion while, at the same time, accelerating ErbB-2-mediated mammary tumorigenesis.[18] It has also been reported that the Akt1 pathway is constitutively active in PDAC cells.[17] Therefore, an association between the overexpression or activation of Akt1 and the clinicopathological behaviour of PDAC is postulated.

In this study, the expression levels of Akt (pan) and p-Akt1 were examined in 74 patients with PDAC, and any relationship to clinicopathological variables was evaluated. Survival analysis was performed to define the prognostic significance of Akt and p-Akt1 expression levels.

Materials and Methods

Patients

This study included 74 patients with PDAC who underwent radical surgery at our hospital between April 1993 and January 2009. The mean patient age was 64.2 ± 9.7 years (40~80 years old). None of the patients had undergone preoperative radiation treatment or chemotherapy. Experienced pathologists provided detailed pathological diagnosis according to UICC TNM classification standards (2002). Patient details and tumour characteristics are summarised in Table 1. Ten normal pancreatic tissue samples were also obtained from patients who underwent pancreatoduodenectomy or distal pancreatectomy for diseases other than pancreatic cancer. Anonymous use of leftover tumour material is part of the standard treatment agreement with patients attending our hospital. Patient survival data was obtained by telephone follow-up and direct home visits.

Table1. Summary of Clinical and Pathological Characteristics

Characteristics	
Age (years)	
Median	66.5
Range	40-80
Gender	
Male	50
Female	24
Location of tumour	
Head	65
Body or tail	9
TNM stage	
Ia	4
Ib	15
IIa	27
IIb	23
III	0
IV	5
T stage	
T1	4
T2	24
T3	46
Lymph node status	
Negative	46
Positive	28
Grade	
G1	15
G2	47
G3	10
Not classified	2
Margin status	
Negative	62
Positive	8
Undetermined	4
Nerve invasion	
Negative	34
Positive	30

Immunohistochemistry

Formaldehyde-fixed and paraffin-embedded cancer tissue samples were retrieved from the archives of the Department of Pathology at our hospital. Normal pancreatic tissues were also fixed in formaldehyde and paraffin-embedded. To confirm the diagnosis, a representative haematoxylin-eosin stained slide from each patient was re-evaluated by two pathologists before the study commenced. Tissue sections (4 μm) were deparaffinised in xylene and rehydrated through graded ethanol. Slides were heated in 0.01 M citrate buffer for 16 minutes in a microwave oven and then cooled for 20 minutes. Slides were then washed in PBS. Endogenous peroxidase was blocked using 3% hydrogen peroxide in methanol for 5 minutes followed by incubation with 10% normal goat serum for 30 minutes. Sections

were then incubated with an anti-Akt rabbit monoclonal antibody (4691, Cell Signaling,USA) or an anti-p-Akt1 rabbit polyclonal antibody (abcam ,USA) at a dilution of 1:50 at room temperature for 60 minutes. Visualisation of bound antibodies was achieved by a two-step procedure using a Histostain-plus kit (Anti-Mouse/Rabbit) (HRP) (LHK611, Jingmei Biotech Co., Ltd., China) according to the manufacturer's instructions. Counter staining was performed with haematoxylin. For the negative controls, the primary antibody was omitted and replaced by PBS.

Semi-quantitative Assessment of the Expression of Akt and p-Akt1 Proteins

Results were evaluated independently by two investigators with no prior knowledge of the patient data. The percentage of positive cells was used to determine both Akt and p-Akt1 expression levels and was categorised according to a published method,[19] with a slight modification. Briefly, specimens were divided into four groups according to the percentage of positive cells: - = negative; + = up to 10% positive cells; ++ = 11% to 50% positive cells; and +++ = >50% positive cells. For statistical reasons, tumours were classified into a low expression group, (-) and (+) and a high expression group, (++) and (+++).

Where independent scoring of a case differed, it was re-checked and the final score was determined by recounting using a multi-headed microscope, with both observers viewing the slides simultaneously. If the deviation was <30% in a continuous variable, the mean value was adopted as the final score. Otherwise, the case was re-evaluated until a consensus was achieved.

Statistical Analysis

Categorical variables were assessed by χ^2 or Fisher's exact test. The Spearman rank correlation coefficient was used to test the association between ordinal variables. Univariate analysis of overall survival and survival curves was performed using the Kaplan-Meier method, and the Cox proportional hazards model was used for multivariate analysis. A value of P <0.05 was considered statistically significant. All analyses were performed using SPSS17.0.

Results

Expression of Akt and p-Akt1 Proteins in PDAC

Neither Akt nor p-Akt1 expression was detected in the exocrine portion of any of the normal pancreatic tissue samples (Fig. 1A, 1B). Akt- and p-Akt1-positive cells were detected in 65/74 (87.8) and 62/74 (83.8) tumour blocks, respectively. Both Akt and p-Akt1 expression was observed in the cytoplasm of the tumour cells (Fig. 1C-1F, 1G-1J). Low (-,+) and high (++,+++) expression levels of Akt were seen in 20 (27.0%) and 54 (73.0%) cases, respectively. Thirty (40.5%) and 41 (55.4%) cases showed low and high expression levels of p-Akt1, respectively.

Relationship between the expression of Akt and p-Akt1 proteins

Spearman rank correlation showed a significant, positive linear correlation between the expression of Akt and p-Akt1(r = 0.274, P = 0.018) (Table 2).

Expression of Akt and p-Akt1 Proteins in Relation to Clinicopathological Parameters

Akt had no significant correlation with any clinicopathologic variables. However, high p-Akt1 expression correlated significantly with a lower T stage (P = 0.002), and TNM stage (P = 0.007), as shown in Table 3. High p-Akt1 expression levels were seen in 78.6% of T1/T2-stage tumours (limited to the pancreas), while 41.3% of T3-stage tumours (invading the surrounding organs) showed high p-Akt-1 expression levels. No other correlations were observed.

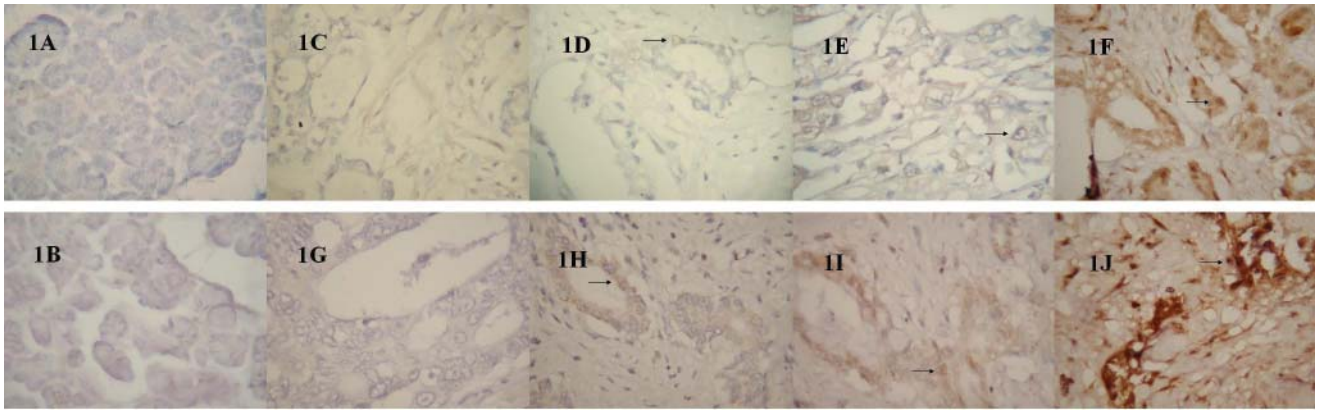

Fig. 1. Expression of Akt and p-Akt1 in normal and cancer pancreatic tissues.
(A) Negative expression of Akt in normal pancreatic tissues; (B) Negative expression of p-Akt1 in normal pancreatic tissues; (C) Negative expression of Akt in pancreatic cancer tissues (-); (D~F) Positive expression of Akt in pancreatic cancer tissues (+, ++, +++, respectively); (G) Negative expression of p-Akt1 in pancreatic cancer tissues (-); (H~J) Positive expression of p-Akt1 in pancreatic cancer tissues (+,++,+++, respectively). All sections magnified × 400.

Table 2. Correlation between the Expression of Akt and p-Akt1 Protein

	Akt expression				Spearman rank correlation	
	(-)(n = 9)	(+)(n = 11)	(++)(n = 33)	(+++)(n = 21)	r	*P* value
p-Akt1 expression						
(-)(n = 12)	4	2	5	1		
(+)(n = 21)	3	3	9	6		
(++)(n = 25)	1	4	13	7		
(+++)(n = 16)	1	2	6	7	0.274	0.018

A significant positive linear correlation between Akt and p-Akt1 was discovered by Spearman rank correlation (r = 0.274, *P* = 0.018).

Survival Analysis

Overall survival was defined as the period from the day of surgery until the death of the patient. Death from a cause other than cancer relapse, or survival until the end of the observation period, was considered as a censoring event. Follow-up data were available for 68 patients. The other 6 patients were lost to follow-up during the observation period. The median follow-up time was 16.4 months and the survival rate was 44.5% at 1 year and 14.9% at 2 years. Forty-one patients died of pancreatic cancer at the end-point of the observation period. Only 3 patients were still alive 5 years after surgery. The median overall survival was 14.3 ± 1.4 months. As shown in Figure 2, Kaplan-Meier survival analysis shows that higher expression levels of both Akt and p-Akt1 are correlated with a more favourable prognosis (*P* = 0.007 and *P* = 0.004, respectively). As a result, an advanced TNM stage significantly predicts decreased patient survival rates, whereas age, gender, histological grade, margin status and nerve invasion have no predictive value. Multivariate Cox regression analysis identified p-Akt1 expression (HR, 0.421, *P* = 0.010) and TNM stage (HR, 1.436, *P* = 0.041) as significant prognostic variables, in which low expression of p-Akt1, coupled with an advanced TNM stage, were associated with poor outcome (Table 4).

Discussion

This study supports the growing body of evidence that both Akt and p-Akt1 are expressed in PDAC, and can be detected using immunohistochemistry. We observed p-Akt1 staining localised to both the cytoplasm and the nucleus of PDAC. In the present study, 87.8% of the pancreatic cancer tissue samples were Akt-positive, with 73.0% having high Akt expression levels. Looking at the expression levels of p-Akt1, 83.8% of the samples were positive, and 55.4% showed a high expression level. The high expression levels of Akt seen in this study are consistent with a report by Chadha et al[13] that showed Akt to be higher expressed in 71.8% of pancreatic cancer tissue samples. It has also been reported that Akt and p-Akt1 are highly expressed in some pancreatic cancer cell lines.[20] Our findings add to the evidence that Akt and p-Akt1 may play important roles in the tumorigenesis and development of PDAC. The present study shows a significant correlation between Akt and p-Akt1 expression in PDAC (*P* = 0.018), suggesting a close association between overexpression of Akt and its increased activity. Additional investigation is needed to clarify the mechanism by which Akt expression and activation are involved in tumorigenesis and disease progression.

A significant association between p-Akt1 levels and

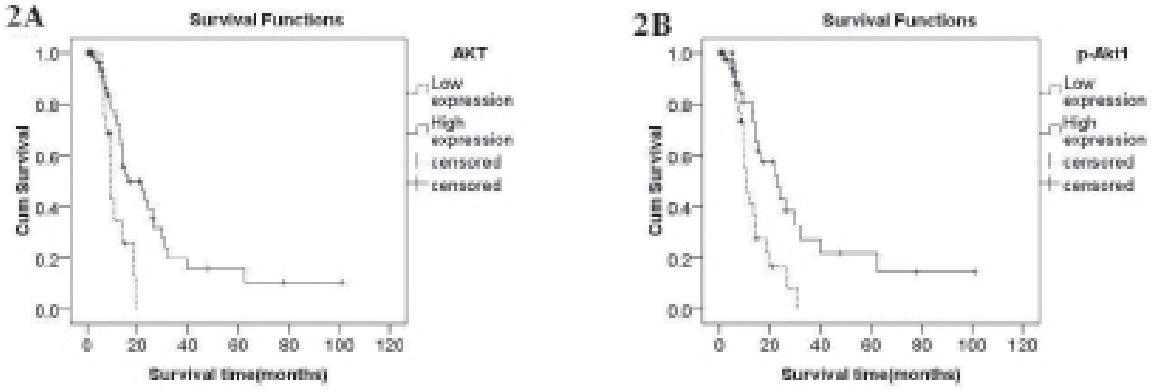

Fig. 2. Kaplan-Meier survival results based on the median value of Akt and p-Akt1. (A) Akt: high versus low. Median survival: high expressers (16.0 ± 5.7 months; n = 54) and low expressers (9.3 ± 0.2 months; n = 20), *P* = 0.007. (B) p-Akt1: high versus low. Median survival: high expressers (23.0 ± 5.5 months; n = 41) and low expressers (11.1 ± 1.8 months; n = 33), *P* = 0.004.

Table 3.Correlation between the Expression of Akt, p-Akt1 Protein and Clinicopathological Data

parameter	case	Expression of Akt (n = 74)			Expression of p-Akt1 (n = 74)		
	Low	High	*P* value		Low	High	*P* value
Age (years)							
≤60	24	4	20		12	12	
>60	50	16	34	0.164[a]	21	29	0.517[a]
Gender							
Male	50	11	39		24	26	
Female	24	9	15	0.160[a]	9	15	0.395[a]
Location of tumour							
head	65	17	48		29	36	
Body and tail	9	3	6	0.696[b]	4	5	1.000[b]
Grade							
G1	15	4	11		4	11	
G2	47	13	34		23	24	
G3	10	3	7	0.983[a]	6	4	0.201[a]
LN status							
Negative	46	11	35		22	24	
Positive	28	9	19	0.439[a]	11	17	0.473[a]
T stage							
T1 +T2	28	6	22		6	22	
T3	46	14	32	0.398[a]	27	19	0.002[a]
TNM stage							
Ia+Ib	19	5	14		4	15	
IIa	27	6	21		18	9	
IIb+III+IV	28	9	19	0.707[a]	11	17	0.007[a]
Nerve invasion							
Negative	34	10	24		16	16	
Positive	30	7	23	0.583[a]	13	17	0.443[a]
Tumour size							
≤2cm	10	1	9		2	8	
>2cm	64	19	45	0.270[b]	31	33	0.169[b]

High p-Akt1 expression was related significantly to lower T stage ($P = 0.002$) and TNM stage ($P = 0.007$); Akt had no obvious correlations with any clinicopathologic parameters. (a: χ^2 test; b: Fisher's exact test.)

T-stage was observed in this study ($P = 0.002$). T1/T2-stage tumours, which are limited to the pancreas, had higher levels of p-Akt1 (78.6) than T3-stage tumours (41.3%), which invade the surrounding organs. This observation suggests that the activation of Akt1 may be associated with decreased invasiveness, and that the tumour may be more aggressive in the absence of p-Akt1. This phenomenon has been reported for other tumours.[15,18] Hutchinson et al[18] found that activation of Akt-1 can suppress tumour invasion, while accelerating ErbB-2-mediated mammary tumorigenesis. Maroulakou et al[15] showed that the highest degree of invasiveness is seen in the tumours arising in Akt1 knockout mice. As Akt1 promotes cell survival,[21] we speculate that these phenomena may be due to a reduced growth rate as a result of increased Akt1 activity (high levels of p-Akt1).

In this study, the expression levels of p-Akt1 in PDAC were shown to correlate with patient prognosis by both univariate and multivariate analysis. However, the correlation between Akt expression and patient prognosis was found only by univariate analysis; a different finding from that of Chadha et al,[13] who showed that either high

Table 4. Univariate and Multivariate Analysis of Overall Survival of 74 Patients with PDAC

Variables	Univariate analysis	Multivariate analysis		
	P value	*P* value	Relative risk	95% CI
Age (≤60 years vs >60 years)	0.937			
Gender (male vs. female)	0.480			
Histological grade (G1/G2/G3)	0.506			
Tumour location (head vs body & tail)	0.247			
Tumour size(≤2cm vs >2cm)	0.111			
Lymph node status (positive vs. negative)	0.306			
TNM stage (Ia/Ib/IIa/IIb/III/IV)	0.024	0.041	1.436	1.015-2.031
Margin status (positive vs. negative)	0.194			
Nerve invasion (positive vs. negative)	0.131			
Akt (high vs. low)	0.010	0.149		
p-Akt1 (high vs. low)	0.006	0.010	0.421	0.217-0.815

Univariate analysis showed that TNM stage, expressions of Akt and p-Akt1 are related to overall survival. p-Akt1 expression (HR, 0.421, $P = 0.010$) and TNM stage (HR, 1.436, $P = 0.041$) as significant prognostic factors were identified by multivariate Cox regression analysis, which low expression of p-Akt1 and advanced TNM stage implied a poor outcome of pancreatic cancer.

Akt or high p-Akt expression was associated with improved survival in PDAC, and Shah et al,[19] who reported that a favourable outcome in non-small-cell lung cancer cases correlated with increased p-Akt expression. Chadha et al[13] hypothesised that surgical resection itself may counter any negative effects due to p-Akt expression, and that tumours expressing low levels of p-Erk require higher p-Akt levels for survival. In our study, we found that the favourable prognosis for high p-Akt1expressers may be associated with less local invasion by the tumour.

It is interesting that there are contradictory results in PDAC regarding the effect of p-Akt on prognosis.[13-14] While Chadha et al[13] reported that a favourable prognosis was associated with high p-Akt expression levels in PDAC, Yamamoto et al[14] showed a significant association between p-Akt expression and a poor prognosis in a study of 65 patients with pancreatic cancer. This finding seemingly contradicts our results. The explanation may be that the three isoforms of Akt (Akt1, Akt2, and Akt3) play different, or even opposite, roles in tumorigenesis.[15-16] In mouse mammary tumour virus (MMTV)-ErbB2/neu and MMTV-polyoma middle T transgenic mice, Akt1 ablation inhibits, but Akt2 ablation accelerates, the development of mammary adenocarcinomas.[15] Activated Akt1 accelerates mammary tumour development but cannot rescue the metastatic phenotype, while p-Akt2 markedly increases the incidence of pulmonary metastases but does not affect the latency of tumour development.[16] Thus, it is desirable to analyse the isoforms of Akt separately in order to assess the prognostic impact of activated Akt on PDAC. To the best of our knowledge, our study is the first to evaluate the effects of p-Akt1 expression on pancreatic cancer *in vivo*.

Conclusions

According to our study, we conclude that p-Akt1 plays an important role in the tumorigenesis of PDAC as detected at high levels in p-Akt1 positive patients, and high levels of p-Akt1 protein are a favourable prognostic factor in PDAC as assessed by immunohistochemical techniques.

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