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KLF9 Is a Prognostic Indicator in Human Pancreatic Ductal Adenocarcinoma

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Abstract. *Background: The aim of this study was to investigate the expression of Krüppel-like factor 9 (KLF9) and its clinicopathological significance and prognostic value in pancreatic ductal adenocarcinoma. Patients and Methods: A total of 149 patients diagnosed with pancreatic ductal adenocarcinoma who underwent curative surgery were enrolled in this study. The expression of KLF9 was examined immunohistochemically. The correlation of KLF9 with clinicopathological parameters and overall survival rate of patients were analyzed. Results: Low expression of KLF9 was observed in 62.82% of tumors, which was related to poor differentiation (p=0.036) and vascular invasion (p=0.017). Furthermore, the overall survival of patients with low KLF9 expression was significantly shorter than that of those with high KLF9 expression (p=0.001). Multivariate analysis confirmed KLF9 as an independent prognostic factor (p=0.000). Conclusion: KLF9 expression was found to be a valuable prognostic factor for patients with pancreatic ductal adenocarcinoma.*

Pancreatic ductal adenocarcinoma (PDAC) is the one of the main causes of cancer-related mortality in the world (1). It is a most lethal disease with a poor prognosis, an overall 5-year survival rate of <1%, and the median survival time after diagnosis is about 6 months (2). At the time of diagnosis, approximately 75% of patients with PDAC have unresectable tumors, and conventional therapies such as chemotherapy and radiotherapy are virtually ineffective. Increasing our understanding of the molecular mechanism of PDAC carcinogenesis is the only strategy to find new markers for early diagnosis and identifying potential targets for therapeutic intervention. Therefore, recent research has concentrated on the molecular alterations occurring in PDAC. New treatment regimens based on molecular classifications of individual tumors may provide improvements in outcome for patients with pancreatic carcinoma.

Krüppel-like factor 9 (KLF9), belongs to the mammalian Sp1/KLF family of transcription factors, which consists of 17 members (3). The members of this family are characterized by the presence of Cys_2/His_2 zinc finger motifs in the carboxyl terminus domains, binding to the GC/GT-rich box in gene promoter and enhancer regions. The *N*-terminal domains of these proteins are quite variable and are associated with different biological functions. KLF9 is involved in various biological processes such as differentiation, proliferation, and development (4-5). KLF9 was found to inhibit breast cancer invasion and is considered a tumor suppressor (6). It has also has been reported that *KLF9* was down-regulated in human colorectal tumors, breast cancer and hepatocellular cancer and the reduction of KLF9 expression is associated with tumor initiation, apoptosis resistance, invasion and migration (6-8). Notably, KLF9 has been identified as having a special role in cancer stem cells, being down-regulated in ovarian cancer stem cell and glioblastoma-derived neurospheres (4, 9).

The evidence for an important role of KLF9 protein in tumor progression prompted us to investigate the relationship between KLF9 expression and patient prognosis in PDAC.

Patients and Methods

Patients and histological evaluation. In all, 149 patients who underwent surgery at Affiliated Hospital of Jiangsu University from 2005 to 2015 were included in this study. No patient had received any presurgical treatment, such as chemotherapy or radiotherapy. Clinical and histopathological data for these patients were extracted

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from electronic medical records. Follow-up began on the date of surgery and ended in October 2015.

Immunohistochemistry. Immunohistochemical analysis was performed on tissue microarray of specimens from 149 patients with PDAC. Formalin-fixed paraffin- embedded tissue sections (4 μm-thick) were stained using the immunoperoxidase method with avidin–biotin complex as described previously (10). The monoclonal antibody to KLF9 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Sections were incubated with the anti-KLF9 at 1:500 dilution overnight at 4˚C. The absence of nonspecific staining was confirmed by omitting the primary antibody. Labeling for marker was carried out using the Envision Plus Detection Kit (DAKO, Carpinteria, CA, USA) following the manufacturer's protocol. Nuclei were counterstained with hematoxylin. Immunohistochemical results were judged by three pathologists who were unaware of the clinical data. The staining results were judged by German semiquantitative scoring system which considers both the staining intensity and the extent of staining; the definition of low and high expression was as previously described (11).

PDACs were staged using the American Joint Committee on Cancer (AJCC) TNM staging system (12). The degree of cancer differentiation was assessed using criteria established by the World Health Organization (13). The study was approved by The Committee of Ethics in Affiliated Hospital of Jiangsu University[Aprroval number:JDFY20140526004] and informed consent was obtained for every specimen examined.

Statistical analysis. Statistical analysis of correlation between KLF9 expression and clinical parameters was performed using chi-squared test. The overall survival (OS) was estimated using the Kaplan–Meier method from the date of surgery.Univariate analysis of the clinicopathological variables was performed using the log-rank test. Multivariate regression was performed using the Cox proportional hazards model. All analyses were performed using SPSS statistical software (version 16.0 for windows; SPSS Inc., Chicago, IL, USA). All *p*-values were derived from two-tailed tests and $p<0.05$ was considered statistically significant.

Results

Patient population. One hundred and forty-nine patients, including 86 men and 63 women, were included in the final analysis. Their average age was 60.56 years (range=34-80 years). All the patients underwent pancreaticoduodenectomy with Whipple's operation (n=112) or distal pancreatectomy (n=37). Most tumors (70.47% were moderately differentiated, and perineural invasion was observed in most (75.17%) (Table I).

Tissue microarray was available for all 149 patients. A total of 38 patients were excluded from the survival analysis due to lack of follow-up; consequently 111 patients were included in the survival analysis.

Expression of KLF9 in PDAC. KLF9 staining was observed predominantly in the nucleus of tumor cells. Immunohistochemical analysis of samples showed that high KLF9 expression was found in 57 tumors (38.26%) and low KLF9 expression in 92 tumors (61.74%) (Figure 1).

Table I. *The relationship between Krüppel-like factor 9 (KLF9) expression and clinicopathological parameters in patients with pancreatic ductal adenocarcinoma (n=149).*

Characteristic	No. of patients	KLF9 expression		*p*-Value*
		High	Low	
Gender				
Male	86	33	53	0.555
Female	63	24	39	
Age				
<60 Years	71	28	43	0.866
≥60 Years	78	29	49	
Tumor position				
Head and neck	112	38	74	0.081
Body and tail	37	19	18	
Differentiation				
Well	5	4	1	**0.036**
Moderately	105	43	62	
Poorly	39	10	29	
T-Stage				
I	37	16	21	0.756
II	89	33	56	
III	23	8	15	
TNM stage				
I	29	14	15	0.397
II	60	23	37	
III	60	20	40	
Perineural invasion				
Absent	37	11	26	0.274
Present	112	46	66	
Vascular invasion				
Absent	132	55	77	**0.017**
Present	17	2	15	
LN metastasis				
Absent	89	37	52	0.390
Present	60	20	40	

TNM: Tumor-node-metastasis; LN: lymph node. *Chi-square test; significant values are shown in bold.

The relationship between KLF9 expression and clinicopathological variable is summarized in Table I. The KLF9 expression level did not significantly differ according to gender, age, tumor position, T-stage, TNM stage, perineural invasion or lymph node metastasis. Low KLF9 expression was significantly associated with poor differentiation and vascular invasion ($p=0.036$ and $p=0.017$, respectively).

Prognostic value of KLF9 expression in PDAC. Overall survival by Kaplan-Meier curves based on KLF9 expression are shown in Figure 2. Patients with low KLF9 expression had a poorer OS (17.9%) than those with high KLF9 expression (24.1%) ($p=0.001$). In addition to KLF9 expression, univariate analysis demonstrated that differentiation, TNM stage, perineural invasion, lymph node

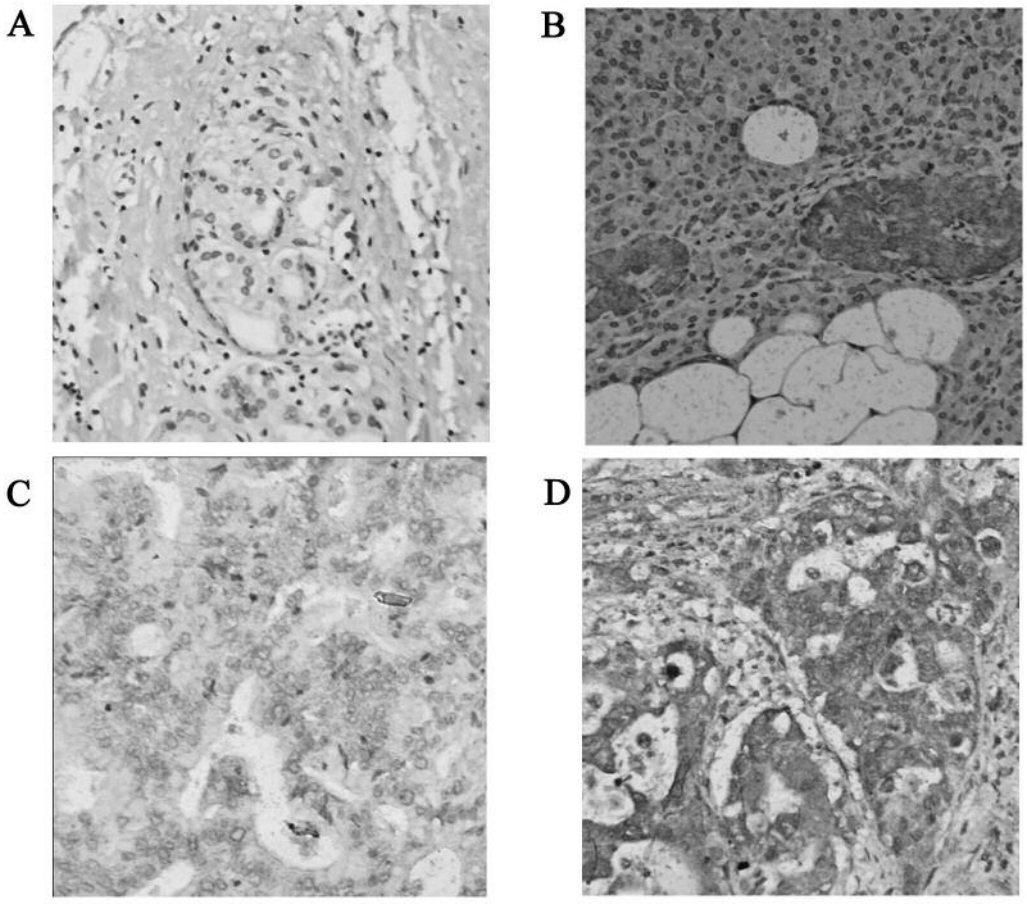

Figure 1. *Immunostaining of Krüppel-like factor 9 (KLF9) in human pancreatic ductal adenocarcinoma (magnification, ×400). A: Negative control; B: normal tissue; C: low KLF9 expression; D: high KLF9 expression.*

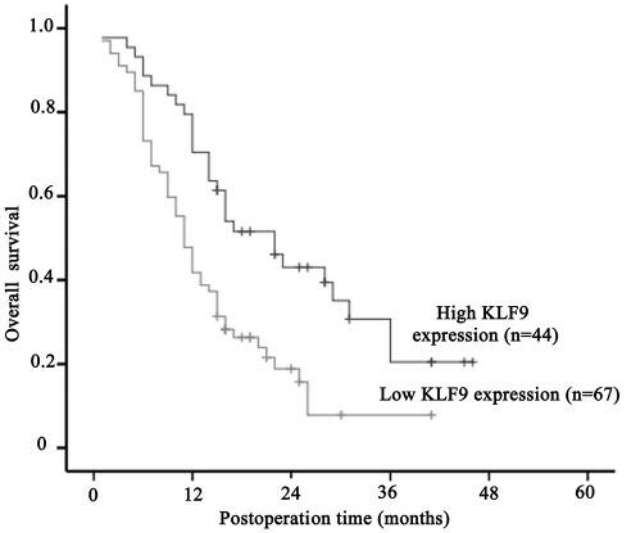

Figure 2. *Overall survival curves of patients with pancreatic ductal adenocarcinoma according to Krüppel-like factor 9 (KLF9) expression. Patients with high expression of KLF9 had a significantly better survival rate than patients with low KLF9 expression (p=0.001).*

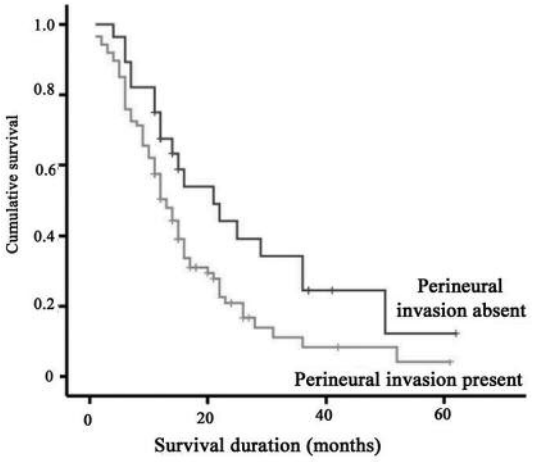

Figure 3. *Overall survival curves of patients with pancreatic ductal adenocarcinoma with perineural invasion. Patients with perineural invasion had a significantly poor survival rate than patients without (p=0.011).*

Table II. *Univariate hazard ratio analysis of prognostic factors after pancreatic resection in patients with pancreatic ductal adenocarcinoma (n=111).*

Variable	No.	HR	95% CI	*p*-Value
Gender				
Male	62	1.091	0.709-1.677	0.692
Female	49			
Age				
<60 Years	47	1.005	0.985-1.026	0.625
≥60 Years	64			
Tumor position				
Head and neck	85	0.652	0.382-1.112	0.116
Body and tail	26			
Differentiation				
Well	5	1.486	1.006-2.197	**0.047**
Moderately	69			
Poorly	37			
T-Stage		1.037	0.744-1.445	0.831
I+II	93			
III+IV	18			
TNM stage		1.560	1.136-2.141	**0.006**
I+II	64			
III+IV	47			
Perineural invasion		2.099	1.149-3.834	**0.016**
Absent	24			
Present	87			
Vascular invasion		1.145	0.704-2.847	0.330
Absent	100			
Present	11			
LN metastasis		2.1	1.360-3.241	**0.001**
Absent	64			
Present	47			
KLF9 expression		2.190	1.373-3.494	**0.001**
Absent	67			
Present	44			

HR: Hazard ratio; CI: confidence interval; TNM: tumor-node-metastasis; LN: lymph node; KLF9: Krüppel-like factor 9. Significant values are shown in bold.

Table III. *Multivariate hazard ratio analysis of prognostic factors after pancreatic resection in patients with pancreatic ductal adenocarcinoma (n=111).*

Variable	No.	HR	95% CI	*p*-Value
Differentiation				
Well	5	1.388	0.949-2.029	0.091
Moderately	69			
Poorly	37			
TNM stage				
I+II	64	1.177	0.609-2.274	0.628
III+IV	47			
Perineural invasion				
Absent	24	2.435	1.311-4.521	**0.005**
Present	87			
LN metastasis				
Absent	64	1.628	0.616-4.303	0.326
Present	47			
KLF9 expression				
Absent	67	2.470	1.536-3.971	**0.0002**
Present	44			

HR: Hazard ratio; CI: confidence interval; TNM: tumor-node-metastasis; LN: lymph node; KLF9: Krüppel-like factor 9. Significant values are shown in bold.

metastasis were also significantly associated with OS, while others were not (Table II).

In order to obtain a more precise estimate, multivariate analysis was performed to identify the independent prognostic factors. Using multivariate analysis, low KLF9 expression was confirmed as an independent prognostic factor for pancreatic ductal adenocarcinoma (p=0.0002, Table III), suggesting that poor expression of KLF9 was a high-risk factor for poor prognosis. Furthermore, perineural invasion (p=0.005) also remained an independent prognostic indicator and the mean survival of patients with perineural invasion was 16.14±1.34 months *versus* 26.79±3.46 months for those without (p=0.011, Figure 3), while differentiation, TNM stage and LN metastasis lost significance.

Discussion

In present study, by comparing the clinical parameters of patients, it was found that KLF9 status was only associated with differentiation and vascular invasion. This may be related to the function of KLF9 during carcinogenesis. KLF9 induced glioma neurosphere cell differentiation, inhibited neurosphere formation, and inhibited neurosphere-derived xenograft growth *in vivo* through binding with the promoter of NOTCH1 (14). KLF9 also promoted the differentiation of dental follicle cells *in vitro* (15). In pericyte-like cell differentiation of glioblastoma stem cells, KLF9 induced a vascularization switch with NOTCH1 stimulation (16).

Furthermore, we demonstrated that patients whose nuclei of pancreatic cancer cells were negative for KLF9 had a mean survival of 14.17±1.39 months compared to 23.95±2.32 months for those whose cells expressed KLF9. The results imply that low KLF9 expression in pancreatic ductal adenocarcinoma may be considered an indicator of poor prognosis in patients who underwent radical resection with curative surgery.

In the multivariable models, KLF9 was confirmed as an independent prognostic factor. Our assessment of KLF9 applied to patients with PDAC who underwent radical surgery. But whether KLF9 status is important in patients with carcinoma of only the pancreatic head or with pancreatic body and tail carcinoma, or with unresectable disease remains unknown.

In our present study, the number of patients recruited was inadequate and there is a possibility of bias. The majority of our cases had PDAC in the pancreatic head. If possible, we would include more patients with PDAC in the body and tail of the pancreas and examine the difference of KLF9 expression pattern. Due to the insidious nature of PDAC, most patients initially presenting with symptoms are diagnosed with locally advanced tumor stage; patients with TNM I stage are very rare. This may have had an underlying effect on the present study. In future studies, it is necessary to include more patients with early-stage disease. However, in the multivariable Cox proportional hazards models, the analyses strongly imply that nuclear KLF9 expression may be a useful biomarker of outcome in patients with PDAC.

The mechanism by which low KLF9 expression portends a worse prognosis is not fully known. Former studies demonstrated that KLF9 induced apoptosis through dysregulation of p53 and programmed cell death protein 5 (PDCD5) (8). Furthermore, KLF9 inhibited the migration and proliferation of cancer stem cells (4, 16). Interestingly, recent studies found that KLF9 inhibited the WNT/β-catenin signaling through binding with transcription factor 4 (17).

In summary, low nuclear KLF9 expression is a marker of poor prognosis in patients with PDAC, independently of common clinical parameters including TNM stage, perineural invasion, and lymph node metastasis. The mechanisms that confer this malignant phenotype need additional study.

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