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Original Article

FOXO3a expression and its diagnostic value in pancreatic ductal adenocarcinoma

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Abstract: FOXO3a (FKHRL1) is an important regulator of cell apoptosis, proliferation, metabolic state and longevity. FOXO3a expression can be measured and has been regarded as a tumor suppressor factor in many cancers. However, the expression and role of FOXO3a in PDAC have not been defined. We evaluated the expression of FOXO3a in PDAC and the relationship of its expression with clinicopathological features and patient outcomes. We found that compared with normal tissues, the expression of FOXO3a was significantly higher in tumor tissues (P<0.001). FOXO3a expression correlates significantly with tumor differentiation and with the primary location of the tumor (P<0.001 and P=0.005, respectively). In a univariate analysis, we found that FOXO3a expression has a strong relationship with survival (P=0.013). In addition, Kaplan-Meier survival curves indicated that a low expression of FOXO3a in tumor tissues has a significantly shorter OS compared with patients with high expression of FOXO3a (P=0.013). In conclusion, the expression of FOXO3a is significantly higher in PDAC compared with normal pancreatic tissues and has a low expression or negative staining in poorly differentiated PDAC, which seems to indicate that FOXO3a expression in tumor tissues may be related to the pathological progression stage and may be used as a diagnostic indicator with early tumors.

Keywords: FOXO3a, pancreatic ductal adenocarcinoma, immunohistochemistry, diagnostic marker, prognosis

Introduction

Pancreatic ductal adenocarcinoma (PDAC), which makes up almost 85% of all pancreatic malignancies, is one of the most aggressive and malignant tumors of the digestive tract [1]. Although surgical approaches and radiochemotherapy have improved, the prognosis of PDAC patients remains poor, and the 5-year survival rate is less than 5% [2]. In China, the incidence of PDAC has gradually increased, with a mortality rate as high as 88% [3]. Furthermore, the diagnosis and treatment of PDAC is not satisfactory, so new therapeutic biomarkers for early diagnosis and prognosis are urgently needed.

FOXO3a (FKHRL1) is a member of the forkhead box (Fox) transcription factor, which works as an important regulator of cell apoptosis, proliferation, metabolic state and longevity [4-7]. The transcriptional activity and subcellular localization of FOXO3a are tightly regulated, including phosphorylation, acetylation, methylation, and ubiquitination [8-10]. The 3-kinase (PI3K)/Akt pathway, which is constitutively activated in pancreatic cancer [11-13], is a key signaling mechanism that can lead FOXO3a to ubiquitylation-mediated proteasome degradation and regulate the subcellular localization of FOXO3a [14-16]. FOXO3a expression can be detected and has been regarded as a tumor suppressor factor in many cancers. FOXO3a has been shown to be a potential prognostic marker in breast cancer [17], gastric cancer [18], colorectal cancer [19] and other carcinomas [20, 21]. However, the expression and role of FOXO3a in PDAC have not been defined.

In this study, we investigated the expression of FOXO3a in PDAC using immunohistochemistry (IHC). Furthermore, we assessed the relationship of its expression with clinicopathological features and patients' outcomes to evaluate the diagnosis and prognostic value of FOXO3a in PDAC.

Table 1. The expression of FOXO3a in normal tissues and tumor tissues

	Tumor tissues (n=61)	Normal tissues (n=13)	X^2 value	*P* Value
Expression			17.896	<0.001*
None	5	7		
Low	28	5		
High	28	1		
Staining			13.248	<0.001*
Negative	5	7		
Positive	56	6		
Total positive rate	91.8%	46.2%		

*P<0.05 was considered significant. A Pearson X^2 test was used to perform the statistical analyses.

Materials and methods

Clinical features and pathological samples

A total of 61 patients undergoing surgical treatment for pancreatic malignancies or undergoing postoperative pathology confirmed as PDAC between January 2013 and January 2016 in the Second Hospital of Tianjin Medical University were enrolled in this study. The patients' clinical, pathological, epidemiological, and prognostic data were collected. Among them, the average age was 61.6 years (range 39 to 79 years), the average size of the tumor was 3.8 cm (range 1 to 11 cm) and the median survival time was 21.0 months (range 3 to 45 months). All surgeries were completed successfully, and the average operation time and intraoperative blood loss were 308.9 minutes (range 90 to 600 minutes) and 352.5 ml (range 50 to 1500), respectively. 48 (78.7%) patients underwent pancreatoduodenectomy, and 13 (21.3%) patients received a partial resection. Only 1 (1.6%) patient received neoadjuvant chemotherapy (gemcitabine + gimeracil and oteracil potassium) and 38 (62.3%) patients received adjuvant chemotherapy or radiotherapy. Meanwhile, 13 samples of normal tissues were obtained from the Department of Pathology of the Second Hospital of Tianjin Medical University. All tissues were fixed in 13% buffered formalin and paraffin embedded for immunohistochemistry.

All the patients recruited for this study signed an informed consent and were followed up by phone, e-mail or outpatient review. This study was approved by the Research Ethics Committee of the Second Hospital of Tianjin Medical University.

Immunohistochemistry (IHC)

All tissue sections (4 um) were stained for H&E to confirm the pathologic diagnosis. Subsequently, we used the avidin-biotin-peroxidase method to stain the tissues, and we also used the anti-FOXO3a rabbit monoclonal antibody (CST D19A7; dilution 1:200) as primary antibody. After routine deparaffinization and rehydration with graded alcohol, the antigen retrieval was performed by microwave heating set at medium-high for 5 minutes and at medium-low for 10 minutes in an ethylene diamine tetra-acetic acid buffer (pH 8.0), then the sections were cooled naturally to room temperature. The sections were incubated with the primary antibody overnight at 4°C. A negative control was processed using a phosphate buffer saline (PBS) solution instead of the primary antibody. The samples were then incubated with a biotin-labeled secondary antibody for 30 minutes. Then the tissue samples were developed with diaminobenzidine (DAB) and counterstained with hematoxylin. Finally, the slides were dehydrated and coverslipped.

Immunohistochemical data analysis

The staining results were evaluated by two independent observers who were blinded to the patients' clinical and pathological information. A semi-quantitative analysis based on the percentage of positive cells and staining intensity, the criteria were as follows: Randomly select 5 high-power field (400×) views and count at least 100 cells per field. The score of the percentage of positive cells ranged from 0 to 4 (0 represents negative staining, 1 represents 0% to 25% stained, 2 is 26% to 50%, 3 is 51 to 75%, 4 represents more than 75%). As for staining intensity, 0 represents no staining, and 3 means strong staining. The product of the two is the final score, and we classified the expression intensities of FOXO3a into three levels: no expression (score 0), low expression (score 1-4) and high expression (score 5-12).

Statistical analysis

The χ^2 test was used to analyze FOXO3a expression in normal tissues and tumor tissues and to

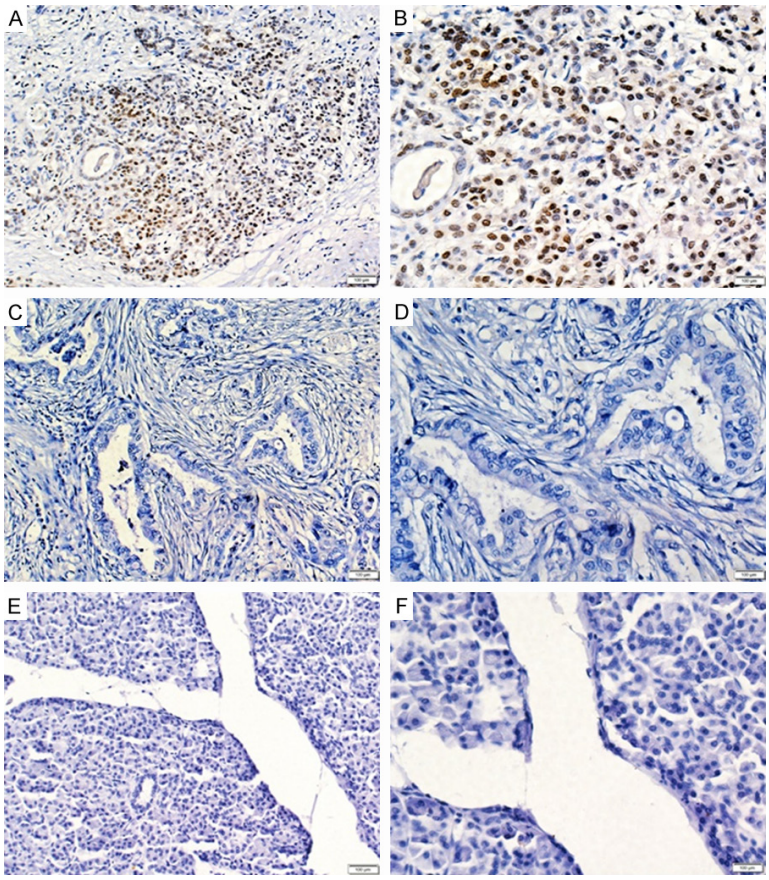

Figure 1. Normal pancreatic tissue and PDAC were stained with antibodies (Ab) specific to FOXO3a. (A, B) FOXO3a over-expression in PDAC. (C, D) Tumor tissue stained with PBS. (E, F) FOXO3a negative staining in normal pancreatic tissue (A, C, E: ×200, B, D, F: ×400).

compare the relationship between FOXO3a expression with the clinical and pathological variables of the PDAC patients. Additionally, we used the Kaplan-Meier and Cox regression analyses to identify the correlation of the clinicopathological features with overall survival (OS). Any variable with P<0.2 on the univariate analysis was included in the multiple Cox regression models. A two-sided P<0.05 was defined as statistically significant, and all analyses were performed using SPSS software (SPSS Version 20, Chicago, IL).

Results

FOXO3a expression in normal tissues and tumor tissues

We used immunohistochemistry to detect the expression of FOXO3a in normal pancreatic tissue and PDAC. The total positive rates of tumor and normal tissue were 91.8% and 46.2%, respectively (**Table 1**). Compared with normal tissues, the expression of FOXO3a was significantly higher in tumor tissues (P<0.001). However, we did not find any significant expression differences in the nuclear and cytoplasmic expressions in the tumor tissue. A cytoplasmic expression of FOXO3a in tumor tissue occurred in only 10 cases (16.4%), and in the normal tissue, the FOXO3a expression was located exclusively in the nucleus (**Figure 1**).

The relationship between FOXO3a and clinicopathological features in patients with PDAC

We examined the relationship between FOXO3a and the clinicopathological features. In this part of the experiment, the expressions of FOXO3a were divided into two groups, of which the low expression group (n=33) included 5 cases that did not express FOXO3a. We investigated whether FOXO3a expression in tumor tissues showed a significant difference between tumor differentiation and the primary location of the tumor (P<0.001 and P=0.005, respectively). Surprisingly, tumors which were located in the pancreatic body and tail were all in the low expression groups, and among them, 7 cases (70%) were poorly differentiated, and 8 (80%) patients died from the tumor. However, as for lymph nodes (P=0.063) and other clinicopathological features, the expressions of FOXO3a were not statistically significant (**Table 2**). Meanwhile, the frequency of FOXO3a overexpression seems to be higher in metastasis (both local and distant) and late-stage (III+IV) than in cases without invasion and with an early-stage (I+II) tumor, suggesting that FOXO3a might involve tumor progression rather than initiation.

The expression of FOXO3a as a prognostic factor in patients with PDAC

To find out whether the expression of FOXO3a is related to the prognosis of patients, we per-

Table 2. The relationship between FOXO3a expression in tumor tissues and clinicopathologic features

Variables	Total	Expression of FOXO3a		X^2 value	*P* value
		Low[1] (n=33)	High (n=28)		
Sex				0.075	0.800
Male	36	20	16		
Female	25	13	12		
Age (years)				1.130	0.414
<60	21	14	7		
≥60	40	21	19		
Primary tumor				8.058	0.005*
Pancreatic head	51	23	28		
Body and tail	10	10	0		
Level of differentiation				24.305	<0.001*
High	4	0	4		
Moderate	27	8	19		
Low	30	25	5		
Lymph node				3.454	0.063
(-)	46	28	18		
(+)	15	5	10		
Tumor size (cm)				0.399	0.527
<3.8	30	15	15		
≥3.8	31	18	13		
Distant metastasis				<0.001	0.993
(-)	24	13	11		
(+)	37	20	17		
TNM stage[2]				<0.001	0.993
I+II	24	13	11		
III+IV	37	20	17		

[1]Low expression contains no expression and low expression. [2]According to the American Joint Committee on Cancer's *AJCC staging system* (8th edition). *P<0.05 was considered significant. The Pearson X^2 test was used to perform the statistical analyses.

formed an average of 21.4 months (range 3 to 45 months) of follow-up on the postoperative patients. All the patients had a complete follow-up, with the information collected by phone or e-mail. In a univariate analysis, we found that FOXO3a expression in tumors (P=0.013), tumor differentiation (P<0.001), tumor size (P=0.058) and postoperative adjuvant treatment (P=0.052) had a strong relationship with survival, and the median survival time was significantly different between the low expression (18.0 months) and the high expression (27.0 months) cases (**Table 3**). In addition, the Kaplan-Meier survival curves indicated that the cases with a low expression of FOXO3a in tumor tissues had a significantly shorter OS compared with the patients with a high expression of FOXO3a (P=0.013, **Figure 2A**). However, a multivariate Cox regression model indicated that tumor differentiation and postoperative adjuvant treatment were strong predictors of survival (P=0.004 and 0.006, respectively), but the expression of FOXO3a could not serve as an independent predictor of prognosis in spite of a relative risk of 2.107 (95% CI: 0.870-5.104, P=0.099) (**Table 4**).

Discussion

In this study, we examined the expression of FOXO3a in PDAC and the relationship between its expression and clinicopathological parameters; then we analyzed the correlation of FOXO3a expression with prognosis. FOXO3a has been regarded as a tumor suppressor and is deregulated in a variety of tumors [19, 22, 23]. However, to our knowledge, the expression of FOXO3a in PDAC and the relationship between its expression and clinicopathological features had not yet been reported.

Bullock et al. reported that, compared with normal tissue, the expression of FOXO3 was significantly reduced in colorectal cancer, and the mean disease-free survival of the low expression group (28 months) was significantly shorter than that of the high expression group (64 months) [19]. Fei et al. showed that FOXO3 is over-expressed in normal tissue but under-expressed in ovarian cancer. In addition, a low expression of FOXO3 is associated with poor prognosis in ovarian cancer patients [24]. In the present study, we also found that a low expression of FOXO3a in patients with PDAC had a significantly shorter OS compared with the high expression group (P=0.013). Multiple studies have shown that the FOXO3a gene is related to human longevity [25, 26]. Combined

Table 3. Univariate analysis of the overall survival of patients with PDAC

Variable	Total	Survival state		Median survival time (mons)	*P* value
		Alive	Dead		
FOXO3a expression					0.013*
Low	33	8	25	18.0	
High	28	12	16	27.0	
Sex					0.917
Male	36	11	25	21.0	
Female	25	9	16	22.0	
Age (years)					0.454
<60	21	7	14	23.0	
≥60	40	13	27	21.0	
Primary tumor					0.507
Pancreatic head	51	18	33	21.0	
Body and tail	10	2	8	23.0	
Level of differentiation					<0.001*
High	4	3	1	33.0	
Moderate	27	14	13	28.0	
Low	30	3	27	14.0	
Lymph node					0.931
(-)	46	15	31	23.0	
(+)	15	5	10	21.0	
Tumor size (cm)					0.058
<3.8	30	17	13	23.0	
≥3.8	31	24	7	21.0	
Distant metastasis					0.365
(-)	24	8	16	26.0	
(+)	37	12	25	21.0	
TNM stage					0.365
I+II	24	8	16	26.0	
III+IV	37	12	25	21.0	
Operation time (mins)					0.577
>300	30	11	19	21.0	
<300	31	9	22	24.0	
Intraoperative blood loss (ml)					0.726
>350	23	8	15	21.0	
<350	38	12	26	22.0	
Postoperative adjuvant treatment					0.052
With	38	15	23	24.0	
Without	24	5	18	18.0	

Mons=months. *P<0.05 was considered significant. A Kaplan-Meier analysis was used to perform the statistical analyses.

with our experimental results, it suggests that the selective expression of FOXO3a in tumors may be associated with tumor malignancy. A decreased expression of FOXO3a may increase tumor malignancy, which leads to a shorter survival time. Thus, upregulating FOXO3a expression could be a promising treatment for PDAC and even for other tumors. However, perhaps due to the high degree of the malignancy of pancreatic cancer, including a limited follow-up time and the fact that some patients received postoperative adjuvant therapy, an independent risk factor that affects the prognosis in multivariate analysis (**Table 4**), there was no statistically significant multiparametric regression analysis of FOXO3a expression and OS (P=0.099), despite the fact that the median survival time of the high expression group (27.0 months) was significantly higher than that of the low expression group (18.0 months). Surprisingly, the expression of FOXO3a was significantly associated with prognosis in the multivariate analysis of patients without postoperative adjuvant therapy (P=0.008) (**Table 5** and **Figure 2B**). This suggests that FOXO3a can be used as an independent prognostic indicator for patients without postoperative adjuvant therapy in PDAC.

Furthermore, unlike earlier studies, our results indicate that FOXO3a expression in PDAC was significantly higher than in normal pancreatic tissue. Our results are consistent with Shan et al [18]. Regrettably, the mechanism of FOXO3a expression in normal tissues and tumors is still

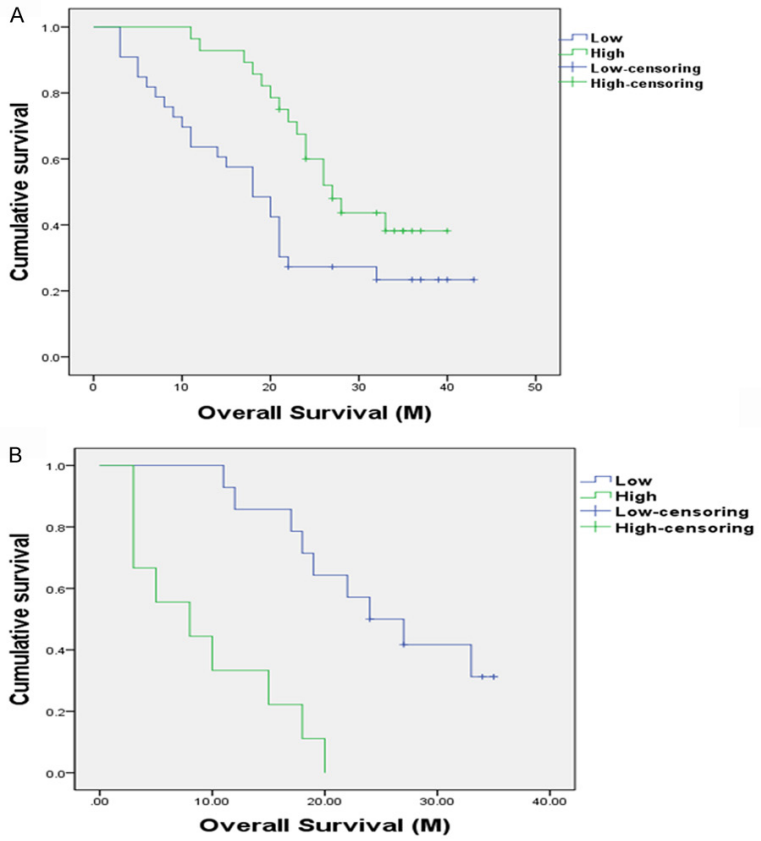

Figure 2. Kaplan-Meier survival curves of FOXO3a expression in PDAC (log-rank test). A. All patients (P=0.013). B. Patients without postoperative adjuvant therapy (P<0.001).

Table 4. Multivariate analysis of overall survival

	Relative risk, RR	95% Confidence interval	*P* value
FOXO3a expression	2.107	0.870-5.104	0.099
Tumor size	1.749	0.902-3.393	0.098
Tumor differentiation	2.730	1.382-5.396	0.004*
Postoperative adjuvant treatment	0.325	0.147-0.720	0.006*

*P<0.05 was considered significant. Cox regression analysis was used to perform the statistical analyses.

Table 5. Multivariate Analysis of Overall Survival of patients without postoperative adjuvant therapy

	Relative risk, RR	95% Confidence interval	*P* value
FOXO3a expression	2.162	1.775-42.551	0.008*
Tumor size	1.393	1.154-14.055	0.029*
Tumor differentiation	0.581	0.695-4.595	0.228

*P<0.05 was considered significant. Cox regression analysis was used to perform the statistical analyses.

unclear. FOXO3a is involved in regulating cell cycle progression [27] and cellular apoptosis, which induces cell death [4]. In normal tissues, FOXO3a protects quiescent cells from oxidative stress and repairs DNA damage [28]. In our study, perhaps because it was limited to a small number of samples (13 cases), a high expression of FOXO3a was observed in only one normal tissue. In addition, we theorize that a high expression of FOXO3a in normal tissues is a self-protection mechanism in response to injury, but under normal circumstances, it may not be expressed or under-expressed.

FOXO3a residues are released from DNA and transported to the cytoplasm after phosphorylation, which can be triggered by many factors [27, 29]. However, in the current study, FOXO3a expression was mostly located in the nucleus, and we did not find a significant nuclear exclusion, something that has been observed in previous studies [30, 31]. Since immunohistochemistry is only a semi-quantitative experiment, further studies are needed to verify the subcellular localization of FOXO3a in pancreatic cancer.

When we analyzed the relationship between FOXO3a expression and patients' clinicopathological parameters, we found that the expression of FOXO3a is related to the location of the tumor (P= 0.005) and the degree of differentiation (P<0.001), but there was no statistical significance between FOXO3a expression and other prognostic factors such as lymph node (P=0.063) and tumor metastasis (P=0.993). We found that tumors located in the pancreatic body and tail were all in the low expression groups, with a poor differentiation and short overall survival. In fact, Only 20 to 25% of PDAC cases are located in

the body and tail of the pancreas [32], and the presenting signs and symptoms there are not obvious compared with tumors in the head of pancreas [1]. Therefore, the lack of any clinical symptoms may lead to the progression of the tumor, so the tumor is then highly malignant at the time of diagnosis. Moreover, FOXO3 had a low expression or showed negative staining in poorly differentiated PDAC, which may indicate that the progressive down-regulation of FOXO3a may be related to the pathological progression stage. The reason for this phenomenon may be that the constitutive activation of FOXO3 can inhibit cell proliferation, tumorigenesis, and cell cycle progression [30], which play an important role in tumor initiation and progression. Furthermore, Usami et al. indicated that upregulating FOXO3a activity induces cell cycle arrest in pancreatic cancer cells [33]. Thus, this may indicate that FOXO3a could be used as a diagnostic indicator for early tumors and as a marker of malignancy.

Conclusion

In conclusion, the expression of FOXO3a is significantly higher in PDAC compared with normal pancreatic tissues. In addition, FOXO3 shows low expression or negative staining in poorly differentiated PDAC, which seems to indicate that FOXO3a expression in tumor tissues may be related to the pathological progression stage and may be used as a diagnostic indicator for early tumors. However, we have not found any statistical significance of the relationship between FOXO3a and overall survival, in spite of a high relative risk. Thus, further research is urgently needed to prove whether FOXO3a can serve as an independent predictor of prognosis and to clarify the mechanism of overexpression of FOXO3a in PDAC.

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Disclosure of conflict of interest

None.

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