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Original Article

Clinicopathological significances of Feline sarcoma-related protein and β2-adrenoceptor expression in pancreatic ductal adenocarcinomas

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Abstract: Introduction: Pancreatic ductal adenocarcinoma (PDAC) is a highly malignant tumor with a high mortality, but biomarkers for its diagnosis, target therapy, and prognosis are not clinically available. Materials and methods: In the present study, Feline sarcoma-related protein (Fer) and ADRB2 protein expression was detected by immunohistochemistry. Results and discussion: Comparing with the peritumoral tissues, benign pancreatic tissues, and normal pancreatic tissues, Fer and ADRB2 protein was overexpressed in PDAC tumor tissues ($P < 0.01$). The percentage of patients with positive Fer and ADRB2 expression were significantly lower in PDAC without lymph node metastasis, without invasion to surrounding tissues and organs, and with low TNM stage (I/II stage) disease compared to PDAC patients with metastasis, invasion, and high TNM stage (III/IV) disease. PDAC patients with positive Fer or ADRB2 protein expression survived significantly shorter time than patients with negative Fer or ADRB2 protein expression ($P = 0.000$). Positive Fer and ADRB2 protein expression was an independent factor for poor prognosis of PDAC patients and ROC curve analysis showed that positive Fer and ADRB2 protein expression was sensitive and specific marker for the PDAC diagnosis. In conclusion, positive Fer and ADRB2 expression is associated with carcinogenesis of PDAC, disease progression, and poor prognosis of PDAC patients.

Keywords: ADRB2, Fer, immunohistochemistry, PDAC, pancreatic tissue

Introduction

It is widely known that pancreatic ductal adenocarcinoma (PDAC) is a highly malignant tumor originating from the pancreas [1]. Pancreatic cancers are reported the fourth-leading cause of cancer-related deaths in the USA with the 5-year survival rate less than 5% [1-3]. The poor prognosis of pancreatic cancer is commonly thought to be associated with its high potential of metastasis and resistance to radiotherapy and chemotherapy [1]. It is estimated that about 90% of patients with pancreatic cancers dying from metastatic diseases [1, 4]. Therefore, the seeking for biomarkers to improve the diagnosis and targeting therapy of PDAC remains to be continued.

Protein tyrosine phosphorylation (pTyr) is a post-translational modification important for cell proliferation, survival, apoptosis, mobility, adhesion, etc [5]. Protein-tyrosine kinases are responsible for the protein tyrosine phosphorylation. Feline sarcoma (Fes) and Fes-related protein (Fer) are the members belonging to a distinct subgroup of the non-receptor protein tyrosine kinases [6]. Fer is ubiquitously expressed in a variety of tissues and revealed to regulate the proliferation, invasion, and metastasis of various tumor cells [7-9]. The increased Fer expression has recently been demonstrated to be associated with the disease progression and poor prognosis in patients with gastric cancer [10], hepatocellular carcinoma [5], prostate cancer [7, 8], colon cancer [11], NSCLC [12], and breast cancer [13]. However, the role of Fer expressions in PDAC has not been reported.

β-adrenoceptors (ADRBs) belong to a large group of the GPCRs superfamily that initiate multiple signaling cascades, including the adenylate cyclase (AC)/cAMP pathway [14]. Several

lines of evidence indicate that catecholamines mediate tumor cell migration via ADRBs. This process was inhibited by the β-adrenergic receptor antagonists [15, 16]. Recent studies have revealed that ADRB exerts an important regulatory role in the pathogenesis, progression, and malignancy of hepatocellular carcinoma [16, 17], NSCLC [18], breast cancer [19, 20], prostate cancer [21], and oral squamous cell carcinoma [22]. Only a most recent study in small sample revealed an association of β-adrenoceptor overexpression with advanced disease and poor prognosis in PDAC patients [23].

In the present study, Fer and ADRB2 protein expressions were stained by immunohistochemistry in the tumor tissues of 106 PDAC patients, 35 peritumoral tissues, 55 benign pancreatic tissues, and 13 normal pancreatic tissues.

Materials and methods

Ethics

The use of tissues samples and medical records was pre-approved by the Ethics Committee of Second Xiangya Hospital, Central South University. The study was conducted in accordance with the ethical standards of the Declaration of Helsinki (revised in Brazil 2013).

Case selection

One hundred and six pancreatic ductal adenocarcinomas, thirteen normal pancreatic tissues, thirty-five peritumoral tissues, fifty-five precursor pancreatic tissues, and were collected at the Second and Third Xiangya Hospitals. Among the one hundred and six adenocarcinomas, sixty-one came from male patients (57.5%) and forty-five from female patients (42.5%) with an average age of 54.50 ± 11.53 years. Histopathologic subtypes of the one hundred and six adenocarcinomas included: thirty-eight well-differentiated adenocarcinomas (35.8%), thirty-five moderately-differentiated adenocarcinomas (33%), and thirty-three poorly-differentiated adenocarcinomas (31.1%). Invasion and metastases were evaluated by following the published standard criteria [20]. Among the one hundred and six adenocarcinomas, eleven cases (10.4%) were T1, forty-one cases (39.6%) were T2, thirty-seven cases (34.9%) were T3, and sixteen cases (15.1%) were T4 stage tumors. Regional lymph node metastasis was found in twenty-nine of the one hundred and six adenocarcinomas (27.5%), and sixty-four cases (60.4%) had invasion to surrounding organs and tissues. The peritumoral tissues were collected over 2 cm from the tumors of the one hundred and six adenocarcinoma patients. Twelve of the thirty-five peritumoral tissues were normal, ten were PanINs (pancreatic intraepithelial neoplasms, PanINs) grade I, eight were PanINs grade II, and five were PanINs grade III. Survival information was obtained from all PDAC patients.

Fifty-five precursor pancreatic tissues were collected from twenty-nine (52.7%) males and twenty-six (47.3%) females. Of the fifty-five patients who supplied the precursor specimens, thirteen (23.6%) had an age ≤ 45 years and forty-two (76.4%) had an age > 45 years. The fifty-five precursor tissues included twenty chronic pancreatitis tissue (36.4%), twenty adenomas (36.4%), and fifteen PanINs (27.3%). Ten, six, and four of the twenty chronic pancreatitis tissues exhibited mild, moderate, and severe pancreatitis, respectively. Five and fifteen of the twenty adenomas exhibited mucinous adenomas and serous adenomas, respectively. Four, three, and two of the twenty adenomas exhibited mild, moderate, and severe dysplasia, respectively. Among the fifteen PanINs, six had grade I, five had grade II, and four had grade III PanINs. Thirteen normal pancreatic tissues were collected from twenty pancreatic adenomas during surgery. Tissues were rinsed in 4% formaldehyde for 24 to 48 hours followed by 10% formalin solution, embedded in paraffin, and sectioned at four μm-thick.

Immunohistochemistry

Fer and ADRB2 polyclonal antibodies (rabbit anti-human) and EnVision™ Detection Kit were obtained from Dako Corporation (Carpentaria, CA, USA). Positive controls were provided with the kit. EnVision immunohistochemistry of Fer and ADRB2 was performed according to the manufacture manual. After deparaffinized, the sections were incubated with 3% H_2O_2 for 15 min in the dark. Antigen retrieval was conducted with 10 mM Sodium citrate with 0.05% Tween-20 (pH 6.0) for 30 minutes at 96°C. The sections were incubated with Fer and ADRB2 primary antibody (1:100 dilution) for 2 hrs fol-

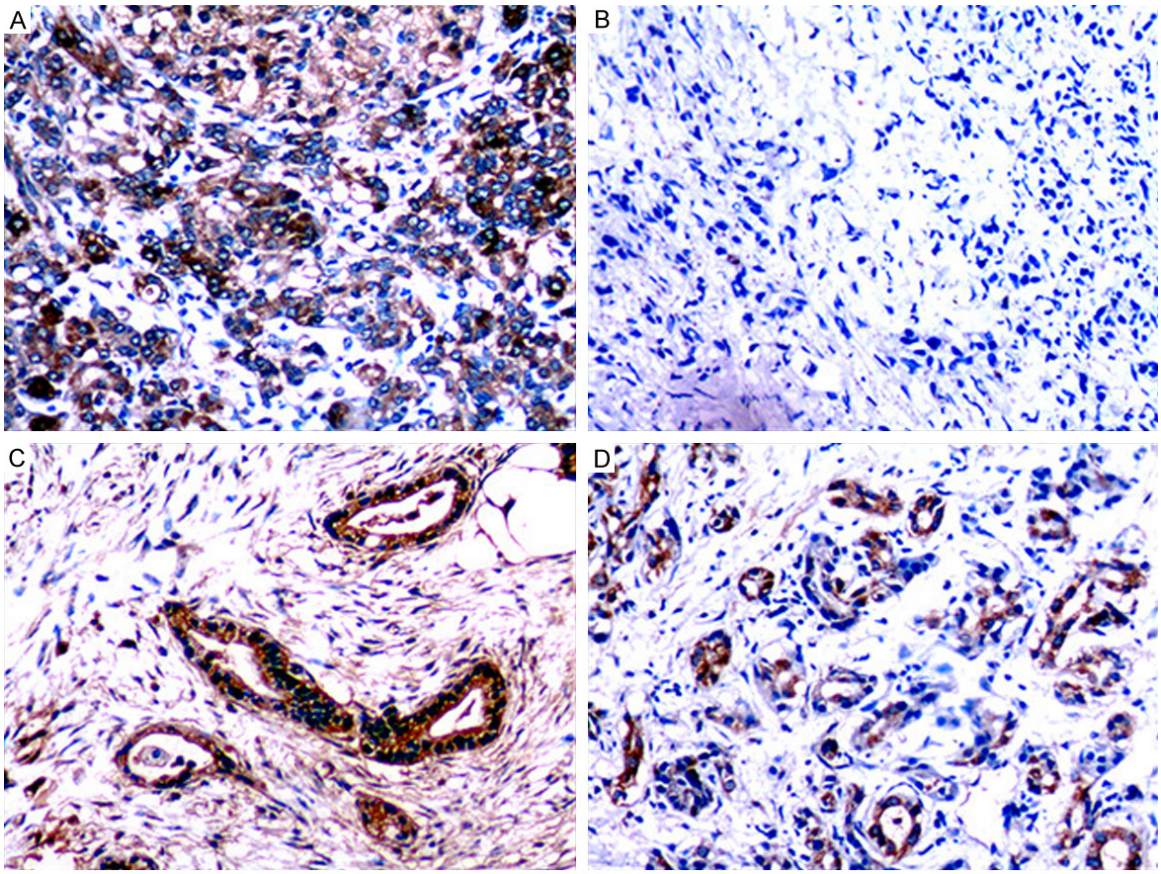

Figure 1. Immunohistological staining of Fer protein. A. Positive expression of Fer in poorly differentiated PDAC, ×200. B. Negative expression of Fer in poorly differentiated PDAC, ×200. C. Positive expression of Fer in chronic pancreatitis, ×200. D. Positive expression of Fer in adenoma, ×200.

lowed by incubation with several drops of Solution A (ChemMate™ EnVision+/HRP) for 30 min, DAB staining, and hematoxylin counterstaining. After dehydrated, sections were soaked in xylene and mounted. Five hundred cells were examined per section by two observers, independently. An average of the percentages from these two observers was used for final evaluation. A cutoff of 25% positive cells was used as a standard. Patients with ≥ 25% cells being Fer and ADRB2 positive were considered positive.

Statistical analysis

Data were analyzed with the statistical package for the Social Sciences (Version 17.0). The $\chi 2$ test or Fisher's exact test was used to analyze the inter-relationship between Fer and ADRB2 protein expression and histological or clinical factors. Kaplan-Meier univariate survival analysis and log-rank tests were used to evaluate the overall survival of PDAC patients. Cox proportional hazards model was used for multivariate analysis and the calculation of 95% confidence interval. AUC for Fer and ADRB2 was performed with ROC (receiver operating characteristic curve). Statistical significance was designed at $P < 0.05$.

Results

Fer and ADRB2 protein expression in adenocarcinoma, peritumoral, precursor, and normal pancreatic tissues

Fer and ADRB2 protein expression was stained using immunohistochemistry. Positive Fer and ADRB2 expressions were mainly seen in the cytoplasm of cells (**Figures 1**, **2**). However, Fer and ADRB2 were negatively stained in all thirteen normal tissues. In the one hundred and six adenocarcinomas, sixty-nine and sixty-five were Fer (65.1%) and ADRB2 (61.3%) positive, respectively. In the thirty-five peritumoral tissues, eleven and ten were Fer (31.4%) and

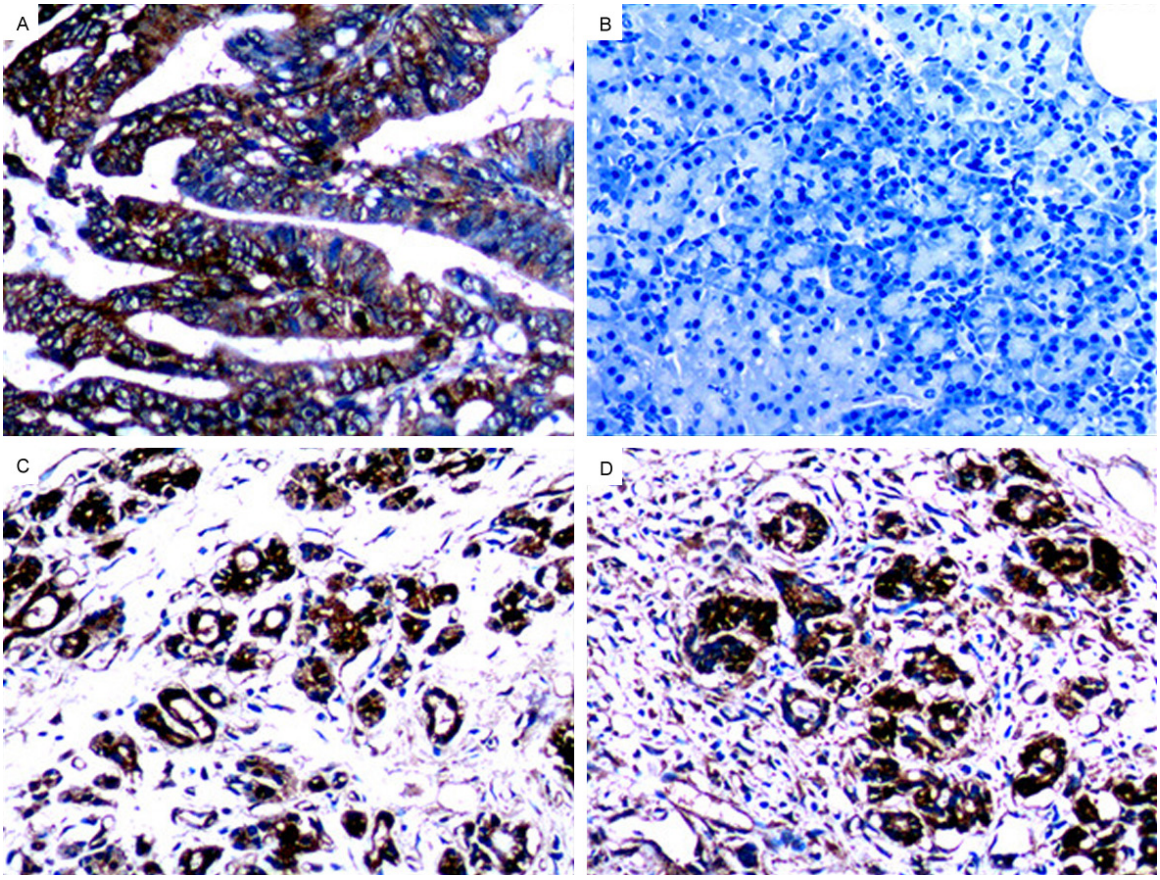

Figure 2. Immunohistological staining of ADRB2 protein. A. Positive expression of ADRB2 in moderately-differentiated PDAC, ×200. B. Negative expression of ADRB2 in poorly differentiated PDAC, ×200. C. Positive expression of ADRB2 in intraepithelial neoplasia II, ×200. D. Positive expression of ADRB2 in peritumori tissue, ×200.

Table 1. Comparison of Fer and ADRB2 expression in normal, benign, and malignant pancreatic tissues

Tissue type	Case No.	Fer positive (%)	ADRB2 positive (%)
Pancreatic ductal adenocarcinoma	106	69 (65.1)	65 (61.3)
Peritumoral tissues	35	11 (31.4)*	10 (28.6)*
Benign tissues	55	15 (27.8)*	12 (21.8)*
Normal pancreatic tissues	13	0 (0.0)*	0 (0.0)*

Compared to pancreatic ductal adenocarcinoma: *P < 0.01.

ADRB2 (28.6%) positive, respectively. In fifty-five precursor pancreatic lesions, fifteen and twelve were Fer (27.8%) and ADRB2 (21.8%) positive, respectively. The percentage of positive Fer or ADRB2 was significantly higher in PDAC tumors than that in normal and precursor pancreatic tissues as well as peritumoral tissues (P < 0.01) (**Table 1**). Peritumoral and precursor tissues with positive Fer and/or ADRB2 expression exhibited moderate to severe dysplasia and grade II or III PanINs. Among the fifty-five precursor le-sions, the percentage of positive Fer and ADRB2 protein expression were 20.0% (4/20) and 15.0% (3/20), 30.0% (6/20) and 25.0% (5/20), 33.3% (5/15) and 26.7% (4/15) in chronic pancreatitis, adenomas, and PanINs, respectively. However, no significant differences in the percen-tage of positive Fer and ADRB2 protein expression were found between three types of precursor lesions.

Fer and ADRB2 protein expressions were associated with clinicopathological characteristics of PDAC

The percentage of positive Fer and ADRB2 protein expression were significantly lower in patients without metastasis in lymph node, without invasion, and with low TNM stage (I/II) disease compared to patients with metastasis, invasion, and high TNM stage (III/IV) disease (P < 0.05, P < 0.01). The percentage of positive Fer expression was significantly lower in PDAC

Table 2. Correlations of Fer and ADRB2 protein expression with the clinicopathological characteristics of PDAC

CPC	Case No.	Fer			ADRB2		
		Pos No. (%)	x^2	*P* value	Pos No. (%)	x^2	*P* value
Age (year)							
≤ 45 years	22	16 (72.7)	0.712	0.399	16 (72.7)	1.523	0.217
> 45 years	84	53 (63.4)			49 (58.3)		
Sex							
Male	61	37 (60.7)	1.246	0.264	37 (60.7)	0.027	0.870
Female	45	32 (71.1)			28 (62.2)		
Differentiation							
Well	38	25 (65.8)	0.716	0.699	19 (50.0)	4.979	0.083
Moderately	35	21 (60.0)			21 (60.0)		
Poorly	33	23 (69.7)			25 (75.8)		
Tumor size							
≤ 3 cm	13	4 (30.8)	7.737	0.021	8 (61.5)	0.694	0.723
3-5 cm	68	48 (70.6)			40 (58.8)		
> 5 cm	25	17 (68.0)			17 (68.0)		
Lymph node metastasis							
No	77	44 (57.1)	7.832	0.005	41 (53.2)	7.736	0.005
Yes	29	25 (86.2)			24 (82.8)		
Invasion							
No	42	16 (38.1)	22.317	< 0.001	18 (42.9)	9.998	0.002
Yes	64	53 (82.8)			47 (73.4)		
TNM stage							
I	11	2 (18.2)	22.865	< 0.001	7 (63.6)	14.460	0.002
II	42	22 (52.4)			17 (40.5)		
III	37	31 (83.8)			27 (73.0)		
IV	16	14 (87.5)			14 (87.5)		

patients with tumor diameter ≤ 3 cm than that in patients with tumor diameter > 3 cm ($P < 0.05$). The expressions of Fer and ADRB2 exhibited no significant association with tumor differentiation, sex, and age of patients. Among the sixty-nine cases with positive Fer expression, forty-nine cases had positive ADRB2 expression (**Table 2**). Among the thirty-seven cases with negative Fer expression, twenty-one cases had negative ADRB2 expression. The Fer protein expression positively correlated with ADRB2 protein expression in PDAC tissues ($\chi^2 = 7.832$, $P = 0.005$).

Fer and ADRB2 expression correlated with the overall survival in PDAC patients

Twenty-nine patients survived over one year, but seventy-seven patients died within one year. Kaplan-Meier survival analysis results were presented in **Table 3**. Tumor differentiation, size, invasion, lymph node metastasis, and TNM stage were significantly associated with the mean overall survival time in PDAC patients ($P < 0.05$, $P < 0.01$). Mean overall survival time was significantly shorter in Fer and ADRB2 positive patients than that in Fer and ADRB2 negative patients ($P < 0.001$) (**Figure 3A**, **3B**). Cox multivariate analysis results were presented in **Table 4**. Poor differentiation, lymph node metastasis, and high TNM stage (III/IV) negatively correlated with mean overall survival. Positive Fer and ADRB2 expression negatively correlated with overall survival and positively correlated with mortality. Both Fer and ADRB2 positive expressions were independent prognostic factors (**Table 4**). The AUC analysis showed that Fer (AUC = 0.689, 95% CI: 0.603-0.705) and ADRB2 (AUC = 0.670, 95% CI: 0.583-0.758) had high sensitivity and specificity in diagnosis of PDAC (**Figure 3C**, **3D**).

Table 3. Correlations of clinicopathological characteristics, Fer and ADRB2 expression with the mean survival in patients with PDAC

Group	Case No. (n)	Mean survival (month)	Chi-square	*P* value
Sex				
Male	61	9.98 (2-24)	1.656	0.198
Female	45	8.61 (2-21)		
Age (year)				
≤ 45	22	8.18 (3-19)	2.144	0.143
> 45	84	9.73 (2-24)		
Differentiation				
Well	38	11.27 (3-24)		
Moderately	35	9.74 (3-21)	17.786	< 0.001
Poorly	33	6.86 (2-14)		
Tumor size				
< 3 cm	13	13.46 (5-21)	7.504	0.023
3-5 cm	68	9.34 (2-22)		
> 5 cm	25	7.40 (3-24)		
TNM stage				
I	11	16.46 (11-24)		
II	42	11.37 (3-22)	80.807	< 0.001
III	37	7.14 (2-17)		
IV	16	4.56 (2-8)		
Lymph node metastasis				
no	77	10.64 (2-24)	27.120	< 0.001
yes	29	6.35 (2-12)		
Invasion				
no	42	13.33 (5-24)	46.949	< 0.001
yes	54	6.83 (2-17)		
Fer				
-	37	12.84 (5-24)	25.725	< 0.001
+	69	7.52 (2-19)		
ADRB2				
-	41	11.91 (3-22)	12.357	< 0.001
+	65	7.84 (2-24)		

Discussion

This study compared Fer and ADRB2 protein expression in PDAC tumors with its expression in normal pancreatic tissues, benign pancreatic lesions, and peritumoral tissues using immunohistochemistry. Significantly high percentage of positive Fer and ADRB2 expression was observed in PDAC tumors compared to other tissues. Positive Fer and ADRB2 expression significantly correlated with the severe clinical symptoms and poor prognosis in PDAC patients.

Fer has been demonstrated to involve in the cell proliferation, adhesion, and migration [24-26]. Dysregulation of Fer expression has been proposed to involve in the carcinogenesis and cancer development [5, 10-13] and associate with the clinicopathological features of several human tumors [5, 10-13]. However, Fer expression in PDAC has not been reported. Our study first showed that positive Fer protein expression significantly correlated with tumor progression and survival in PDAC patients. In addition, Fer protein expression was revealed to be an independent prognostic biomarker in PDAC patients. Moreover, the percentage of positive Fer protein expression was significantly higher in PDAC tissues than that in benign pancreatic lesions, suggesting the involvement of Fer expression in the carcinogenesis. Thus, Fer may involve in the carcinogenesis of PDAC, and a biomarker for the poor prognosis in PDAC patients.

ADRB2 is important in the initiation and progression of cancer [27, 28] through regulating inflammation, angiogenesis, epithelial mesenchymal transition, and apoptosis [27]. Beta-adrenergic receptors have also been demonstrated to involve in the cell migration [29] and proliferation of tumor cells [30, 31]. ADRB2 has been revealed to regulate the stress-induced pancreatic tumor growth and angiogenesis [32], pancreatic cancer cell invasion [33], cell cycle, and apoptosis [34, 35]. Only on study measured ADRB2 protein expression in a small sample of PDAC tissues with comparison with its expression in peritumoral tissues [23]. This study measured ADRB2 protein expression by immunohistochemistry in one hundred and six PDAC tumor tissues, thirty-five peritumoral tissues, fifty-five precursor, and thirteen normal pancreatic tissues. A significant correlation was found between positive ADRB2 protein expression, the clinical severity, and survival in PDAC patients. In addition, this study first suggested that ADRB2 is involved in the carcinogenesis of PDAC. Consistent with a previous study [23], positive ADRB2 expression is an independent prognostic biomarker in PDAC patients. Intere-

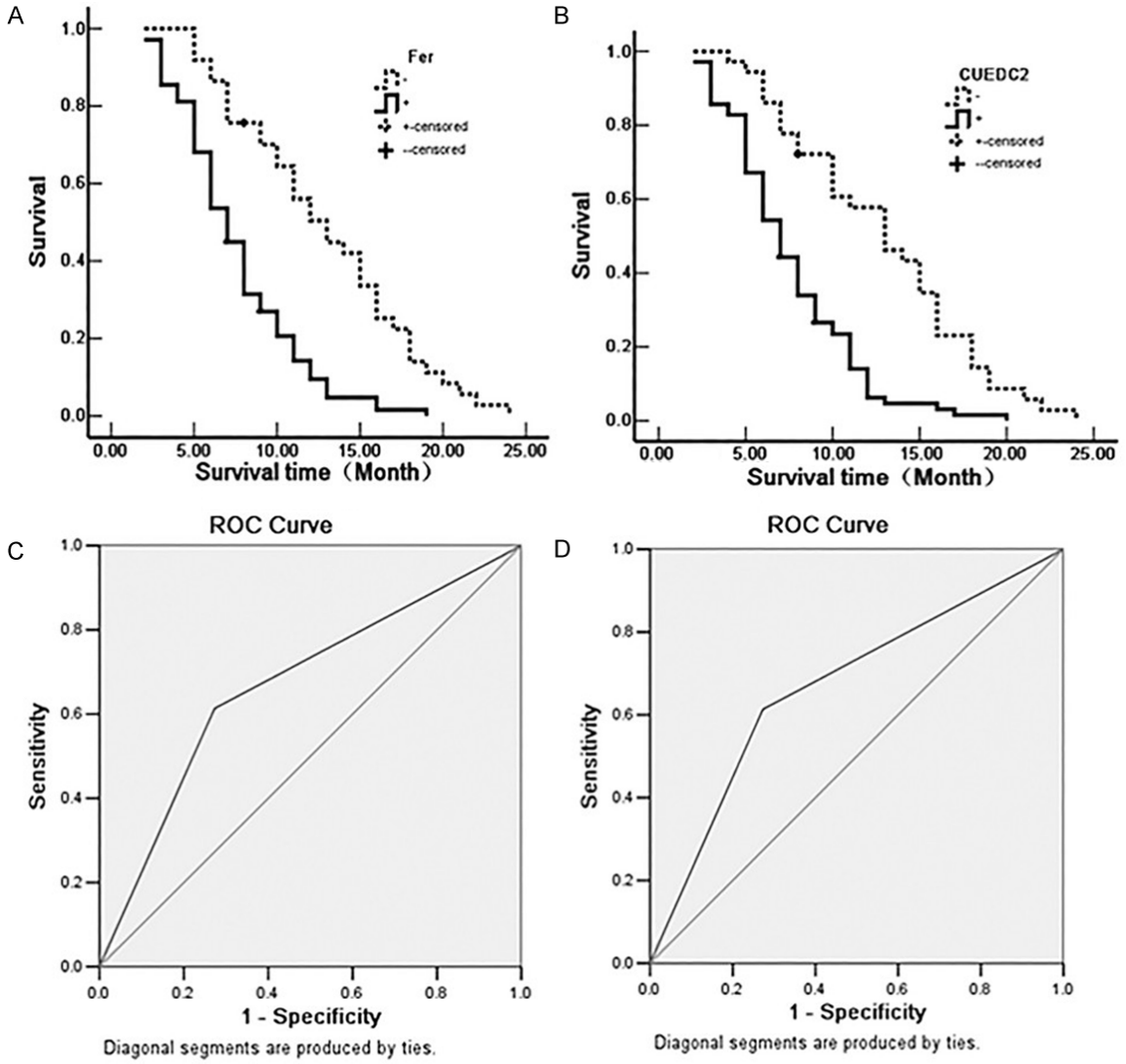

Figure 3. Kaplan-Meier plots of overall survival and receiver operating characteristic curve analysis. A. Kaplan-Meier plots of overall survival in patients with PDAC and with positive and negative Fer expression. B. Kaplan-Meier plots of overall survival in patients with PDAC and with positive and negative ADRB2 expression. C. ROC analysis of the diagnosis ability of Fer. D. ROC analysis of the diagnosis ability of ADRB2.

Table 4. Multivariate Cox regression analysis of survival rate in patients with pancreatic ductal adenocarcinoma and Fer and ADRB2 expression

Groups	Factors	B	SE	wald	P	RR	95% CI	
							Lower	Upper
Differentiated degree	Well/moderately/poorly	.314	.148	4.501	.034	1.369	1.024	1.830
Tumor size	< 3 cm/3-5 cm/> 5 cm	.116	.203	.327	.568	1.123	.754	1.672
Lymph node metastasis	No/yes	.601	.286	4.416	.036	1.824	1.041	3.195
Invasion	No/yes	.390	.355	1.207	.272	1.477	.737	2.962
TNM stage	I/II/III/IV	.639	.233	7.521	.006	1.895	1.200	2.991
Fer	-/+	.597	.253	5.568	.018	1.817	1.106	2.983
ADRB2	-/+	.478	.226	4.473	.034	1.613	1.036	2.512

stingly, this study demonstrated that Fer protein expression positively correlated with ADRB2 protein expression in PDAC tumors (P = 0.005), suggesting that these two molecules may play a collaborative effect in the development of PDAC may through regulating same signaling pathways.

In conclusion, Fer and ADRB2 may play a crucial role in the tumorigenesis and progression of PDAC. Positive Fer and ADRB2 protein expression could be a biomarker for the poor prognosis in PDAC patients.

Disclosure of conflict of interest

None.

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