

Original Article

Expression of FXR and HRG and their clinicopathological significance in benign and malignant pancreatic lesions

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Abstract: Aims: The following study examines the FXR and HRG expression in benign and malignant lesions of the pancreas and evaluates the association between FXR and HRG expression with clinicopathological features and prognosis of pancreatic cancer. Materials and methods: Immunohistochemistry of FXR and HRG was performed with EnVision™ in 106 pancreatic ductal adenocarcinoma (PDAC) specimens, 35 paracancer samples (2 cm away from the tumor, when possible or available), 55 benign lesions and 13 normal tissue samples. Results: The percentage of cases with positive FXR and negative HRG expression was significantly higher in PDAC compared to pericancerous tissues, benign lesions and normal tissues ($P < 0.05$ or $P < 0.01$). In pancreatic tissues with benign lesions, tissues with positive FXR and/or negative HRG protein expression exhibited dysplasia or intraepithelial neoplasia. The percentage of cases with positive FXR and negative HRG expressions was significantly higher in PDAC with lymph node metastasis, invasion, and TNM stage III+IV disease ($P < 0.05$ or $P < 0.01$). The expression of FXR was negatively correlated with HRG ($P < 0.05$). In addition, the univariate Kaplan-Meier analysis showed that positive FXR and negative HRG expression, poor differentiation, large tumor size, high TNM stage, lymph node metastasis, and invasion were closely associated with decreased overall survival in PDAC patients ($P < 0.05$ or $P < 0.01$). Moreover, multivariate Cox regression analysis identified that positive FXR and negative HRG expression were independent factors for poor prognosis in PDAC. The AUC for FXR was (AUC=0.709, 95% CI: 0.632-0.787), and for HRG was (AUC=0.719, 95% CI: 0.643-0.796) in PDAC compared to benign lesions. Conclusions: Positive FXR and negative HRG expression are closely associated with the carcinogenesis, clinical, pathological and biological behaviors, and poor prognosis in PDAC.

Keywords: Pancreatic ductal adenocarcinoma, dysplasia, pancreatic intraepithelial neoplasia, FXR, HRG, immunohistochemistry

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a highly malignant tumor of the pancreas and the fourth-leading cause of cancer-related deaths in the USA with an estimated 39,590 pancreatic cancer deaths in 2014 [1]. The 5-year survival rate of PDAC is less than 5% [2], and annual deaths from PDAC continue to rise [1]. This poor prognosis results from late diagnosis due to the lack of specific signs and biomarkers for early diagnosis, the metastatic potential of PDAC, and resistance to chemotherapy. Despite recent advancements in diagnostic technology, over 85-90% of patients present with advanced and metastatic disease, thus losing the option

of surgical resection [3]. Unfortunately, most surgical patients still relapse despite adjuvant systemic therapies [4]. Metastasis is a natural feature of pancreatic cancer, responsible for over 90% of patients' deaths [5]. The search for diagnostic and targeting therapy biomarkers continues, since few of them have reached clinical application.

The Farnesoid X-receptor (FXR; NR1H4) is an adopted nuclear receptor with oxysterols, such as primary and secondary bile acids, as its endogenous ligands [6, 7]. Bile acids are the metabolic products of cholesterol that can be toxic to the body at high concentrations. FXR prevents the accumulation of bile acids by

inducing expression of a second nuclear receptor small heterodimer partner (SHP, NROB2), which in turn inhibits expression of CYP7A1, the rate-limiting enzyme in bile acid synthesis [8]. The main sites of bile acid production and secretion are the liver and intestine, respectively, which is mirrored by high levels of FXR expression in these organs [9]. Yet, as approximately 70% of bile acids are re-absorbed by the body, they can reach micromolar concentrations in the plasma, which may underlie the expression of FXR in a number of other tissues [10, 11]. FXR is highly expressed in the enterohepatic system where it transcriptionally regulates bile acid and lipid metabolism [12]. Recently, some studies have demonstrated that FXR is overexpressed in multiple human cancers, including breast cancer [13], lung cancer [14], gastric cancer [15, 16], colorectal cancer [17, 18], pancreatic cancer [19] and hepatocellular carcinoma [20-22]. Nonetheless, some authors have found that overexpression of FXR could inhibit the carcinogenesis and progression of prostate cancer [23, 24].

Histidine-rich glycoprotein (HRG) is a heparin-binding plasma protein that modulates immune, hemostatic, and vascular functions and the activity of degradative enzymes such as the plasminogen/plasmin to regulate angiogenesis [25]. HRG is a 75 kDa single chain heparin-binding plasma protein produced by the liver and is present at high levels in plasma (100-200 $\mu\text{g/ml}$) [26]. HRG, which is a host-produced antiangiogenic and immunomodulatory factor, regulates tumor vessel abnormalization and inflammation for various reasons. First, HRG is a multidomain protein that binds thrombospondins (TSPs), heparin, Fc γ R receptors and other molecules implicated in tumorigenesis [27]. Second, HRG is deposited in the tumor stroma from plasma or platelets; nonetheless, the tumor HRG levels have been analyzed in only a few human cancers [28]. Third, binding of HRG to its ligands is facilitated by Zn^{2+} and low pH, conditions found in the tumor milieu [29]. Fourth, HRG stimulates phagocytosis of dying cells [30], but it is unknown if it regulates TAM polarization. Fifth, HRG inhibits tumor growth [31], while its precise underlying mechanisms remain incompletely understood. Moreover, a role for HRG in metastasis has not yet been documented.

Some authors have found that HRG expression is significantly decreased in breast cancer [32], lung cancer [33], gastric cancer [34], colorectal cancer [35], hepatocellular cancer [36], pancreatic ductal adenocarcinoma [37], ovarian cancer [38] and prostate cancer [39]. HRG might be an independent diagnostic indicator and improved prognostic indicator.

The role of FXR and HRG expression in PDAC remain to be clarified. In this study, FXR and HRG expressions in benign and malignant pancreatic lesions were measured by immunohistochemistry. The clinicopathologic significance of FXR and HRG expressions and their associations with the prognosis of PDAC were analyzed.

Material and methods

Case selection

One hundred and six PDACs, thirty-five peritumoral tissues, fifty-five precursor pancreatic tissues, and thirteen normal pancreatic tissues were obtained at the Second and third Xiangya Hospitals, Central South University. This study was pre-approved by the Ethics Committee for Human Study of Central South University. Among the one hundred and six PDACs, sixty-one came from male patients (57.5%) and forty-five came from female patients (42.5%) with an average age of 54.50 ± 11.53 years. Histopathologic subtypes of PDAC include: thirty-eight well-differentiated adenocarcinomas (35.8%), thirty-five moderately-differentiated adenocarcinomas (33%), and thirty-three poorly-differentiated adenocarcinomas (31.1%). Invasion and lymph node metastases were evaluated according to standard criteria. Among the one hundred and six PDAC, eleven cases (10.4%) were TNM stage I, forty-one cases (39.6%) were TNM stage II, thirty-seven cases (34.9%) were TNM stage III, and sixteen cases (15.1%) were TNM stage IV tumors. Among the one hundred and six PDACs, twenty-nine cases (27.5%) had regional lymph node metastasis, and sixty-four cases (60.4%) had invasion to surrounding organs and tissues. Survival information was obtained through letters and phone calls from all patients with PDAC.

Thirty-five peritumoral tissues were collected ≥ 2 cm from the tumors of above mentioned

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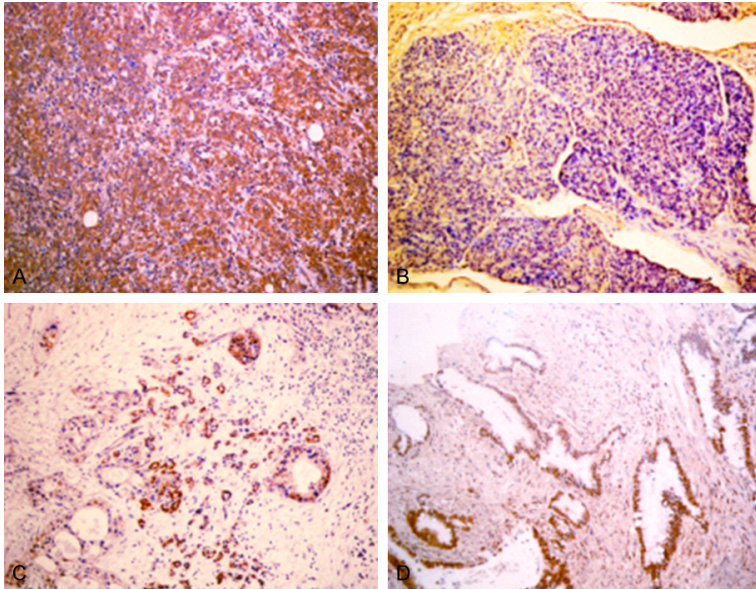


Figure 1. A. Positive expression of FXR, poorly differentiated PDAC, $\times 200$. B. Negative expression of FXR, moderately differentiated PDAC, $\times 200$. C. Positive expression of FXR, chronic pancreatitis, $\times 200$. D. Positive expression of FXR, adenoma, $\times 200$.

PDAC patients. Twelve of the 35 peritumoral tissues were normal, ten were PanINs (pancreatic intraepithelial neoplasms) grade I, eight were PanINs grade II, and five were PanINs grade III.

Fifty-five precursor pancreatic tissues were collected from twenty-nine (52.7%) males and twenty-six (47.3%) females. Among the fifty-five patients who supplied the precursor specimens, thirteen (23.6%) were ≤ 45 years old and forty-two (76.4%) were > 45 years old. The fifty-five precursor tissues included twenty chronic pancreatitis tissues (36.4%), twenty adenomas (36.4%), and fifteen intraepithelial neoplasias (27.3%). Ten, six, and four of the twenty chronic pancreatitis tissues were mild, moderate, and severe pancreatitis, respectively. The twenty adenomas included five mucinous adenomas and fifteen serous adenomas. Four, three, and two of the twenty adenomas had mild, moderate, and severe dysplasia, respectively. Among the fifteen intraepithelial neoplasias, six had grade I, five had grade II, and four had grade III intraepithelial neoplasia. Thirteen normal pancreatic tissues were collected during surgery of the twenty pancreatic adenomas.

All tissues were treated with 4% formaldehyde for 24 to 48 hours, with 10% formalin solution, and were then embedded in paraffin.

Immunohistochemistry

Rabbit anti-human FXR and HRG polyclonal antibody were purchased from Dako Corporation (Carpentaria, CA, USA). EnVision™ Detection Kit was purchased from Dako Laboratories (CA, USA). Positive controls were provided with the EnVision™ Detection Kit. EnVision immunohistochemistry of FXR and HRG was performed following the user manual. Briefly, 4 μm -thick sections were cut from paraffin-embedded tissues. The sections were deparaffinized and then incubated with 3% H_2O_2 in the dark for 15 min. The heat-induced epitope retrieval was conducted with sodium citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH

6.0) at 96°C for 30 min. The sections were incubated with rabbit anti-human FXR and HRG primary antibody (1:100 dilution) for 1 hr after they were soaked in PBS for 3×5 min. The sections were incubated with several drops of Solution A (ChemMate™ EnVison+ /HRP) for 30 min followed by DAB staining and hematoxylin counter-staining. The sections were dehydrated, soaked in xylene, and mounted with neutral balsam. Five hundred cells from ten random fields were examined per section by 2 observers independently. An average of the percentages from these two observers was used for final evaluation. Cases with positive cells $\geq 25\%$ were considered positive whereas other cases were considered negative.

Statistical analysis

Data were analyzed using the SPSS 17.0 (statistical package for the Social Sciences, Version 17.0). The inter-relationship of FXR and HRG with histological or clinical factors was analyzed using χ^2 test or Fisher's exact test. The overall survival of patients with PDAC was analyzed using Kaplan-Meier univariate survival analysis and log-rank tests. Multivariate analysis was performed with Cox proportional hazards model and the 95% confidence interval was calculated. A $P < 0.05$ was considered significant.

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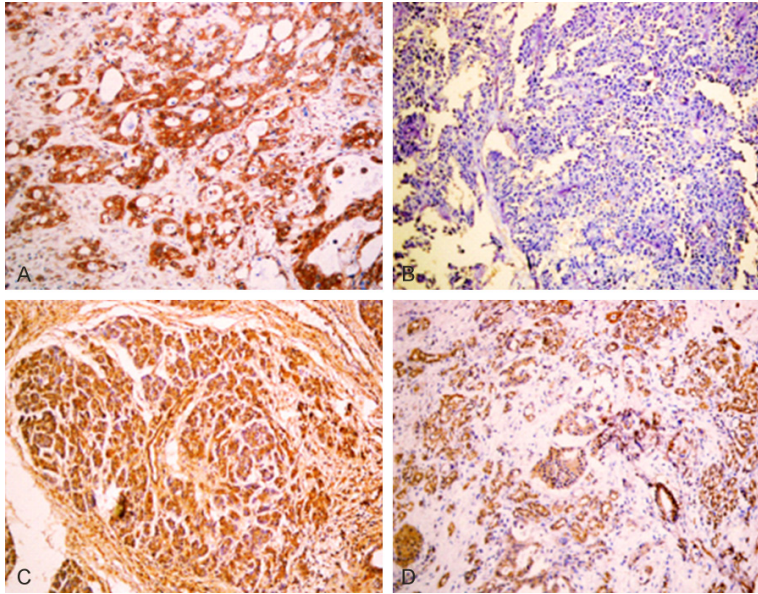


Figure 2. A. Positive expression of HRG, well differentiated PDAC, $\times 200$. B. Negative expression of HRG, poorly differentiated PDAC, $\times 200$. C. Positive expression of HRG, intraepithelial neoplasia II, $\times 200$. D. Positive expression of HRG, pericancerous tissue, $\times 200$.

Table 1. Comparison of FXR and HRG expression in normal, benign, and malignant pancreatic tissues

Tissue type	Case No.	FXR positive (%)	HRG positive (%)
PDAC	106	60 (56.6)	47 (44.3)
Peritumoral tissues	35	10 (28.6)*	28 (80.0)*
Benign tissues	55	10 (18.2)**	47 (85.5)**
Normal pancreatic tissues	13	0 (0.0)**	13 (100.0)**

Compared to PDAC: * $P < 0.05$; ** $P < 0.01$.

Results

FXR and HRG protein expression in adenocarcinoma, peritumoral tissues, precursor, and normal pancreatic tissues

Immunohistochemical staining showed that positive FXR and HRG expression were located in the cytoplasm (**Figures 1 and 2**). In one hundred and six PDACs, sixty and forty-seven were FXR (56.6%) and HRG (44.3%) positive, respectively. In the thirty-five peritumoral tissues, ten and twenty-eight were FXR (28.6%) and HRG (80.0%) positive, respectively. In fifty-five precursor pancreatic lesions, ten and forty-seven were FXR (18.2%) and HRG (85.5%) positive, respectively. In all thirteen normal tissues, FXR was negative and HRG was positive. The positive rates of FXR were significantly higher in

PDAC than those in peritumoral, precursor, and normal pancreatic tissues ($P < 0.05$ or $P < 0.01$; **Table 1**). The positive rate of HRG was significantly lower in PDAC than in peritumoral, precursor, and normal pancreatic tissues ($P < 0.05$ or $P < 0.01$; **Table 1**). Peritumoral tissues and precursor pancreatic lesions with positive FXR and/or negative HRG expression exhibited moderate to severe dysplasia and grade II or III intraepithelial neoplasia. Among the fifty-five precursor lesions, the positive rate of FXR in chronic pancreatitis, adenomas, and intraepithelial neoplasia were 10.0% (2/20) and 20.0% (4/20), 26.7% (4/15); the positive rate of HRG in chronic pancreatitis, adenomas, and intraepithelial neoplasia were 90.0% (18/20) and 85.0% (17/20), 80.0% (12/15), respectively. No significant differences in the positive rate of FXR and HRG were observed between three types of precursor lesions ($P > 0.05$).

FXR and HRG protein expression was associated with clinicopathological characteristics

of PDAC

As shown in **Table 2**, the positive rates of FXR and negative rates of HRG expression were significantly lower in cases with no metastasis in lymph node, no invasion to surrounding tissues and organs, and TNM I+II stage disease compared to cases with lymph node metastasis, invasion, and TNM III or IV stage disease ($P < 0.05$ or $P < 0.01$). The expressions of FXR and HRG exhibited no significant association with sex, age, differentiation degree, or tumor diameter ($P > 0.05$). Among the sixty cases with positive FXR expression, twenty-one cases had positive HRG expression. Among the forty-six cases with negative FXR expression, nineteen cases had negative HRG expression. The expression of FXR was negatively correlated with HRG ($\chi^2 = 4.887$, $P < 0.05$).

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Table 2. Correlations of FXR and HRG protein expression with the clinicopathologic characteristics of pancreatic ductal adenocarcinoma

CPC	Case No.	FXR			HRG		
		Pos No. (%)		P value	Pos No. (%)		P value
Age (year)							
≤45 years	22	16 (72.7)	2.938	0.087	8 (36.4)	0.716	0.398
>45 years	84	44 (52.4)			39 (46.4)		
Sex							
Male	61	35 (54.7)	0.035	0.852	31 (50.8)	2.445	0.118
Female	45	25 (55.6)			16 (35.6)		
Differentiation							
Well	38	17 (44.7)	4.486	0.106	18 (47.4)	0.221	0.895
Moderately	35	20 (57.1)			15 (42.9)		
Poorly	33	23 (69.7)			14 (42.4)		
Tumor size							
≤3 cm	13	6 (46.2)	5.136	0.077	9 (69.2)	4.807	0.090
3-5 cm	68	35 (51.5)			30 (44.1)		
>5 cm	25	19 (76.0)			8 (32.0)		
Lymphnode metastasis							
No	77	33 (42.9)	21.652	0.000	41 (53.2)	9.048	0.003
Yes	29	27 (93.1)			6 (20.7)		
Invasion							
No	42	12 (28.6)	22.253	0.000	28 (66.7)	14.051	0.000
Yes	64	48 (75.0)			19 (29.7)		
TNM stage							
I+II	53	20 (27.3)	15.362	0.000	33 (81.8)	13.799	0.001
III+IV	53	40 (40.5)			14 (57.1)		

FXR and HRG protein expressions correlated with overall survival in patients with PDAC

Survival information of all patients was collected. Twenty-nine patients survived over one year, but seventy-seven patients died within one year with a mean overall survival time of 9.44 ± 0.69 months. Kaplan-Meier survival analysis revealed that the differentiation, tumor size, lymph node metastasis, invasion, and TNM stage were significantly associated with the average overall survival time of patients with PDAC ($P < 0.05$ or $P < 0.01$) (**Table 3**). Average overall survival time for FXR positive patients or HRG negative ones was significantly lower than in those with negative FXR or positive HRG expression ($P = 0.000$) (**Figure 3**). Cox multivariate analysis showed that tumor mass > 5 cm, poor differentiation, lymph node metastasis, invasion, and high TNM stage (III or IV) were negatively correlated with overall survival and positively correlated with mortality. Positive FXR and negative HRG expression negatively

correlated with overall survival and positively correlated with mortality. Both the positive FXR and negative HRG expressions were independent prognostic factors (**Table 4**). Finally, we calculated the AUC for FXR (AUC=0.709, 95% CI: 0.632-0.787), or HRG (AUC=0.719, 95% CI: 0.643-0.796) in PDAC compared to benign lesions, respectively (**Figure 4**).

Discussion

The expression of FXR and HRG in PDAC has not been previously reported, although their expression has been associated with the progression and prognosis of a variety of tumors. This study investigated FXR and HRG protein expression in PDAC tumors, peritumoral tissues, benign pancreatic lesions, and normal pancreatic tissues using immunohistochemistry. A significant increase in FXR and decrease in HRG expression in PDAC tumors were observed. The positive FXR and negative HRG expressions were associated with TNM stage,

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Table 3. Correlations of clinicopathologic characteristics, FXR, and HRG 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)	17.786	0.000
Moderately	35	9.74 (3-21)		
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)	80.807	0.000
II	42	11.37 (3-22)		
III	37	7.14 (2-17)		
IV	16	4.56 (2-8)		
Lymph node metastasis				
No	77	10.64 (2-24)	27.120	0.000
Yes	29	6.35 (2-12)		
Invasion				
No	42	13.33 (5-24)	46.949	0.000
Yes	54	6.83 (2-17)		
FXR				
-	46	12.74 (6-24)	38.310	0.000
+	60	6.83 (2-18)		
HRG				
-	59	7.23 (2-19)	24.956	0.000
+	47	12.07 (3-24)		

invasion, metastasis, and poor prognosis of PDAC.

Most of the studies identified bile acids as risk factors in human cancers, especially for the gastrointestinal tract cancers [40], despite some of the existing data arguing that bile acids have anti-tumoral factors [41]. FXR is a nuclear receptor responsible for bile acid homeostasis by regulating the expression of genes involved in the process [8-11]. However, various reports have shown that FXR is elevated in multiple human cancers responsible for their initiation and progression [13-22]. For instance, it has been proven that FXR can suppress the prolif-

eration of liver cancer by inhibiting mTOR/S6K signaling [42]. On the other hand, some of the reports have proposed the opposite; i.e. they have shown that FXR promotes the carcinogenesis and progression of prostate cancer [23, 24] and esophageal cancer growth [43]. The role of FXR in gallbladder cancer remains largely unknown, which is why the identification of its functional significance may shed new light into its pathogenic mechanism.

HRG was initially identified from proteomic studies on identification of breast cancer biomarkers [44]. HRG, which is a 2-glycoprotein synthesized by liver and present in plasma and platelets, is potentially involved in numerous biologic processes. It can inhibit rosette formation and interact with heparin, thrombospondin and plasminogen. There have been conflicting reports on both enhancing and inhibitory effects of HRG in angiogenesis [45].

Proteomics approaches and other experimental methods have been employed to determine that HRG could be useful as a prognostic tool in some types of cancers [32-39].

Previous studies have confirmed the importance of HRG in hemostasis, angiogenesis, and immunity, which in turn can greatly affect tumor control and metastasis. *In vivo*, HRG can significantly affect tumor growth and metastasis through modulation of TAMs towards an M1 phenotype, activation of platelets, and normalization of angiogenesis. Some studies have tested whether HRG might be used as an independent diagnostic and improved prognostic indicator in patients [32-39].

In the present study, the percentage of cases with positive FXR and negative HRG expression was significantly higher in PDAC patients than

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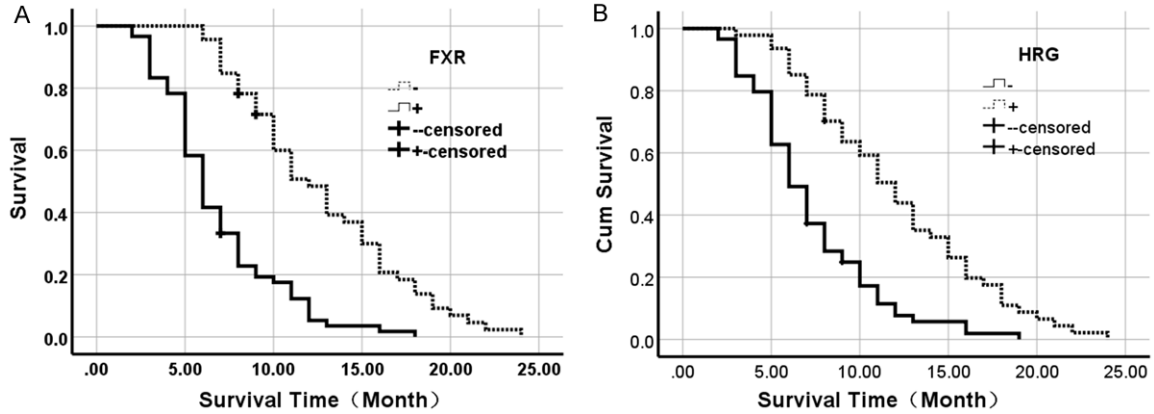


Figure 3. Kaplan-Meier plots of overall survival in patients with PDAC and with positive and negative FXR and HRG expression. A. Kaplan-Meier plots of overall survival in patients with PDAC and with positive and negative FXR expression. B. Kaplan-Meier plots of overall survival in patients with PDAC and with positive and negative HRG expression.

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

Groups	Factors	B	SE	wald	P	RR	95% CI	
							Lower	Upper
Differentiated degree	Well/moderately/poorly	.456	.153	8.883	.003	1.578	1.169	2.129
Tumor size	<3 cm/3~5 cm/>5 cm	.240	.205	1.371	.242	1.271	.851	1.900
Lymph node metastasis	No/yes	.818	.301	7.385	.007	2.266	1.256	4.088
Invasion	No/yes	.929	.348	7.126	.008	2.532	1.280	5.008
TNM stage	I/II/III/IV	.728	.243	8.975	.003	2.071	1.286	3.334
FXR	-/+	.840	.258	10.600	.001	2.316	1.397	3.841
HRG	-/+	-.749	.242	9.579	.002	.473	.294	.760

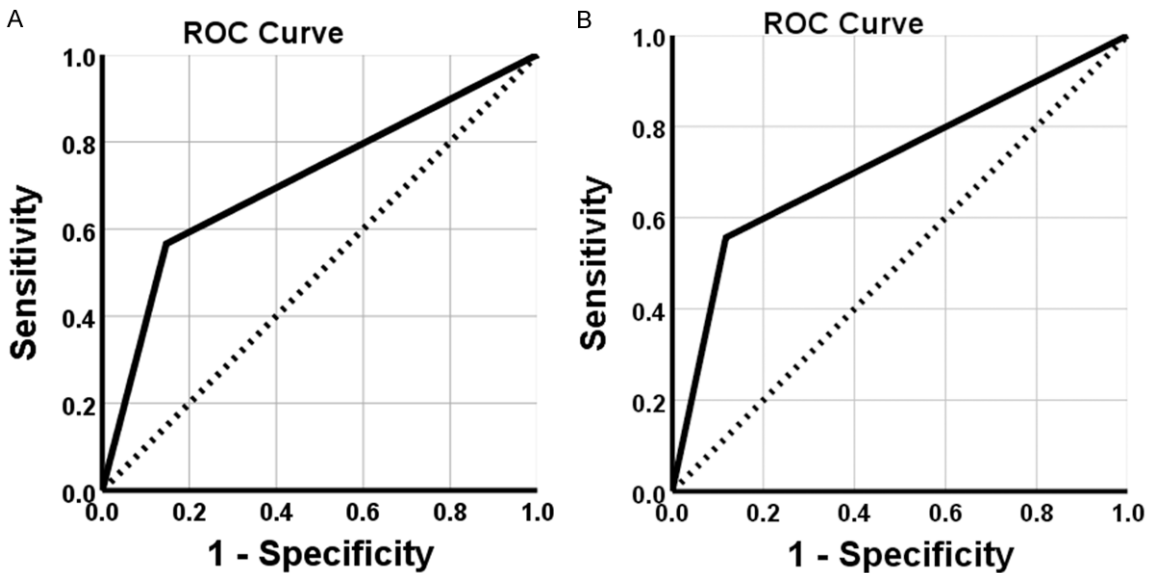


Figure 4. Receiver operating characteristic curve analysis. A. ROC shows the ability of FXR in diagnosis of PDAC. B. ROC shows the ability of HRG in PDAC diagnosis.

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that in pericancerous tissues, benign lesions and normal tissues. In pancreatic tissues with benign lesions, tissues with positive FXR and/or negative HRG protein expression exhibited dysplasia or intraepithelial neoplasia. The percentage of cases with positive FXR and negative HRG expression was significantly higher in PDAC patients with poor-differentiation, lymph node metastasis, invasion, and TNM stage III+IV disease than in patients with well-differentiation, no lymph node metastasis and invasion, and TNM stage I+II disease. The expression of FXR was negatively correlated with HRG in PDAC. The univariate Kaplan-Meier analysis showed that positive FXR and negative HRG, poor-differentiation, large tumor size, high TNM stage, lymph node metastasis, invasion and surgical curability, were closely associated with a decreased overall survival in PDAC patients. The multivariate Cox regression analysis identified that positive FXR and negative HRG expression were independent factors for poor-prognosis with PDAC. Yet, the AUC for FXR was (AUC=0.709, 95% CI: 0.632-0.787), and for HRG was (AUC=0.719, 95% CI: 0.643-0.796) in PDAC compared to benign lesions, respectively. These findings strongly suggested that FXR and HRG might have important roles in the carcinogenesis, progression, biological behaviors and prognosis of PDAC.

In conclusion, the elevated expression of FXR and lowered expression of HRG in PDAC samples indicated that they were significant markers for progression, clinical biologic behavior, and prognosis. The involvement of FXR and HRG in chemoresistance indicated that these two markers have a strong potential to be developed as targets for gene therapy, which may sensitize chemotherapy and radiotherapy. In addition, patients with positive FXR and negative HRG expression in their tumors were more likely to suffer from invasion and metastatic recurrence. These patients may require close monitoring for clinical signs of relapse, so that therapeutic interventions can be applied early enough for optimal outcomes.

Disclosure of conflict of interest

None.

Abbreviations

PDAC, Pancreatic ductal adenocarcinoma; FXR, Farnesoid X-receptor; HRG, Histidine-rich glycoprotein.

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