

# Pancreatic progenitor cell differentiation and proliferation factor predicts poor prognosis in hepatocellular carcinoma

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## Abstract

The aim of this study is to investigate the expression of pancreatic progenitor cell differentiation and proliferation factor (PPDPF) and its relationship with clinicopathological factors in hepatocellular carcinoma (HCC).

A total of 135 patients diagnosed with HCC who underwent curative surgery were enrolled in this study. The expression of PPDPF was examined by real time-polymerase chain reaction (RT-PCR), western blot, and immunohistochemistry. The prognostic value for each sample was explored.

Both RT-PCR and western blot revealed PPDPF expression was upregulated in HCC. Higher PPDPF expression was also observed in HCC (54.07%) detected by immunohistochemistry (IHC), which was significantly associated with tumors size ( $P = .003$ ), Edmondson-Steiner Grading ( $P = .021$ ), recurrence ( $P = .010$ ), and Dismal complete ( $P = .023$ ). Patients with higher PPDPF expression had increased cancer progression and poorer prognosis than those with lower expression ( $P = .043$ ). Multivariate analysis indicated PPDPF as an independent prognostic factor ( $P = .014$ ).

Aberrance PPDPF expression might be a useful predictor and could serve as a potential therapeutic target for HCC.

**Abbreviations:** HBV = hepatitis B virus, HCC = hepatocellular carcinoma, HCV = hepatitis C virus, IHC = immunohistochemistry, PPDPF = pancreatic progenitor cell differentiation and proliferation factor, RT-PCR = real time-polymerase chain reaction.

**Keywords:** hepatocellular carcinoma, pancreatic progenitor cell differentiation and proliferation factor, prognosis

## 1. Introduction

Hepatocellular carcinoma (HCC) is considered as the most common cause of cancer-related mortality in worldwide.<sup>[1]</sup> HCC incidence is highest in East Asia and Africa due to the hepatitis B virus (HBV) infection and rapid increases in prevalence have occurred in Western countries caused by hepatitis C virus (HCV) infection and alcohol abuse.<sup>[2,3]</sup> Despite great progress of the complicated treatment acquired in the decade, HCC patients still

have a poor prognosis, and the average survival time since initial diagnosis is dismal.<sup>[4]</sup> Tumor recurrence and metastasis after liver resection seem unavoidable, which are major contributor to the unfavorable prognosis.<sup>[5]</sup> Therefore, therapeutic intervention is needed for targeting new clinically applicable molecules for an early treatment of HCC.

Pancreatic progenitor cell differentiation and proliferation factor (PPDPF) locates in chromosome 20. The role of PPDPF is rarely explored, and the main molecular function is reported as a key regulator of exocrine pancreas development controlled by retinoic acid and ptf1a; PPDPF could promote the exocrine and inhibit the endocrine development through regulating the expression of cell cycle genes: cyclinG1, cyclinD1, p21<sup>Cip</sup>, p27<sup>Kip</sup>.<sup>[6]</sup>

In the National Center for Biotechnology Information (NCBI) database, the human PPDPF ortholog is expressed in relatively high levels in multiple tissues including the pancreas, colon, stomach, prostate, and kidney. Interestingly, the EST expression profile also indicates that higher level of PPDPF ortholog has been detected in several cancers including pancreatic cancer, liver cancer, and kidney cancer. In addition to structural mutations, many growth factor receptors and their ligands are overexpressed in liver cancers.<sup>[7]</sup> Since PPDPF can promote cell proliferation, it is worth studying the role of this gene in liver cancer.

The PPDPF consists with a putative signaling molecule containing 2 SH2 and 2 SH3 domains as well as other conserved domains,<sup>[6]</sup> suggesting that PPDPF may function in response to signals from adjacent mesoderm tissues. Multiple intercellular signals including transforming growth factor beta, Hedgehog, and Notch are critical for the tumor development.<sup>[8]</sup> It is not clear which signal or signals PPDPF responds to in order to promote the carcinogenesis.

In the present study, we evaluate the expression of PPDPF and investigate its relationship with clinicopathological characteristics and patients' prognosis. This study will provide insight into the function of PPDPF genes and liver cancer progression.

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## 2. Materials and methods

### 2.1. Patients and samples

A total of 135 HCC tissue specimens and 91 adjacent normal liver tissue cases were obtained by surgical resection samples from HCC patients admitted at Affiliated Hospital of Jiangsu University from 2008 to 2015. Both kinds of tissues were confirmed by pathological examination, formalin-fixed, and paraffin embedded. Clinicopathological features were collected from the hospital electronic database. Overall survival (OS) was followed up from the date of surgery until the death date or the last follow-up. This study was approved by the Review Board of Hospital Ethics Committee (Approval Number: JDFY20160527007) and the informed consent from each participant was obtained for every specimen examined.

### 2.2. RNA preparation and quantitative real-time PCR

The complementary DNA (cDNA) was prepared using the kit purchased from Promega after the total RNA was extracted using Trizol (Invitrogen, CA). Primers for the genes tested in the present experiments were designed using software PRIMER5 (version 5.0 for windows, CA). The mRNA level of PPDPF in the HCC tissues and paired non-cancerous tissues was examined by quantitative real-time PCR using SYBR Green Realtime PCR Master Mix (TOYOBO, Kita-ku, Osaka, Japan) following the instructions of the manufacturer. Sequences of quantitative real-time PCR primers are listed as follows:

18S Forward primer: 5'-TAAATCAGTTATGGTT CCTT-3'  
18S Reverse primer: 5'-CGACTACCATCGAAAG TTGA-3'  
PPDPF Forward primer: 5'-CGGTCTTCTCTGCAAATGGGC-3'  
PPDPF Reverse primer: 5'-TGGCTGGTGGGATCTGGG-3'

### 2.3. Western blotting

Human polyclonal anti-rabbit PPDPF antibody was purchased from Proteintech (IL) and antibody to actin was purchased from Sigma (St. Louis, MO). All other chemicals were obtained either from Sigma (St. Louis, MO) or Fisher Scientific (Houston, TX).

After being determined the protein concentration, the cellular protein was subjected to dodecyl sulfate, sodium salt–polyacrylamide gel electrophoresis (SDS–PAGE), and the gel-fractionated proteins were transferred to nitrocellulose membranes (Bio-Rad, CA) and reacted with appropriate antibodies. Signals were detected with ECL kit (Pierce, Rockford, IL) by using one dimensional Image analysis.

### 2.4. Immunohistochemistry

Formalin-fixed paraffin-embedded tissue sections (4  $\mu$ m thick) were stained using the immunoperoxidase method with avidin-biotin complex as described previously.<sup>[9]</sup> The monoclonal anti-PPDPF antibody was from Proteintech (IL). Sections were incubated with the anti-PPDPF monoclonal antibody at 1:100 dilutions overnight at 4 °C. The absence of nonspecific staining was confirmed by the control staining omitting the primary antibody. Labeling for marker was carried out using the Envision Plus Detection Kit (DAKO, Carpinteria, CA) followed the manufacturer's protocol. Nuclei were counterstained with hematoxylin. Immunohistochemical results were judged by 3 pathologists who were unaware of the clinical data. The staining results referred to PPDPF high or low expression were judged by

German semiquantitative scoring system which considers both the stain intensity and the area extent as previous described.<sup>[10]</sup>

### 2.5. Statistical analysis

For comparison of PPDPF mRNA in 2 different groups, Student *t* test was used. Statistical analysis of correlation between PPDPF 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). All *P*-values were derived from 2-tailed tests and *P* < .05 was considered statistically significant.

## 3. Results

### 3.1. Expression of PPDPF in HCC

To determine the significance of PPDPF in HCC, we first analyzed microarray data sets in the TCGA database (<http://xena.ucsc.edu/>; data ID: TCGA. LIHC sample Map/HiSeqV2\_exon). As showed in Fig. 1A, we found PPDPF mRNA levels were significantly increased in HCC samples as compared with normal liver tissue (*P* = .036).

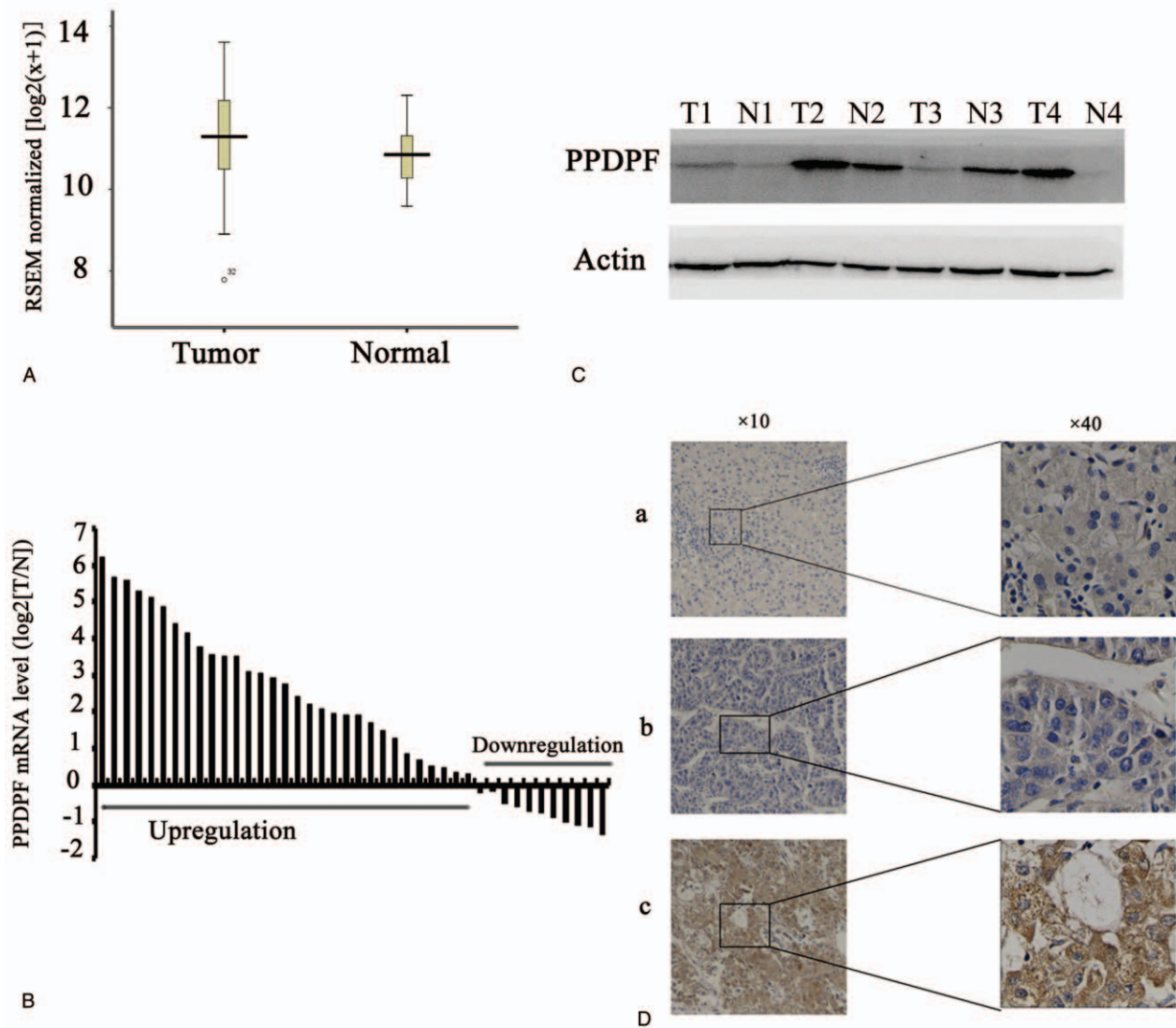
We further assessed the mRNA expression of PPDPF on 42 pair human HCC specimens and their matched normal tissues by real-time RT-PCR test. As shown in Fig. 1B, upregulation of the PPDPF gene occurred in 31 of 42 (73.8%) HCC compared with the paired normal liver tissues. Elevated levels of PPDPF protein were also found in 4 randomly picked pair HCC samples with different expression levels of PPDPF mRNA, as shown by western blot analysis (Fig. 1C).

In addition, in order to further confirm the result demonstrated above, we employed HCC tissues slides with follow-up data from 135 HCC specimens and 91 paired adjacent non-cancerous tissues. The IHC staining demonstrated that PPDPF expression was dramatically enhanced in 54.07% of the HCC samples (73/135) when compared with the adjacent non-tumorous specimens (22/91, 24.17%) (Table 1) (Fig. 1D).

Furthermore, analysis revealed that PPDPF overexpression was significantly correlated with tumors size (*P* = .003), Edmondson-Steiner Grading (*P* = .021), recurrence (*P* = .010), and Diolame complete (*P* = .023). However, no statistical connections were found between PPDPF expression and other clinicopathological parameters, such as age, sex, HBsAg level,  $\alpha$ -fetoprotein (AFP) level, tumor number, cirrhosis, or vascular invasion (Table 2).

### 3.2. Prognostic value of PPDPF expression in HCC

Notably, HCC patients with PPDPF-positive tumor exhibited shorter overall survival than did patients with PPDPF negative tumors (*P* = .043). HCC patients with higher PPDPF expression exhibited shorter median OS time (19 months) compared with patients who had lower levels of PPDPF expression (31.0 months) (Fig. 2). In addition, HCC patients with higher PPDPF expression levels had shorter overall survival than did those with lower PPDPF expression levels (1-, 3-, and 5-year OS: 54.1%, 33.2%, and 19.7% vs 72.1%, 50.6%, and 35.6%, respectively).



**Figure 1.** PPDPF expression is upregulated in HCC. (A) Analysis from the TCGA database (<http://xena.ucsc.edu/>) shows that mRNA expression levels of PPDPF is significantly higher in HCC compared with normal liver tissues. (B) Quantitative real-time PCR results of the relative expression level of PPDPF in 42 pairs of HCC and normal liver tissue samples. All of the reactions were performed in triplicate and results represent the mean  $\pm$  SD ( $P < .05$ ). The expression of PPDPF is normalized to beta-actin. (C) Expression of PPDPF protein in 4 randomly selected paired HCC samples was analyzed using western blot. N, normal; T, primary HCC. (D) Immunohistochemistry (IHC) results of PPDPF in human HCC specimens and adjacent normal tissues. a: expression of PPDPF in the paired adjacent tissues; b: low expression of PPDPF in HCC tissue; c: High PPDPF expression in HCC tissue. HCC=hepatocellular carcinoma, PCR=polymerase chain reaction, PPDPF=pancreatic progenitor cell differentiation and proliferation factor.

In addition to PPDPF expression, univariate analysis demonstrated that tumor size, tumor number, Edmondson grade, vessel invasion, diolame complete were also significantly associated with OS, while others were not (Table 3). Moreover, multivariate

analysis revealed that higher expression of PPDPF, tumor size, tumor number, vessel invasion, diolame complete were independent risk factors associated with decreased survival (Table 4). Collectively, the clinical data indicated that PPDPF is correlated with poor prognosis in HCC patients.

**Table 1**

**High expression of PPDPF in HCC.**

	Case	PPDPF expression		P-value*
		High	Low	
HCC	135	73 (54.07%)	62 (45.93%)	<b>.000</b>
Adjacent tissues	91	22 (24.17%)	69 (75.83%)	

HCC=hepatocellular carcinoma, PPDPF=pancreatic progenitor cell differentiation and proliferation factor.

\* Chi-square test; significant values are shown in bold.

**4. Discussion**

To the best of our knowledge, this study represents the first observation of expression of PPDPF and its prognostic role in patients with liver cancer. NCBI database had reported that PPDPF was highly expressed in a variety of human cancers, including liver, colon, and prostate. Coinciding with this result, we demonstrated that both PPDPF mRNA and protein expression were dramatically increased in HCC tissues. Furthermore we obtained increased expression of PPDPF in HCC tissues

**Table 2****Correlation between PPDPF expression and clinicopathological features of HCC patients.**

Characteristics	Number	PPDPF expression		P-value*
		High (73)	Low (62)	
Age, yr				
<60	81	42	39	.598
≥60	54	31	23	
Gender				
Male	107	61	46	.206
Female	28	12	16	
Size, cm				
<5	61	24	37	<b>.003</b>
≥5	74	49	25	
Location				
Left	42	25	17	.457
Right	93	48	45	
Tumor number				
Single	114	60	54	.483
Multiple	21	13	8	
Edmondson grade				
I+II	97	46	51	<b>.021</b>
III+IV	38	27	11	
Recurrence				
Yes	44	31	13	<b>.010</b>
No	91	42	49	
Vessel invasion				
Absence	108	56	52	.389
Presence	27	17	10	
HBsAg				
Negative	38	23	15	.443
Positive	97	50	47	
Cirrhosis				
Negative	42	20	22	.354
Positive	93	53	40	
AFP, ng/mL				
≤25	52	29	23	.859
>25	83	44	39	
Diolame complete				
Yes	57	24	33	<b>.023</b>
No	78	49	29	

AFP=alpha fetoprotein, HCC=hepatocellular carcinoma, PPDPF=pancreatic progenitor cell differentiation and proliferation factor.

\* Chi-square test; significant values are shown in bold.

by IHC, compared with normal adjacent tissues (54.07% vs 24.17%).

Besides, the increased PPDPF expression was correlated with large tumor size, Edmondson-Steiner Grading, Diolame complete and recurrence, poor prognosis, and overall survival rates. Furthermore, we demonstrated that patients whose liver cancer cells were positive for PPDPF had a mean survival of  $19.17 \pm 1.92$  months compared with  $31.25 \pm 3.4$  months for those whose cells not expressed PPDPF. The results imply that higher PPDPF expression in liver cancer may be considered an indicator of poor prognosis in patients who underwent radical resection with curative surgery.

In our present study, the number of patients recruited was inadequate and there is a possibility of bias. Due to the insidious nature of HCC, most patients diagnosed first time are in advanced tumor stage; patients with early stage are very rare. This may have had an underlying bias on the present study. In future studies, it is necessary to include more patients with early-stage. The majority of our HCC cases accompany with HBV infection. If possible, we would include more patients with HCC

**Table 3****Univariate analysis of risk factors associated with overall survival of HCC patients.**

Characteristics	Number	Univariate analysis		
		HR	95% CI	P-value*
Age, y				
<60	81	0.987	0.569–1.713	.964
≥60	54			
Gender				
Male	107	1.286	0.598–2.765	.520
Female	28			
Size, cm				
<5	61	2.179	1.503–3.158	<b>.000</b>
≥5	74			
Location				
Left	42	1.128	0.812–1.567	.473
Right	93			
Tumor number				
Single	114	0.549	0.423–0.713	<b>.000</b>
Multiple	21			
Edmondson grade				
I+II	97	1.74	1.246–2.430	<b>.001</b>
III+IV	38			
Vessel invasion				
Absence	108	2.887	1.841–4.527	<b>.000</b>
Presence	27			
HBsAg				
Negative	38	1.247	0.879–1.768	.287
Positive	97			
Cirrhosis				
Negative	42	1.373	0.985–1.915	.382
Positive	93			
AFP, ng/mL				
≤25	52	1.927	0.858–4.327	.112
>25	83			
Diolame complete				
Yes	57	0.564	0.375–0.849	<b>.006</b>
No	78			
PPDPF expression		2.12	1.254–3.587	<b>.005</b>
Lower expression	62			
Higher expression	73			

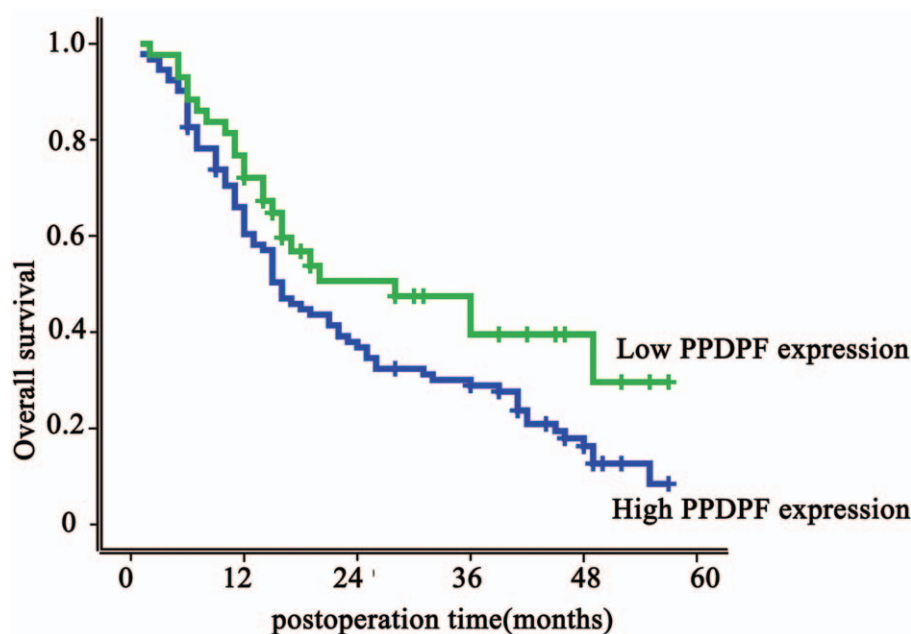
AFP=alpha fetoprotein, HCC=hepatocellular carcinoma, HR=hazard ratio, PPDPF=pancreatic progenitor cell differentiation and proliferation factor.

\* Chi-square test; significant values are shown in bold.

without HBV and examine the PPDPF expression pattern. However, in the multivariable Cox proportional hazards models, the analyses strongly demonstrated that PPDPF expression may be a useful biomarker of predicting outcome of patients with HCC.

The mechanism by which PPDPF expression portends a worse prognosis is not fully known. As previous describe, the PPDPF consist of SH2, SH3 domains, and CLK2 kinase binding site. The former SH domains are considered associating with proteins of signaling pathways involving in the cytoskeleton, the Ras associated protein, and many others.<sup>[11]</sup> The CLK2 kinase has shown to interact with serine/arginine-rich (SR) proteins of the spliceosomal complex, which is responsible for the SR proteins to control RNA splicing.<sup>[12]</sup> This protein kinase is involved in the regulation of several cellular processes and may serve as a link between cell cycle progression, apoptosis, and telomere length regulation.<sup>[13]</sup> In the development of exocrine of pancreas, PPDPF could inhibit the cell cycle arrest through mediating cell cycle inhibitor genes p21<sup>Cip</sup>, p27<sup>Kip</sup>, and cyclin G1, it is likely that the high expression of PPDPF might associated with dysfunction





**Figure 2.** Kaplan–Meier overall survival curves of patients with HCC according to pancreatic progenitor cell differentiation and proliferation factor (PPDPF) expression. Patients with high expression of PPDPF had a significantly poor survival rate than patients with low PPDPF expression ( $P = .043$ ). HCC = hepatocellular carcinoma.

of the cell cycle.<sup>[6]</sup> Given the pivotal roles of PPDPF in the regulation of cell division, abnormal expression of PPDPF may perturb its normal biological functions, which might ultimately induce cancer progression. According to previous study and our results, we can hypothesize that the higher expression of PPDPF might promote tumorigenesis and progression in HCC. Further studies are required to prove our hypothesis.

In conclusion, our observations have provided evidence for the roles of PPDPF in HCC; high expression of PPDPF is related to

unfavorable prognosis in HCC. PPDPF may be considered as a promising therapeutic target in control HCC.

#### Author contributions

**Data curation:** Xi Li.

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**Supervision:** Xuqing Wang.

**Writing – original draft:** Zhengfa Mao.

**Writing – review & editing:** Xin Fan.

**Table 4**

**Multivariate analysis of risk factors associated with overall survival of HCC patients.**

Characteristics	Number	Multivariate analysis		
		HR	95% CI	<i>P</i> -value*
Size, cm				
<5	61	2.090	1.219–3.853	<b>.007</b>
≥5	74			
Tumor number				
Single	114	0.657	0.478–0.903	<b>.009</b>
Multiple	21			
Edmondson grade				
I+II	97	1.336	0.962–1.856	.083
III+IV	38			
Vessel invasion				
Absence	108	1.638	1.124–2.386	<b>.010</b>
Presence	27			
Diolame complete				
Yes	57	0.705	0.508–0.978	<b>.036</b>
No	78			
PPDPF expression				
Lower expression	62	1.884	1.133–3.134	<b>.014</b>
Higher expression	73			

HCC = hepatocellular carcinoma, HR = hazard ratio, PPDPF = pancreatic progenitor cell differentiation and proliferation factor.

\* Chi-square test; significant values are shown in bold.

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