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Expression of Pregnancy Up-regulated Non-ubiquitous Calmodulin Kinase (PNCK) in Hepatocellular Carcinoma

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Abstract. *Background/Aim: Pregnancy up-regulated non-ubiquitous calmodulin kinase (PNCK) is a member of calmodulin kinase, and overexpression of PNCK with involvement in carcinogenesis have been reported in HER-2 amplified breast cancer, clear cell renal cell carcinoma and nasopharygeal carcinoma. However, the expression of PNCK and its clinical implication have not been elucidated in hepatocellular carcinoma (HCC). Materials and Methods: We investigated PNCK expression at both the protein and mRNA level using immunohistochemistry (IHC) and microarray gene expression profiling in HCC tissue samples, and evaluated its association with clinicopathological parameters and their potential prognostic significance. Results: High PNCK protein expression and high PNCK mRNA level was observed in 61.7% and 34.7% of total HCC cases, respectively. PNCK mRNA level was higher in tumor tissues than in background non-tumor tissues, and significantly correlated with protein expression by IHC. High PNCK expression was associated with higher Edmondson grade, intrahepatic metastasis, microvascular invasion and higher AFP levels. Patients with high PNCK expression showed shorter recurrence-free survival and disease-specific survival, and high mRNA expression of PNCK was an independent prognostic factor in disease-specific survival. Conclusion: Up-regulation of PNCK expression as well as its association with poor prognosis was demonstrated in HCC. PNCK might be a prognostic biomarker of HCC, and could be a potential candidate therapeutic target.*

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Hepatocellular carcinoma (HCC) shows a high incidence of tumor recurrence and metastasis, causing a poor prognosis. Sorafenib has been recognized as the most effective treatment for advanced HCC and new targeted agents such as regorafenib and lenvatinib, or programmed cell death protein-1 immune checkpoint inhibitors such as nivolumab and pembrolizumab have also been approved by the US Food and Drug Administration (FDA). However, the application of these therapies remains limited (1-4). Therefore, the investigation of new therapeutic targets and reliable biomarkers is necessary for more effective treatment of HCC (5, 6).

Pregnancy up-regulated non-ubiquitous calmodulin kinase (PNCK) is a calmodulin kinase which is located in Xq28 and is expressed during fetal development and acts in a tissue-specific manner (7). There have been a few previous studies regarding PNCK expression with its involvement carcinogenesis in *human epidermal growth factor receptor-2 (HER-2)* amplified breast cancer, clear cell renal cell carcinoma and nasopharygeal carcinoma (8-10). PNCK expression was increased in tumor tissues compared to non-tumor tissues, and has been associated with proliferation of tumor cells in *HER-2* amplified breast cancer and nasopharyngeal carcinoma (8, 9). In addition, overexpression of PNCK was related with a poor prognosis as well as poor differentiation, large tumor size, and advanced T and N stage in clear cell renal cell carcinoma (10). However, the expression of PNCK and its clinical implications have not been elucidated in HCC.

In this study, we investigated PNCK expression at both protein and mRNA level in HCC tissue samples, and evaluated its association with clinicopathologic parameters and prognostic significance.

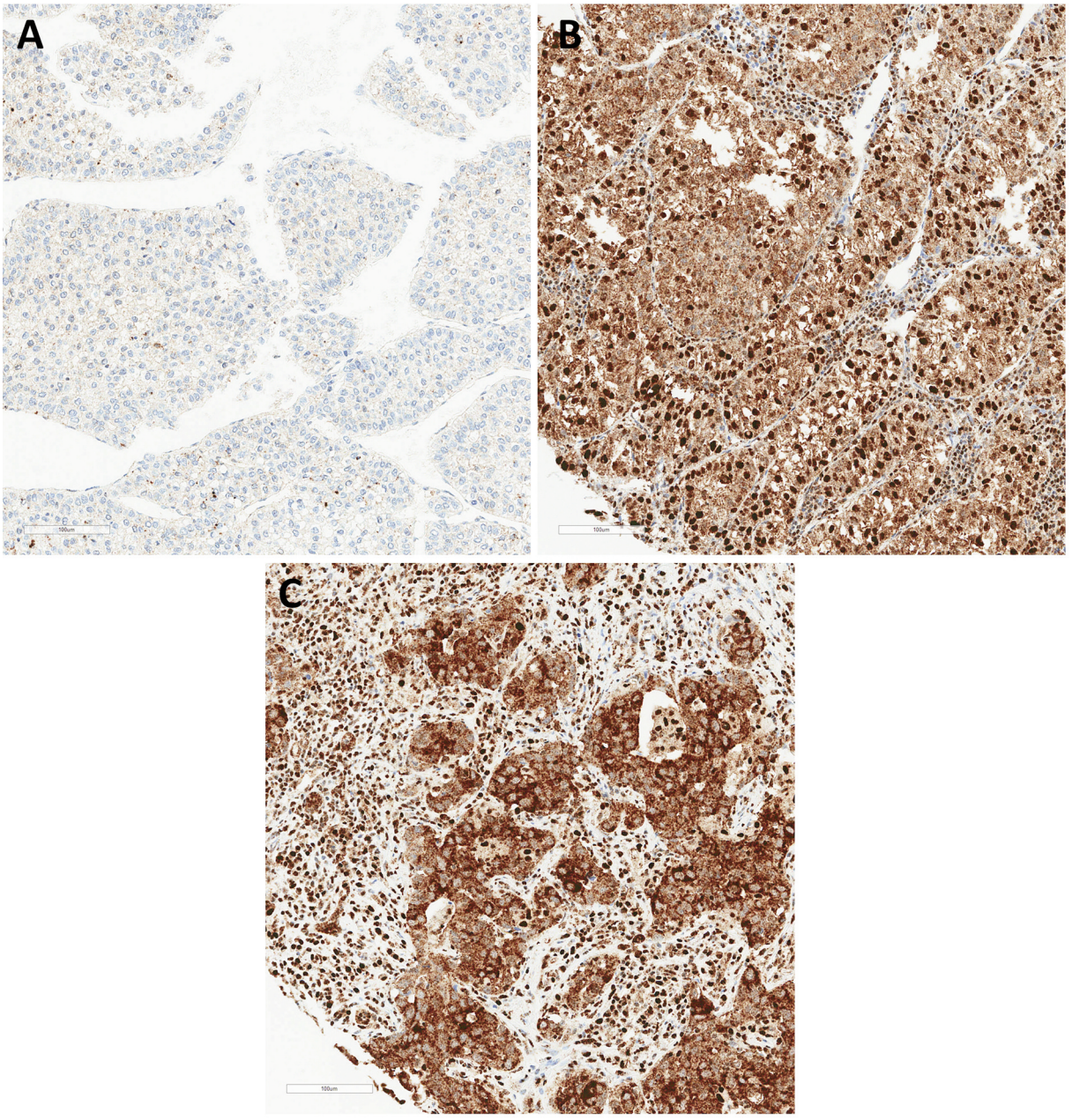

Figure 1. *Representative figures of pregnancy up-regulated non-ubiquitous calmodulin kinase (PNCK) immunohistochemistry (IHC). (A) Tumor cells show negative or faint staining of PNCK IHC. (B-C) Tumor cells show nuclear or cytoplasmic staining of PNCK IHC.*

Materials and Methods

Patients and specimens. Initially, a total of 291 patients with curative hepatectomy for primary HCC from July 2000 to May 2006 at the Samsung Medical Center, Seoul, Korea were enrolled. Eight patients with preoperative local treatment such as radiofrequency ablation, transarterial chemoembolization or radiotherapy and 17 patients with no sufficient tissue on tissue microarray (TMA) were excluded. Finally, 266 patients were included in the study.

Tumors with complete resection margins were confirmed by microscopic examination and no residual tumor one month after surgery was defined as curative resection. All tumor tissues were histologically confirmed. Tumor stages were assessed according to both the American Joint Committee on Cancer (AJCC) staging system, 8th edition (11) and Barcelona Clinic Liver Cancer (BCLC) staging classification (12). Intrahepatic metastasis and multicentric occurrence were defined as in previously reported criteria (13). All patients were followed-up every 3 months after operation, with three

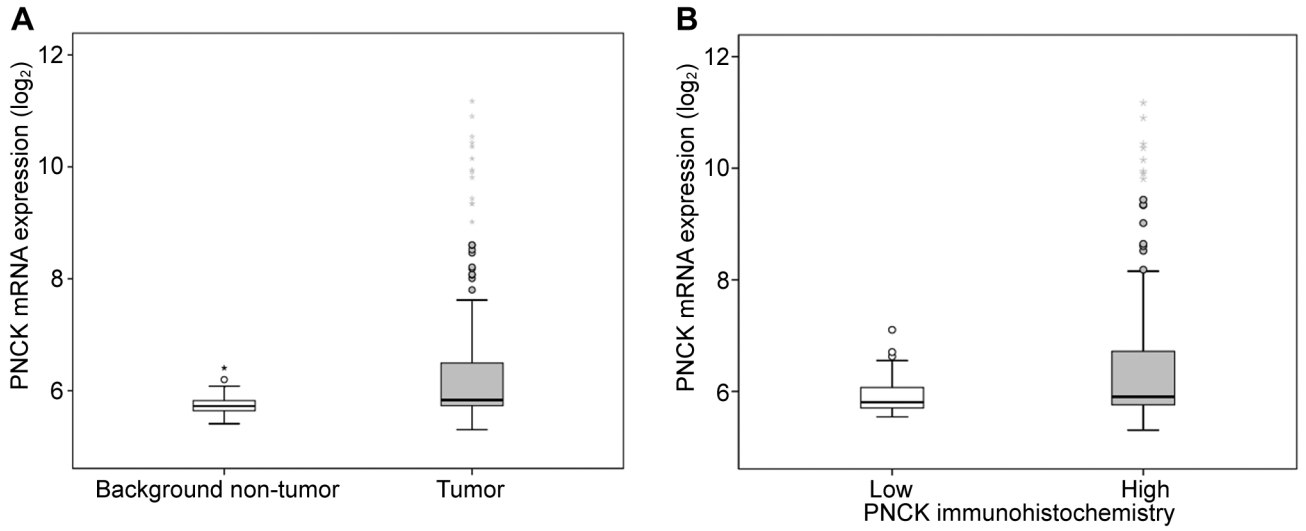

Figure 2. *(A) mRNA expression level of pregnancy up-regulated non-ubiquitous calmodulin kinase (PNCK) in hepatocellular carcinoma tissues and background non-tumor liver tissues. (B) mRNA expression level of PNCK in low expression and high expression group by immunohistochemistry of PNCK.*

phase dynamic computed tomography scans or magnetic resonance imaging and serum α-fetoprotein (AFP) levels. Recurrence-free survival (RFS) or disease-specific survival (DSS) was defined as the difference between the date of surgery and the date of recurrence or HCC-related death, respectively, as previously described (14). Institutional Review Board of Samsung Medical Center approved this study and waived the informed consent.

Immunohistochemical studies. Immunohistochemistry was performed on tissue microarray consisting of two 2 mm cores of HCC tissue as previously described (15). The sections were incubated with a rabbit anti-PNCK antibody (HPA007458, 1:100, Sigma-Aldrich Inc., St. Louis, MO, USA) for 120 min by using the Ventana BenchMark XT Autostainer (Ventana Medical Systems Inc., Tucson, AZ, USA), after Heat Induced Epitope Retrieval (HIER) with CC1 for 92 min. Antigen-antibody chromogenic reactions were developed for 12 min and detected using an OptiView DAB IHC Detection kit (Ventana Medical Systems Inc.). Normal brain tissue was used for positive control. No staining or weak intensity of IHC staining was considered as negative (Figure 1A), while moderate to strong intensity of nuclear or cytoplasmic IHC staining was considered as positive (Figure 1B and C). The proportion of positive PNCK IHC staining among the tumor was evaluated.

mRNA expression of PNCK. We used microarray gene expression profiling data that were obtained by using the same patient cohort (16). The data have been submitted in Gene Expression Omnibus (GSE 36376, http://www.ncbi.nlm.nih.gov/geo/). It consists of 240 HCC tissue and 193 adjacent non-tumor liver tissues. Among them, data from 219 HCC samples and 163 non-tumor liver samples were used for this study. The normalized values of *PNCK* (probe ID: ILMN_1697189) expression with base 2 logarithm were extracted.

Statistical analysis. X-tile bio-informatics software (Yale University, New Haven, CT, USA) (17) was used to determine the cut-off value of PNCK expression with the most significant difference in RFS. Pearson's Chi square tests, Fisher's exact tests or Cochran Armitage test were used to analyze the relationships between PNCK expression and clinicopathologic parameters, as appropriate. The Mann-Whitney *U*-test was performed to compare PNCK mRNA expression between tumor and normal tissue. The Spearman test was used to evaluate correlation between IHC expression and mRNA expression of PNCK. The Kaplan-Meier method was used to analyze survival rates, and differences were compared using the log-rank test. Multivariate regression analysis was performed using a Cox proportional hazards model. Two-sided *p*-values <0.05 were considered statistically significant. Statistical analyses were conducted using IBM SPSS software for Windows (IBM Corp., Armonk, NY, USA).

Results

PNCK IHC staining in HCC in conjunction with clinicopathological features. PNCK IHC expression varied among HCC cases, which showed nuclear or cytoplasmic expression and heterogeneous staining (Figure 1). The proportion of positive staining in HCCs ranged from 0 to 100%, with mean 27.19% and median 8.75%. By using X-tile analysis (17), the best cut-off value for PNCK IHC expression associated with RFS was 5%. High PNCK IHC expression was observed in 61.7% of HCCs, and was significantly related with age younger than 55 years-old (p=0.007), higher Edmondson grade (p=0.040), presence of microvascular invasion (p=0.003), presence of intrahepatic metastasis (p=0.027) and AFP level more than 200 ng/ml (p=0.002) (Table I).

PNCK mRNA expression in HCCs. Mean value of normalized *PNCK* mRNA expression in tumors was 6.39 (range=5.30-11.17). The best cut-off value for *PNCK* mRNA level associated with RFS was 6.09, by X-tile analysis (17). *PNCK*

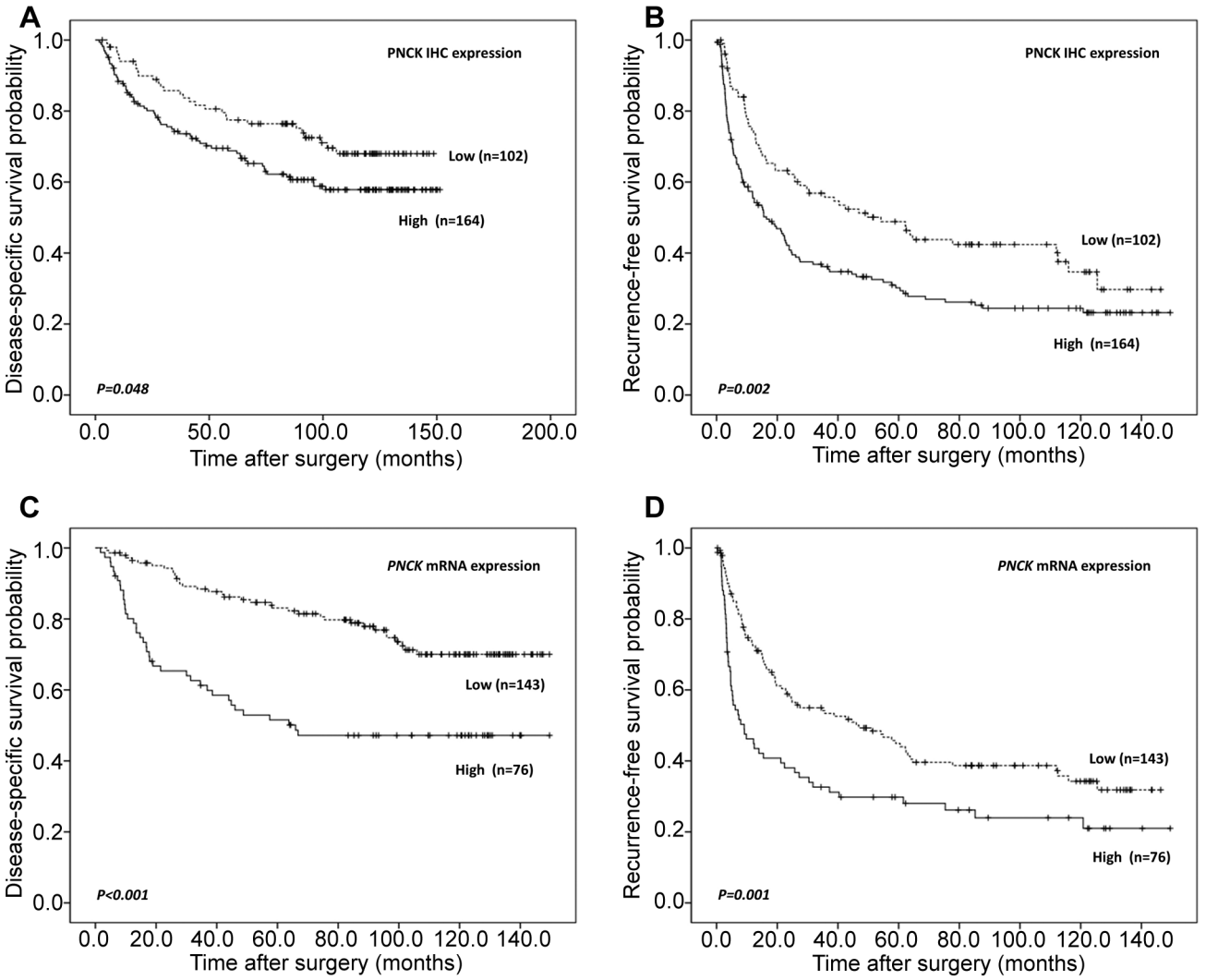

Figure 3. *Kaplan-Meier survival curves according to pregnancy up-regulated non-ubiquitous calmodulin kinase (PNCK) expression; (A-B) PNCK protein expression (C-D) PNCK mRNA expression.*

mRNA expression was significantly higher in tumor than in background liver tissues (6.39±1.17 *vs*. 5.74±0.14, $p<0.001$, Figure 2A). *PNCK* mRNA expression was significantly correlated with PNCK IHC expression (Spearman's coefficient=0.221, $p=0.001$), and *PNCK* mRNA expression was higher in high PNCK IHC expression group than in low group (6.56±1.31 vs 6.12±0.86, $p=0.001$, Figure 2B).

High *PNCK* mRNA expression was observed in 34.7% of HCCs, and was significantly associated with larger tumor size ($p<0.001$), higher Edmondson grade ($p=0.004$), presence of microvascular invasion ($p<0.001$), presence of major portal vein invasion ($p=0.009$), presence of intrahepatic metastasis ($p<0.001$), higher AJCC stage ($p<0.001$), higher BCLC stage ($p<0.001$) and AFP level more than 200 ng/ml ($p<0.001$) (Table I).

Impact of PNCK expression on the survival of HCC patients. Patients with high expression of PNCK IHC showed shorter DSS ($p=0.048$, Figure 3A) and RFS ($p=0.002$, Figure 3B). Patients with high expression of *PNCK* mRNA showed shorter DSS ($p<0.001$, Figure 3C) and RFS ($p=0.001$, Figure 3D). In multivariate analysis, *PNCK* mRNA expression was an independent prognostic factor for DSS [HR=1.908 (95% CI=1.183-3.078), $p=0.008$ by the time-dependent Cox model], in addition to intrahepatic metastasis (Tables II and III).

Discussion

In this study, we elucidated that *PNCK* expression at the mRNA level was up-regulated in HCC samples compared to background non-tumor liver tissue. High expression of

Table I. *The association between PNCK expression and clinicopathological parameters*.

Category	No. of cases (n=266)	PNCK IHC expression				*p*-Value	No. of cases (n=219)	*PNCK* mRNA level				*p*-Value
		High (%) (n=164)		Low (%) (n=102)				High (%) (n=76)		Low (%) (n=143)		
Age (year)												
≤55	158	108	(65.9)	50	(49.0)	0.007	130	50	(65.8)	80	(55.9)	0.194
>55	108	56	(34.1)	52	(51.0)		89	26	(34.2)	63	(44.1)	
Gender												
Male	217	138	(84.1)	79	(77.5)	0.194	179	64	(84.2)	115	(80.4)	0.583
Female	49	26	(15.9)	23	(22.5)		40	12	(15.8)	28	(19.6)	
Tumor size (cm)												
≤5.0	178	109	(66.5)	69	(67.6)	0.894	148	33	(43.4)	115	(80.4)	<0.001
>5.0	88	55	(33.5)	33	(32.4)		71	43	(56.6)	28	(19.6)	
Edmondson grade												
I	29	15	(9.1)	14	(13.7)	0.040	23	2	(2.6)	21	(14.7)	0.004
II	216	131	(79.9)	85	(83.3)		179	64	(84.2)	115	(80.4)	
III	21	18	(11.0)	3	(2.9)		17	10	(13.2)	7	(4.9)	
Microvascular invasion												
Absent	118	61	(37.2)	57	(55.9)	0.003	97	12	(15.8)	85	(59.4)	<0.001
Present	148	103	(62.8)	45	(44.1)		122	64	(84.2)	58	(40.6)	
Major portal vein invasion												
Absent	253	153	(93.3)	100	(98.0)	0.141	210	69	(90.8)	141	(98.6)	0.009
Present	13	11	(6.7)	2	(2.0)		9	7	(9.2)	2	(1.4)	
Intrahepatic metastasis												
Absent	201	116	(70.7)	85	(83.3)	0.027	167	46	(60.5)	121	(84.6)	<0.001
Present	65	48	(29.3)	17	(16.7)		52	30	(39.5)	22	(15.4)	
Multicentric occurrence												
Absent	249	156	(95.1)	93	(91.2)	0.209	207	73	(96.1)	134	(93.7)	0.755
Present	17	8	(4.9)	9	(8.8)		12	3	(3.9)	9	(6.3)	
AJCC T stage												
1	42	25	(15.2)	17	(16.7)	0.304	34	10	(13.2)	24	(16.8)	<0.001
2	133	81	(49.4)	52	(51.0)		111	21	(27.6)	90	(62.9)	
3	77	46	(28.0)	31	(30.4)		64	38	(50.0)	26	(18.2)	
4	14	12	(7.3)	2	(2.0)		10	7	(9.2)	3	(2.1)	
BCLC stage												
0-A	153	93	(56.7)	60	(58.8)	0.122	128	28	(36.8)	100	(69.9)	<0.001
B	98	58	(35.4)	40	(39.2)		81	40	(52.6)	41	(28.7)	
C	15	13	(7.9)	2	(2.0)		10	8	(10.5)	2	(1.4)	
Albumin level (g/dl)												
>3.5	236	143	(87.2)	93	(91.2)	0.426	195	66	(86.8)	129	(90.2)	0.495
≤3.5	30	21	(12.8)	9	(8.8)		24	10	(13.2)	14	(9.8)	
AFP level (ng/ml)[a]												
≤200	157	85	(53.5)	72	(73.5)	0.002	131	29	(39.7)	102	(72.9)	<0.001
>200	100	74	(46.5)	26	(26.5)		82	44	(60.3)	38	(27.1)	
Etiology												
Non-viral	32	20	(12.2)	12	(11.8)	0.828	27	8	(10.5)	19	(13.3)	0.323
HBV	206	129	(78.7)	77	(75.5)		171	64	(84.2)	107	(74.8)	
HCV	24	13	(7.9)	11	(10.8)		18	4	(5.3)	14	(9.8)	
HBV&HCV	4	2	(1.2)	2	(2.0)		3	0	(0.0)	3	(2.1)	
Non tumor liver pathology												
Cirrhosis	133	85	(51.8)	48	(47.1)	0.528	106	36	(47.4)	70	(49.0)	0.887
Others	133	79	(48.2)	54	(52.9)		113	40	(52.6)	73	(51.0)	

Values are presented as number (%). AJCC, American Joint Committee on Cancer; BCLC, Barcelona Clinic Liver Cancer; AFP, α-fetoprotein; HBV, hepatitis B virus; HCV, hepatitis C virus. [a]AFP evaluation was not applicable in 9 cases.

Table II. *Univariate and multivariate analysis for disease-specific survival*.

Category	Disease specific survival					
	Univariate		Multivariate		Multivariate	
	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value
Age (y)*						
≤55	1					
>55	0.972 (0.641-1.474)	0.895				
Gender						
Female	1					
Male	0.838 (0.496-1.418)	0.510				
Tumor size (cm)						
≤5	1		1		1	
>5	3.390 (2.257-5.092)	<0.001	1.688 (1.030-2.766)	0.038	1.478 (0.871-2.507)	0.148
Microvascular invasion						
Absent	1		1		1	
Present	3.536 (2.206-5.667)	<0.001	1.249 (0.675-2.309)	0.479	1.281 (0.645-2.544)	0.479
Major portal vein invasion						
Absent	1		1		1	
Present	5.628 (2.900-10.922)	<0.001	1.248 (0.608-2.561)	0.545	1.481 (0.657-3.336)	0.343
Intrahepatic metastasis						
Absent	1		1		1	
Present	6.431 (4.257-9.715)	<0.001	4.492 (2.556-7.896)	<0.001	5.410 (3.318-8.822)	<0.001
Multicentricity						
Absent	1					
Present	0.417 (0.153-1.136)	0.087				
Edmonson grade						
I-II	1		1		1	
III	2.345(1.249-4.402)	0.008	1.207(0.620-2.347)	0.580	1.020(0.470-2.215)	0.959
AJCC T stage						
T1	1					
T2 to T4	4.659 (1.892-11.475)	0.001				
BCLC stage						
0 to A	1					
B to C	3.506 (2.278-5.397)	<0.001				
Serum albumin level						
>3.5 g/dl	1					
≤3.5 g/dl	1.510 (0.892-2.554)	0.125				
Serum AFP level						
≤200 ng/ml	1		1		1	
>200 ng/ml	1.800 (1.193-2.716)	0.005	0.932 (0.586-1.480)	0.764	0.759 (0.440-1.308)	0.321
Etiology						
Non-viral	1					
Viral	1.731 (0.801-3.740)	0.163				
Non-tumor pathology						
Cirrhosis	1					
Others	0.904 (0.603-1.356)	0.626				
PNCK IHC expression						
Low	1		1			
High	1.554 (1.000-2.413)	0.050	1.214 (0.753-1.956)	0.426		
PNCK mRNA level						
Low	1				1	
High	2.695 (1.712-4.244)	<0.001			1.908 (1.183-3.078)	0.008

HR, Hazard Ratio; CI, Confidence Interval; AJCC, American Joint Committee on Cancer; BCLC, Barcelona Clinic Liver Cancer; AFP, α-fetoprotein; PNCK, pregnancy up-regulated non-ubiquitous calmodulin kinase; IHC, immunohistochemistry.

Table III. *Univariate and multivariate analysis for recurrence-free survival*.

Category	Recurrence free survival					
	Univariate		Multivariate		Multivariate	
	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value
Age (y)*						
≤55	1					
>55	1.045 (0.769-1.420)	0.780				
Gender						
Female	1					
Male	0.971 (0.667-1.412)	0.877				
Tumor size (cm)						
≤5	1		1		1	
>5	1.824 (1.341-2.479)	<0.001	0.997 (0.688-1.444)	0.988	0.846 (0.573-1.248)	0.398
Microvascular invasion						
Absent	1		1		1	
Present	2.341 (1.714-3.196)	<0.001	1.178 (0.798-1.740)	0.410	1.349 (0.891-2.040)	0.157
Major portal vein invasion						
Absent	1		1		1	
Present	4.242 (2.345-7.675)	<0.001	1.030 (0.541-1.960)	0.929	0.779 (0.351-1.731)	0.779
Intrahepatic metastasis						
Absent	1		1		1	
Present	4.323 (3.115-6.000)	<0.001	5.113 (3.647-7.169)	<0.001	5.185 (3.532-7.611)	<0.001
Multicentricity						
Absent	1					
Present	0.640 (0.338-1.212)	0.171				
Edmonson grade						
I-II	1		1		1	
III	2.277 (1.395-3.716)	0.001	1.507 (0.916-2.480)	0.106	1.414 (0.797-2.507)	0.237
AJCC T stage						
T1	1					
T2 to T4	1.656 (1.083-2.531)	0.020				
BCLC stage						
0 to A	1					
B to C	1.697 (1.259-2.287)	0.001				
Serum albumin level						
>3.5 g/dl	1					
≤3.5 g/dl	1.004 (0.652-1.546)	0.985				
Serum AFP level						
≤200 ng/ml	1		1		1	
>200 ng/ml	1.779 (1.314-2.410)	<0.001	1.294 (0.944-1.775)	0.109	1.150 (0.795-1.665)	0.458
Etiology						
Non-viral	1		1		1	
Viral	1.997 (1.134-3.519)	0.017	1.583 (0.871-2.877)	0.132	1.864 (0.938-3.703)	0.076
Non-tumor pathology						
Cirrhosis	1					
Others	0.787(0.583-1.061)	0.116				
PNCK IHC expression						
Low	1		1			
High	1.631 (1.189-2.237)	0.002	1.213 (0.866-1.697)	0.261		
PNCK mRNA level						
Low	1				1	
High	1.747 (1.245-2.452)	0.001			1.078 (0.711-1.634)	0.722

HR, Hazard Ratio; CI, Confidence Interval; AJCC, American Joint Committee on Cancer; BCLC, Barcelona Clinic Liver Cancer; AFP, α-fetoprotein; PNCK, pregnancy up-regulated non-ubiquitous calmodulin kinase; IHC, immunohistochemistry.

PNCK at both the protein and mRNA level was associated with aggressive clinicopathological parameters such as high Edmondson grade, presence of microvascular invasion, presence of intrahepatic metastasis and high AFP level, as well as shorter RFS and DSS, in a large cohort of HCC patients with long-term follow-up.

PNCK is a novel member of calmodulin kinase, which is involved in diverse biological processes including cell-cycle control, transcriptional regulation, neurotransmitter release and muscle contraction (7). *PNCK* is located in Xq28 and its protein expression differs with regards to organs, and is highest in brain, and moderate to low in hormone-related tissues such as uterus, ovary, testis and mammary gland, or other tissues such as stomach, heart and skeletal muscle (7). There have been a few previous studies regarding *PNCK* expression in cancer. *PNCK* overexpression was found in more than 30% of *HER-2* amplified breast cancer, and induced proliferation, cell-cycle progression and clonogenicity as well as trastuzumab resistance through *phosphatase and tensin homologue (PTEN)*-mediated process (8). In clear cell renal cell carcinoma, *PNCK* was one of the mostly overexpressed genes by massive parallel sequencing analysis (18), and PNCK expression at the protein and mRNA levels were higher in tumor than non-tumor samples, and associated with higher Fuhrman grade, larger tumor size and advanced T and N stage, as well as a poor prognosis (10). In a recent study in nasopharyngeal carcinoma, PNCK expression at the protein and mRNA levels was found increased in tumor tissues, and knockdown of *PCNK* inhibited proliferation and induced apoptosis by regulating *PI3K/AKT/mTOR* signaling pathway by *in vitro* and *in vivo* experiments (9) .

In this study, we are the first to report the overexpression of PNCK and its association with poor prognosis in HCC, and these results are highly consistent with results from previous studies in other cancers. Our study provides clinical evidence suggesting PNCK as a potential therapeutic tool targeting HCC. Previous studies revealed that PNCK mediates degradation of epidermal growth factor receptor (EGFR) protein, which might be a promising target for *EGFR*-regulated oncogenesis (19, 20). EGFR overexpression was found in up to 68% of the HCC cohort, and was related with aggressiveness of tumor (21, 22). It was found mainly in poorly differentiated HCC, and was correlated with high Ki-67 proliferative index, advanced stage, presence of intrahepatic metastasis and poorer prognosis (21, 22). However, monotherapy using cetuximab, the monoclonal antibody against *EGFR*, showed disappointing results to date in HCC patients (23). A case report of complete remission of unresectable HCCs treated with another anti-*EGFR* antibody nimotuzumab have been reported, suggesting *EGFR* as a potential therapeutic target in HCC (23). The implication for PNCK expression for prediction for targeted therapy, including anti-*EGFR* drugs, needs further examination.

In conclusion, we demonstrated up-regulation of PNCK expression in HCCs and its association with poor prognosis. PNCK might be a prognostic biomarker of HCCs, and could be a potential candidate therapeutic target.

Conflicts of Interest

None of the Authors have any conflicts of interest to declare regarding this study.

Authors' Contributions

Conception and design: SYH; Acquisition of data: YAC, SC, SP, CP, SYH; Analysis and interpretation of data: YAC, SYH; Drafting the article: YAC, SYH; Revising and final approval of the article to be published: YAC, SC, SP, CP, SYH; All Authors read and approved the final manuscript.

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