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Original Article

Prognostic value of pAMPKa/ADM2/VEGF expression in hilar cholangiocarcinoma

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Abstract: Activation of AMPK by metformin leads toattenuating tumor angiogenesisin various cancers. ADM2 is a novel tumor angiogenic factor. However, its status and its correlation with AMPKand VEGF protein in hilar cholangiocarcinoma (HCC)were rarely investigated. The purpose of the study was sought to detect the expression of pAMPKa, ADM2 and VEGF and to explore their clinical values. Activation of pAMPKa by metformin leaded to inhibition of ADM2 and VEGF. The positive rates of pAMPKa, ADM2 and VEGF proteins expression in HCC were 51.0% (25/49), 61.2% (30/49), and 83.7% (41/49), respectively. Overexpression of ADM2 correlated significantly with highly invasive tumors (P<0.001) and regional LN metastasis (P=0.018). Overexpression of VEGF was significantly associated with regional lymph node metastases (P=0.049). No significance was found between pAMPKa expression and clinical variable. Additionally, increased expression of ADM2 and VEGF proteins and decreased expression of pAMPKa were associated with tumor recurrence and poor outcome for HCC patients. Multivariate Cox regression analysis identified pAMPKa was an independent predictor for tumor recurrence (*P*=0.047) and there is a trend thatpAMPKa might be a promising prognostic factor (*P*=0.083). Taken together, loss expression of pAMPKamight act as a valuable independent marker to predict tumor recurrence of HCC.

Keywords: Hilar cholangiocarcinoma, AMPK, ADM2, VEGF

Introduction

Hilar cholangiocarcinoma (HC), also known as Klatskin tumor, approximately accounts for over 60% of all cholangiocarcinoma. Surgical margin-negative (R0) resection offers the only chance for long survival of HC patients [1, 2]. However, more than half of these tumors are unresectable at the time of initial diagnosis. Therefore, the five-year survival of these patients remains unfavorable. Prediction of the prognosis of HC patients is crucial in monitoring and evaluating the treatment effectiveness. Previous studies revealed that lymph node invasion, tumor grade and negative margins are the most important prognostic indicators [2]; however, the grading system remains controversial [3].

Epidemiological studies suggested an association between metformin use and reduced cancer risk, including intrahepatic cholangiocarcinoma [4-6]. Mechanically, metformin activates AMP-activated protein kinase (AMPK), a key player in the regulation of energy homeostasis, and inhibits the mammalian target of rapamycin complex 1 (mTORC1) resulting in decreased cancer cell proliferation [5]. Recent studies showed that AMPK activation by metformin markedly inhibited angiogenesis in various tumors. ADM2 is a calcitonin gene-related peptide which was supposed to be a novel tumor angiogenesis factor, acting via vascular endothelial growth factor/vascular endothelial growth factor-2 signaling pathways [7]. Thus we hypothesized that metformin might target AMPKa/ADM2/VEGF pathway in hilar cholangiocarcinoma. Moreover, underexpression of p-AMPKa and overexpression of ADM2 and VEGF were detected in several malignant tumors and theirs expression was reported to be associated with malignant potential [8-11]; however, the correlation between the expression

Table 1. Correlation between the expression of VEGF, ADM2, and pAMPKa and clinicopathological parameters of hilar cholangiocarcinoma

	N	VEGF+(%)	*P*	ADM2+(%)	*P*	pAMPKa+(%)	*P*
Age							
≤55 y	35	30 (85.7)	0.541	22 (62.9)	0.711	20 (57.1)	0.175
>55 y	14	11 (78.6)		8 (57.1)		5 (35.7)	
Gender							
Male	25	21 (84.0)	0.95	17 (68.0)	0.32	12 (48.0)	0.666
Female	24	20 (83.3)		13 (54.2)		13 (54.2)	
Size							
≤3 cm	18	15 (83.3)	0.961	11 (61.1)	0.99	7 (38.9)	0.196
>3 cm	31	26 (83.9)		19 (61.3)		18 (58.1)	
T stage							
T1-3	7	5 (71.4)	0.344	0 (0.0)	<0.001	4 (57.1)	0.726
T4	42	36 (85.7)		30 (71.4)		21 (50.0)	
N stage							
N0	16	11 (68.8)	0.049	6 (37.5)	0.018	9 (56.2)	0.61
N1-2	33	30 (90.9)		24 (72.7)		16 (48.5)	
Differentiation							
High/moderate	37	30 (81.1)	0.389	21 (56.8)	0.26	21 (56.8)	0.158
Low/undifferentiated	12	11 (91.7)		9 (75.0)		4 (33.3)	
TNM							
I/II	20	16 (80.0)	0.563	10 (50.0)	0.181	9 (45.0)	0.484
III/IV	29	25 (86.2)		20 (69.0)		16 (55.2)	

profiles of p-AMPKa/ADM2/VEGF and HC remains uncharacterized.

In the present study, we detected the expression of p-AMPKa/ADM2/VEGF in cholangiocarcinoma cells after treated by metformin and then examined their expressions in HC samples by immunohistochemistry and results were correlated with patients' survival data.

Materials and methods

Cell lines and culture conditions

The hilar cholangiocarcinoma cell line QBC939 was obtained from the Cell Center of Chinese Academy of Sciences, Shanghai, China. QBC939 cell lines were maintained in DMEM with 10% fetal bovine serum (FBS) (Invitrogen Corp., Grand Island, NY) and were cultured in a 37°C humidified atmosphere containing 95% air and 5% CO_2. Metformin was purchased from Sangon Biotechnology (Shanghai, China).

MTT assay

QBC939 Cells were digested and seeded into 96-well plates at a density of 5,000 cells per well. Then, cells were treated with metformin at concentrations of 0, 20, or 50 mM. At 0 and 48 hours, cells were incubated with MTT (Sigma Chemical Co., USA, 5 mg/ml) for 4 hours before test. Then the supernatant was removed and 150 μl DMSO was added. Absorbance at 490 nm (A570) (Sigma Chemical Co., USA) was measured with a microplate reader (KHB ST-360, Kehua Bio. Co., China). Actual absorbance = absorbance of the experimental group-absorbance of DMSO. The experiment was repeated three times independently. The ratio of cell proliferation was determined by comparing the absorbance at 48 h to that at 0 h.

Western blot analysis

CCA cell lines treated with metformin were collected and the lysates were prepared for Western blot analysis. Standard Western blotting was performed using a rabbit antibody against human p-AMPKα (1:1000), ADM2 (1:1000), and VEGF (1:1000) and an anti-rabbit IgG antibody, which was a horseradish peroxidase linkedF (ab') 2 fragments obtained from a donkey (Amersham) as previously described. The immunoreactive proteins were detected by the enhanced chemiluminescene (ECL) kit (Santa Cruz, CA, USA).

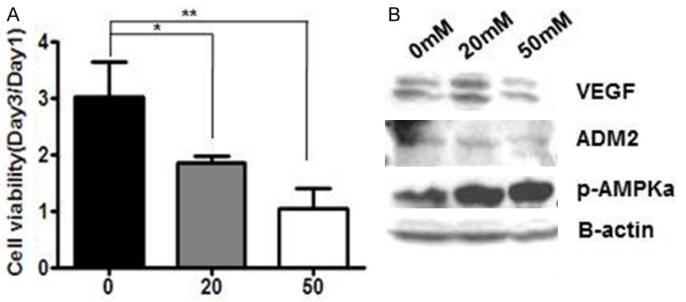

Figure 1. Metformin treatment leads to inhibition of cholangiocarcinoma cell proliferation by regulating pAMPKa/ADM2/VEGF pathway. A. QBC939 cells were treated with metformin (0, 10, 50 mM) for 48 hours and MTT assay was performed to analyze the proliferation ratio by comparing the results at 48 h and that at 0 h. B. Western blotting was used to detect the expression of pAMPKa, ADM2, and VEGF. **P*<0.05, ****P*<0.001, compared with untreated group.

Tissue samples and tissue microarray construction

Forty-nine patients receivedresection of hilar cholangiocarcinomaat the Department of Surgery, Eastern Hepatobiliary Hospital and the Department of Medical Oncology, Changzheng Hospital, Shanghai, China from 2004 to 2008 were enrolled in this study. Hematoxylin and eosin (HE) stained slides were evaluated by two pathologists (G.Y. and Y.C.) and tissues with enough tumor cells were selected for tissue microarray construction. **Table 1** presented the detailed information of these patients. 25 were male and 24 were female. The mean ageat tumor resection was 55 years old. The median follow-up duration for these patients was 16 months, while one patient was lost during follow-up. The Institutional Review Board of both Eastern Hepatobiliary Hospital and Changzheng Hospital approved the use of the tissues and clinical information and a inform consent was obtained from each patient or their guardians. Three paraffin-embedded tissue microarray blocks were created using a manual arrayer (Beecher Instruments, Sun Prairie, WI, USA) as previously described, which contained 1.5 μm core of normal and two or three 1.5 μm cores tumor tissue specimens from eachpatient.

Immunohistochemistry

Antibodies to phospho-AMPKα (pAMPKα, Thr-172) were purchased from Cell Signaling Technology. VEGF (C-1) and ADM2 (G-13) were from Santa Cruz Biotechnology. Consecutive tissue sections (4 μm) of paraffin-embedded sections from the tissue microarrays were prepared as described previously and processed for phospho-AMPKα (1:100), ADM2 (1:100), and VEGF (1:150) proteins immunohistochemical staining. An S-p (Streptavidin-Biotin) kit (#KIT-9720, MAIXIN, Fuzhou, China) was used to visualize antibody binding to the tissues. Haematoxylin was performed as a counterstaining. Control sections were incubated with PBS by omitting the primary antibodies.

Review and scoring of immunohistochemically stained sections

Two individuals (G.Y. and Y.C.) evaluated the stained TMA sections under an Olympus CX31 microscope (Olympus, Center Valley, PA) without any knowledge of clinical information. The discrepancy was dissolved by re-evaluating the sections by two pathologists. A semiquantitative scoring system was used as described previously. The mean percentage of positive tumor cells was assigned from 0% to 100%, while the intensity of immunostaining was scored as follows: negative, 0; weak, 1; moderate, 2; and intense, 3. Therefore, a weighted score was generated for each case: ranging from 0 (0 of cells staining) to 3 (100% of the cells staining at 3+ intensity) [12].

Statistical analysis

Statistical analysis was performed using the SPSS 16.0 statistical software and GraphPad Prism5.0 software. Categorical data were analyzed using χ^2 statistics tests. The Kaplan-Meier method was used to estimate survival rates. The Cox proportional hazards model for multivariate survival analysis was used to assess predictors related to survival. The significance of the in vitro results was determined by using the Student t test (two tailed). Two-sided *P* value <0.05 was considered statistically significant [13].

Results

Treatment of metformin leads to inhibition of cell proliferation, increased expression of pAMPKa and reduced expression of ADM2 and VEGF

To investigate the effects of metformin on cell proliferation, we treated QBC939 cells with

metformin (0 mM, 20 mM, and 50 mM) and measured cell viability at 0 and 48 hours. As shown in **Figure 1A**, metformin significantly inhibited the proliferation ratio of QBC939 cell lines in a dose-dependent manner. Next, we investigated the levels of phophorylated-AMPK at Thr172 after metformin treatment. Use of metformin remarkably activated AMPK (**Figure 1B**). However, metformin treatment leaded to inhibition of the expression of ADM2 and VEGF (**Figure 1B**). Together, these data strongly suggest that metformin exerted its anti-tumor role partially through ADM2/VEGF pathway.

pAMPKa, ADM2 and VEGF expression in patients with hilar cholangiocarcinoma

We further evaluated the predictive role of metformin-targeted genes in hilar cholangiocarcinoma. As shown in **Figure 2**, positive staining of pAMPKa, ADM2 and VEGF was preferentially cytoplasm-localized. pAMPKa was moderately expressed in the epithelium of normal bile ducts, while ADM2 and VEGF were weakly or negatively expressed in these tissues. In tumor, pAMPKa was weakly expressed or lost in the tumor cells. However, the levels of ADM2 and VEGF were evaluated in the cancer tissues. The positive rates of pAMPKa, ADM2 and VEGF were 51.0% (25/49), 61.2% (30/49), and 83.7% (41/49), respectively. Moreover, the mean values of pAMPKa, ADM2 and VEGF in tumor tissues were 0.64 ± 0.62, 1.24 ± 1.07, and 1.66 ± 0.95, respectively; while their values in normal tissue were: 1.16 ± 0.85, 0.48 ± 0.72, and 0.38 ± 0.58 (**Figure 2D1-D3**).

Correlation between pAMPKa, ADM2 and VEGF expression and clinicopathologic characteristics of hilar cholangiocarcinoma

Table 1 presented the association between pAMPKa, ADM2 and VEGF expressionand clinicopathological parameters of hilar cholangiocarcinoma. Evaluated expression of ADM2 occurred more frequently in highly invasive tumors (T4, 71.4%) than less invasive tumors (T1-3, 0; *P*<0.001); more frequently in patients with regional LN metastasis (72.7%) than in N0-stage tumors (37.5%; *P*=0.018). Meanwhile, overexpression of VEGF was more often observed in patients with regional LN metastases than those without regional LN metastases (90.9% *vs* 68.8%; P=0.049). However, no significant association was found between pAMPKa expression and clinical variable.

Relationship of pAMPKa, ADM2 and VEGF expression with poor outcome in patients with hilar cholangiocarcinoma

Factors that associated with tumor recurrence in univariate analysis were tumor invasion (52.2 mo for T1-T3 tumors vs. 16.0 mo for T4 tumors; *P*=0.002), surgical types (39 mo for R0 vs. 11 mo for R1-2; *P*=0.004), lymph node metastasis (12.0 mo for LN-positive tumors vs. 39 mo for LN-negative tumors; *P*=0.023), pAMPKa (pAMPKa negative vs pAMPKa positive: 15 months vs 32 months; P=0.034) ADM2 (ADM2 negative vs ADM2 positive: 39 months vs 11 months; P<0.001) and VEGF (VEGF negative vs VEGF positive: 46 months vs 15 months; P=0.008) (**Figure 3A-C**). Multivariate analysis showed that tumor invasion and pAMPKawas independent prognostic factors (**Table 2**).

As for overall survival, the valuable factors in univariate analysis were tumor invasion (50 mo for T1-3 tumors vs. 16 mo for T4 tumors; *P*=0.006), LN metastasis (14 mo for LN-positive tumor vs. 42 mo for LN-negative tumors; *P*= 0.018), surgical types (46 mo for R0 vs. 14 mo for R1-2; *P*=0.002), tissue differentiation (23 mo for well/moderate differentiated tumors vs. 14 mo for poor/un-differentiated tumors; *P*=0.056), pAMPKa (pAMPKa negative vs pAMPKa positive: 16 months vs 40months; P= 0.026), ADM2(ADM2 negative vs ADM2 positive: 46 months vs 14 months; P<0.001) and VEGF (VEGF negative vs VEGF positive: 48 months vs 16 months; P=0.006) (**Figure 3D-F**). Multivariate analysis showed that tumor invasion was an independent prognostic factor (**Table 3**).

Discussion

In present study, we firstly confirmed that metformin treatment could lead to increased expression of pAMPKa and reduction of ADM2 and VEGF, highlighting the mechanism underlying metformin inhibiting cell growth. We then explored the predictive and prognostic value of pAMPKa, ADM2, and VEGF by immunohistochemistry and tissue microarray. Our data showed inactivation of pAMPKa and activation of ADM2 and VEGF in human hilar cholangiocarcinoma. Moreover, pAMPKa positive expression was associated with better survival, while ADM2 and VEGF positive expression was associated with poor survival for patients with HCC.

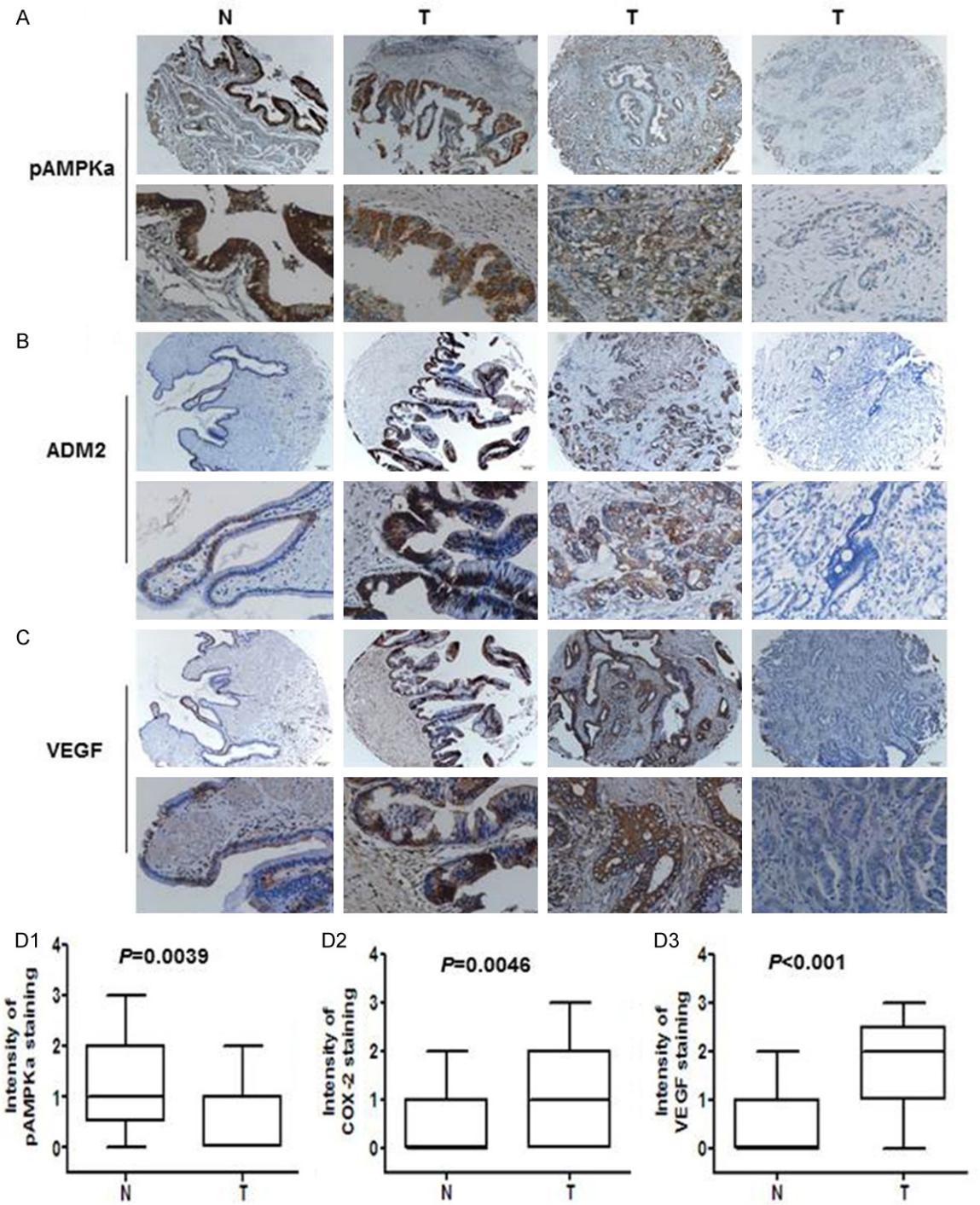

Figure 2. Analysis of pAMPKa, ADM2, and VEGF expression in human hilar cholangiocarcinoma and non-cancerous bile duct tissues. (A-C) Positive expression of pAMPKa (A), negative expression of ADM2 (B) and VEGF (C) in non-cancerous tissues (N) (left panel), positive expression of pAMPKa (A), ADM2 (B), and VEGF (C) in cancer tissues (T) (middle panels), and negative expression of pAMPKa (A), ADM2 (B), and VEGF (C) in cancer tissues (right panel). Original magnification of upper panel of (A-C, 40×); Original magnification of lower panel of (A-C, 200×). *(D1-3) Graphical* representation of the differences of pAMPKa (D1), ADM2 (D2), and VEGF (D3) staining in nonneoplastic (N) and cancer tissues (T).

Specifically, pAMPKa was identified as an independent predictor for tumor recurrence.

Emerging experimental data supported the anti-cancer effects of metformin and clinical

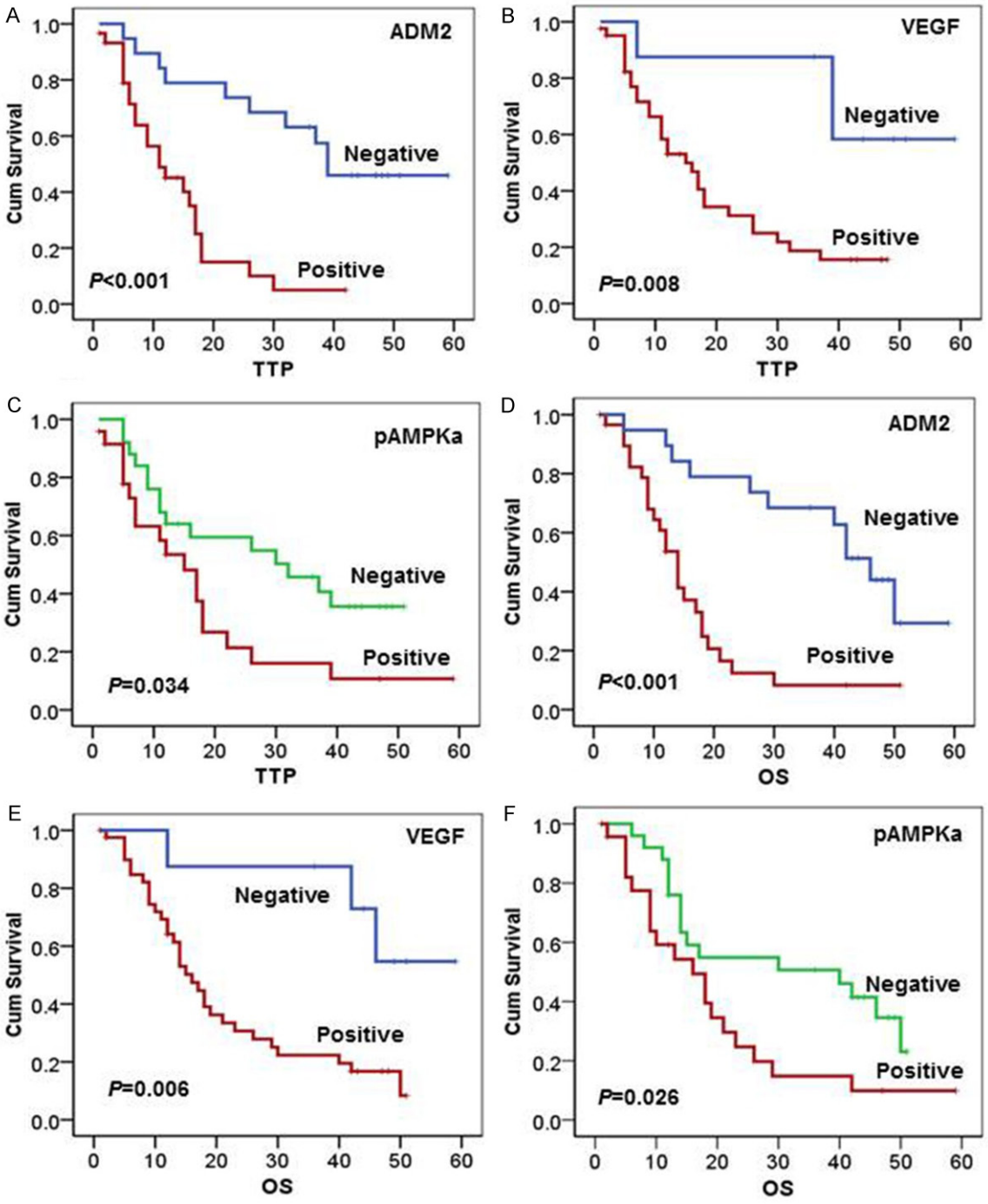

Figure 3. Kaplan-Meier curves of time to recurrence and overall survival durations in patients with hilar cholangiocarcinoma according to pAMPKa, ADM2, and VEGF expression. A. Patients with pAMPKa overexpression had a longer time to recur than those without pAMPKa expression (32 months vs 15 months; *P*=0.034); B. Patients with ADM2 overexpression had a shorter time to recur than those without ADM2 expression (11 months vs 39 months; *P*<0.001); C. Patients with VEGF overexpression had a shorter time to recur than those without VEGF expression (15 months vs 46 months; *P*=0.008). D. Survival durations were significantly better in patients with high expression of pAMPKa (median survival, 40 mo) than in those with low expression of pAMPKa (median survival, 16 mo; *P*=0.001). E. Survival durations were significantly worse in patients with high expression of ADM2 (median survival, 14 mo) than in those with low expression of ADM2 (median survival, 46 mo; *P*=0.006). F. Survival durations were significantly better in patients with low expression of VEGF (median survival, 16 mo) than in those with low expression of VEGF (median survival, 48 mo; *P*<0.001).

Table 2. Univariate and Multivariate Analysis of Variables Associated With TTP in HC Patients

Variable	No.	TTP (months)	Range	*P* (univariate)	*P* (multivariate)	HR	95% CI
Tumor stage							
T1-3	7	52.2		0.002	0.022	0.078	0.009-0.889
T4	42	16.0					
Regional lymph nodes positive							
No	16	39.0		0.023	0.160	0.526	0.215-1.288
Yes	33	12.0					
R							
R0	19	39		0.004	0.676	1.206	0.501-2.899
R1-2	30	11					
VEGF							
Negative	8	46		0.008	0.088	0.266	0.058-1.216
Positive	41	15					
ADM2							
Negative	19	39		<0.001	0.285	0.594	0.228-1.543
Positive	30	11					
pAMPKa							
Negative	24	15		0.034	0.047	2.161	1.009-4.626
Positive	25	32					

Table 3. Univariate and Multivariate Analysis of Variables Associated With OS in HC Patients

Variable	No.	TTP (months)	Range	*P* (univariate)	*P* (multivariate)	HR	95% CI
T stage							
T1-3	7	50	13-59	0.006	0.045	0.184	0.035-0.962
T4	42	16	2-51				
Regional lymph nodes positive							
No	16	42	2-59	0.018	0.461	0.710	0.286-1.763
Yes	33	14	1-51				
Differentiation							
High/moderate	37	23	1-59	0.056	0.336	1.243	0.798-1.936
Low/undifferentiated	12	14	2-50				
R							
R0	19	46	1-59	0.002	0.955	1.028	0.295-2.675
III/IV	30	14	2-51				
VEGF							
Negative	8	48	12-59	0.006	0.111	0.301	0.069-1.317
Positive	41	16	1-51				
ADM2							
Negative	19	46	5-59	<0.001	0.402	0.674	0.268-1.695
Positive	30	14	1-51				
pAMPKa							
Negative	24	16	1-59	0.026	0.083	1.990	0.914-4.331
Positive	25	40	6-51				

studies were trying to establish its use a synergistic therapy. Undoubtedly, metformin exhibited a dose-dependent anti-proliferation effect on HCC cells. AMP-activated protein kinase (AMPK) can be activated by metformin and the phosphorylation and activation of AMPK results in decreased cancer cell proliferation. Loss of AMPK expression is correlated with aggressive

clinicopathologic features and is associated with poor survival in multiple human cancers, such as melanoma [14], gastric cancer [15], and hepatocellular carcinoma [16]. Another study discovered that metformin sensitized intrahepatic cholangiocarcinoma cells to certain chemotherapeutic agents, such as sorafenib, 5-fluorouracil and As2O3 by targeting the AMPK pathway [6]. These data indicate a crucial role of activation of AMPK in inhibition of tumor growth. In the present study, we showed that pAMPKa was decreased in HCCA compared with non-cancerous tissues. Moreover, patients with pAMPKa expression had longer time to recur and better survival than those without pAMPKa expression. Multivariate analysis revealed that pAMPKa was an independent predictor for tumor recurrence and there is a trend that pAMPKa was an independent prognostic factor for HCCA patients. Together, pAMPKa could be a useful marker to guide clinical outcome in patients with hilar cholangiocarcinoma.

Tumor angiogenesis is one of the hallmarks of tumor growth and metastasis. Targeting tumor angiogenic factors will provide analternativestrategy for cancer treatment. Adrenomedullin-2 (ADM2) is a novel tumor angiogenesis factor and was suggested to play a role in angiogenesis and cancer. ADM2 is highly expressed in various cancer cell lines and blockade of ADM2 impaired blood supply and eventually inhibited tumor growth *in vivo* [7]. Previous studies have shown that metformin could inhibit angiogenesis through AMPK pathway in various tumors [17]. These results indicated the potential role of AMPK-ADM2-VEGF signaling in tumor growth. ADM2 was overexpressed in pancreatic carcinoma, hepatocellular carcinoma, and colorectal adenocarcinoma [18-20]. Moreover, high levels of ADM2 expression predict a poorer survival in patients with pancreatic adenocarcinoma. Whether ADM2 is apotential prognostic marker needs further studies. Our results indicated that ADM2 expression in HCCA was significantly higher than that in the non-cancerous tissues. There was a significant association between ADM2 overexprssion and tumor invasion and regional lymph node metastasis. Most importantly, patients with ADM2 overexpression were more easily to recur and had a poor outcome than those without ADM2 overexpression. Similarly, VEGF was upregulated in more than half of patients with cholangiocarcinoma, but unlikely to be an independent prognostic marker [10, 21]. Our results confirmed upregulation of VEGF in HCCA compared that in the non-cancerous tissues and VEGF overexpression with regional lymph node metastasis. Additionally, patients with VEGF overexpression had a poor outcome than those withoutVEGF overexpression. However, in this small cohort, ADM2 and VEGF was not independent valuable biomarker for HCCA patients. Definitely, further studies will be required to evaluate the predictive and prognostic value of ADM2 and VEGF in HCCA.

In summary, we reported that decreased expression AMPKa and increased expression of ADM2 and VEGF in HCCA were associated with future tumor recurrence and the poor overall survival rates of HCCA patients. Furthermore, multivariate analysis indicated that loss of pAMPKa might be regarded as an independent predictive factor for tumor recurrence and a promising prognostic factor for poor prognosis in HCCA patients.

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Disclosure of conflict of interest

None.

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