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Expression of Phosphorylated AMP-Activated Protein Kinase Predicts Response to Transarterial Chemoembolization in Postoperative Cases of Hepatocellular Carcinoma

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Abstract: Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies in the world. Transcatheter arterial chemoembolization (TACE) was commonly used for HCC patients postoperatively. However, the survival benefits of adjuvant TACE were controversial due to the extensive heterogeneity of HCC. Hence, there is a critical need to explore potential biomarkers that can predict the clinical response to TACE. The AMP-activated protein kinase (AMPK) is a highly conserved heterotrimeric serine/threonine kinase that plays a central role in linking metabolism and cancer development. In this study, we aimed at evaluating the association of pAMPKα (Thr172) status with clinical outcomes in HCC patients treated with or without postoperative adjuvant TACE.

pAMPKα (Thr172) expression was assessed using immunohistochemical analysis in a cohort of 378 Chinese HCC patients who had undergone tumor resection. Kaplan–Meier analysis and multivariate Cox proportional hazards models were used to study the impact on clinical outcomes.

High pAMPKα (Thr172) expression was associated with improved disease-free and overall survival and was an independent prognostic factor for overall survival by multivariate analysis. Furthermore, low pAMPKα (Thr172) expression level was correlated with high percentage of $OV6^+$ tumor-initiating cells (T-ICs) in HCC specimens.

To our knowledge, it can be demonstrated for the first time that pAMPKα (Thr172) status is associated with response to postoperative adjuvant TACE. High pAMPKα (Thr172) level in HCC may serve as a positive predictor of survival in HCC patients undergoing TACE.

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Abbreviations: AMPK = AMP-activated protein kinase, CK19 = cytokeratin 19, HCC = hepatocellular carcinoma, TACE = transcatheter arterial chemoembolization, T-ICs = tumor-initiating cells.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common solid cancer in the world and the third leading cause of cancer-related mortality globally, with an annual incidence of approximately 780,000 cases per year worldwide.[1] Although the short-term survival of HCC patients has been much improved due to recent advances in diagnosis and treatment, partial hepatectomy and liver transplantation remain the main curative treatments for HCC.[2] Even after radical excision, the long-term prognosis of HCC remains dismal, largely due to high frequency of recurrence. The overall recurrence rate was 50% to 80% in 5 years after resection of HCC.[3]

Transcatheter arterial chemoembolization (TACE) was one of the most commonly used postoperative adjuvant therapies for preventing recurrence and prolonging the survival of HCC patients.[3] However, due to the extensive heterogeneity of HCC, the survival benefits of adjuvant TACE were controversial in different groups. Therefore, it is important to explore potential biomarkers which identify individuals most likely to benefit from TACE.

The AMP-activated protein kinase (AMPK) is a highly conserved heterotrimeric serine/threonine kinase. As an energy sensor in all eukaryotic cells, AMPK plays a central role in linking metabolism and cancer development.[4,5] AMPK is consisted of a catalytic α subunit and regulatory β and γ subunits, and is activated by an increase in the cellular AMP/ATP ratio, adiponectin, leptin, and the antidiabetic drug metformin. Growing evidence suggested that AMPK has critical tumor suppressor activities in both in vitro experimental models and in vivo mice model in various types of cancers.[6,7] Altered levels of AMPK have been linked with many human diseases including cancer.[8–11] In our previous study, we revealed that AMPK is dysfunctional in HCC patients, and AMPK activity is inversely correlated with clinicopathologic features and prognosis.[11] However, whether the phosphorylation status of AMPK can predict the response to postoperative adjuvant TACE in HCC patients is unknown.

Accumulating evidence has shown that liver tumor-initiating cells (T-ICs) or cancer stem cells (CSC) contribute to HCC initiation, progression and chemoresistance.[12] Previous studies have identified $OV6^+$ HCC cells as liver T-ICs. $OV6^+$ liver T-ICs exhibited higher tumorigenicity and chemoresistance ability than the corresponding marker negative cells in HCC cell lines and HCC specimens.[13–15] Therefore, accumulation of liver T-ICs might be associated with a poor response to TACE.

To address this issue, in this study, we investigated the association of pAMPKα (Thr172) status with survival in a Chinese cohort of 378 HCC patients treated with or without postoperative adjuvant TACE.

METHODS

Patients

A total of 378 HCC patients were recruited at the Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai, China, from January 2003 to December 2006, with the inclusion criteria: preoperative World Health Organization performance status of 0–1; Child–Pugh class A; no distant metastases, visualizable ascites, or encephalopathy; no chemotherapy or radiotherapy before surgery; no metformin prescriptions; curative resection (The Chinese University Prognostic Index (CUPI)[16] and Japan Integrated Staging (JIS)[17] guidelines were referenced to guide patient management); and the diagnosis of HCC was confirmed by pathological results. The study was approved by the institutional ethics committee. Informed consent was obtained from each participant before surgery.

Transarterial Chemoembolization Procedure

Patients with risk factors (multiple tumors, tumor size exceeding 5 cm, vascular invasion, or incomplete tumor encapsulation) were selected for postoperative adjuvant TACE.[18–20] Adjuvant TACE treatment was performed 1 to 2 months after hepatectomy. The treatment regimen was comprised of an emulsion of 50 mg epirubicin (Pharmorubicin; Pfizer, Wuxi, China) and 5 ml of lipiodol (Lipiodol Ultra-Fluide; Guerbet Laboratories, Aulnay-Sous-Bois, France).

Follow-Up

Patients were observed once every 2 months in the first 2 years after surgery and then every 3 to 6 months thereafter. Overall survival (OS) was defined as the interval between partial hepatectomy and death or the last date of follow-up. Disease-free survival (DFS) was defined as the dates of surgery and first recurrence or the last follow-up if recurrence was not diagnosed.

Immunohistochemical Evaluation

Tissues were cut into 5-μm-thick sections and the following primary antibodies were used: rabbit antiphospho-AMPK (Thr172) (Cell Signaling Technology, USA), mouse anti-OV6 (R&D, USA). The slides were incubated with primary antibodies overnight at 4°C, and detection was performed using Vector ABC kit (Vector Laboratories, CA) and DAB reagent (Dako Comp, Japan). All the slides were observed and photographed under an Olympus microscope (IX-70 OLYMPUS, Tokyo, Japan). All sections were then evaluated by 2 independent observers who were blind to the clinical and pathological data of the tumors. As described previously,[21] the German immunoreactive score (IRS) was scored semi-quantitatively for both the staining intensity and the proportion of positive cells. The intensity was grouped into score of 0–4 points (0: no staining (no color reaction), 1: weak staining (mild reaction), 2: moderate staining (moderate reaction), 3: strong staining (intense reaction)). The proportion of positive cells was given scores 1 to 6 (1: 0–5%, 2: 6–20%, 3: 21–40%, 4: 41–60%, 5: 61–80%, and 6: 81–100%). The 2 scores were multiplied to obtain the final immunoreactive score (range 0–18). High expression of p-AMPK in tumor was defined as immunoreactive score ≥4. All 378 patients were subdivided into 2 groups: high p-AMPK expression group (n = 120) and low p-AMPK expression group (n = 258). In addition, samples with OV6 expression on more than 30% of the cancer cells were recorded as having high percentage of $OV6^+$ cells.

Statistical Analysis

Survival analysis was performed using the Kaplan–Meier method and compared using a log-rank test. χ^2 test was applied to determine statistical significance. A value of $P < 0.05$ was considered significant. Data analysis was performed with the SPSS software (version 16; SPSS, USA).

RESULTS

Clinical and Pathological Characteristics of Study Participants

Of the 378 patients with HCC included in this study, 327 were male (86.5%) and 51 were female (13.5%). The majority of patients were long-term carriers of hepatitis B virus (HBV) (90.7%, n = 343). The follow-up time of the cohort ranged from 1 month to 90 months, and the mean follow-up time was 37 months. In addition, 168 patients received adjuvant TACE (adjuvant TACE group) and 210 patients received no adjuvant therapy (control group). The 2 groups were comparable in demographic data and tumor characteristics (Table 1). There were no significant differences in their age, HBsAg positive rate, alpha-fetal protein (AFP) level, maximal tumor size, tumor multiplicity, tumor encapsulation, Edmondson grade, TNM stage between 2 groups. Moreover, as shown in Figure 1, adjuvant TACE treatment group exhibited a better survival outcome.

p-AMPK Expression in the Tumor Was Associated With the Treatment Response to Adjuvant TACE

To evaluate whether p-AMPK level was associated with the treatment response to adjuvant TACE, we first detected p-AMPK expression in 378 HCC specimens by immunohistochemical analysis. Based on the p-AMPK immunohistochemical staining result (Figure 2A), all patients were subdivided into 2 groups: high p-AMPK expression group (n = 120) and low p-AMPK expression group (n = 258). As shown in Figure 2B, patients with low p-AMPK expression in tumors had no significant improvement in disease-free survival rate (mean DFS for adjuvant TACE versus control: 31.3 (±2.9) vs 23.9 (±2.2) months, $P = 0.067$) and slight improvement in overall survival (mean OS for adjuvant TACE vs control: 42.9 (±2.9) versus 33.8 (±2.3), months, $P = 0.041$) after receiving postoperative adjuvant TACE, as compared with those without adjuvant TACE. In contrast, adjuvant TACE significantly improved the disease-free survival (mean DFS for adjuvant TACE vs control: 56.7 (±3.6) vs 42.2 (±4.1) months, $P = 0.027$) and overall survival (mean OS for adjuvant TACE versus control: 80.7 (±3.0) vs 50.7 (±3.8), months, $P = 9.71 \times 10^{-5}$) of patients with high p-AMPK expression in tumors (Figure 2C). In addition, patients with high p-AMPK expression had a better survival than those presenting low p-AMPK expressions in both adjuvant TACE group (mean DFS for high-p-AMPK vs low-p-AMPK: 56.7 (±3.6) vs 31.3 (±2.9) months, $P < 0.00001$; mean OS for high-p-AMPK versus low-p-AMPK: 80.7 (±3.0) vs 42.9 (±2.9), months, $P < 0.000001$) and control group (mean DFS for high-p-AMPK vs low-p-AMPK: 42.8 (±4.1) vs 23.9 (±2.2) months,

TABLE 1. Clinical and Pathological Characteristics of Study Participants

	Control (n = 210)	Adjuvant TACE (n = 168)	*P*
Age			0.745
<50	89	74	
≥50	121	94	
Gender			0.614
Male	180	147	
Female	30	21	
HBV infection			0.361
Yes	188	155	
No	22	13	
AFP (ng/ml)			0.854
≥200	112	88	
<200	98	80	
Maximal tumor size (cm)			0.811
<5	75	62	
≥5	135	106	
Tumor multiplicity			0.568
Single	165	136	
Multiple	45	32	
Tumor encapsulation			0.129
Incomplete	130	91	
Complete	80	77	
Edmondson grade			0.054
I/II	25	32	
III/IV	185	136	
Pathologic TNM stage			0.813
Early stage (I–II)	130	102	
Late stage (III)	80	66	

AFP = alpha-fetal protein, HBV = hepatitis B virus, TACE = transcatheter arterial chemoembolization.

$P = 0.0003$; mean OS for high-p-AMPK vs low-p-AMPK: 51.4 (±3.7) vs 33.8 (±2.3), months, $P = 0.001$) (Figure 2D).

Univariate and Multivariate Analysis of Prognostic Factors Affecting Overall Survival

Furthermore, univariate and multivariate survival analysis were performed to identify the prognostic factors for overall survival in patients with adjuvant TACE treatment. As shown in Table 2, absent tumor encapsulation, late TNM stage, and low levels of p-AMPK were associated with the shorter survival of HCC patients. Notably, multivariate analysis revealed that low levels of p-AMPK, along with absent tumor encapsulation, late TNM stage, were also independent risk predictors for the poor outcome of HCC. Taken together, these data suggested that p-AMPK expression could serve as a valuable predicting factor for recurrence and poor survival of HCC patients.

p-AMPK Expression Level Was Negatively Associated With Percentage of $OV6^+$ T-ICs in HCC Specimens

Moreover, we determined the frequency of $OV6^+$ liver T-ICs by IHC in 168 HCC patients receiving adjuvant TACE treatment. The pattern of staining for OV6 was variable, some were semi-quantitatively as low as 0% to <30% positive, others as high as ≥30% in HCC cells. Representative staining of HCC specimens are shown in Figure 3A. Interestingly, as shown in Figure 3B, patients with low AMPK expression had much more $OV6^+$ cells in their tumor tissues, suggesting that AMPK inactivation led to expansion of tumorigenic HPCs, and thus contributed to the poor response to transarterial chemoembolization in HCC.

DISCUSSION

Identification of potential biomarkers that predicting the clinical response to TACE is pivotal to improve the therapeutic effect of TACE. In this retrospective cohort study, we found that high pAMPKα (Thr172) expression is associated with improved response to postoperative adjuvant TACE and pAMPKα (Thr172) status may serve as a positive predictor of survival in HCC patients undergoing TACE.

As the metabolic master switch to regulate cellular and whole-body energy homeostasis, AMPK has also been implicated in regulating cancer cell growth, invasion.[7] Multiple lines of evidence suggest AMPK is a critical metabolic tumor suppressor in both humans and animal experimental models.[22] Great efforts have been made to clarify the mechanisms for downregulating AMPK in cancer. LKB1/STK11 was shown to activate AMPK by phosphorylating Thr172 residue of the catalytic α subunit. Mutation or deletion of LKB1 is one mechanism of reducing AMPK activity in cancer. Indeed, LKB1 expression was decreased in the HCC samples and HCC patients with decreased LKB1 expression have a poor prognosis.[23] Recently, melanoma antigen (MAGE)-A3/6-

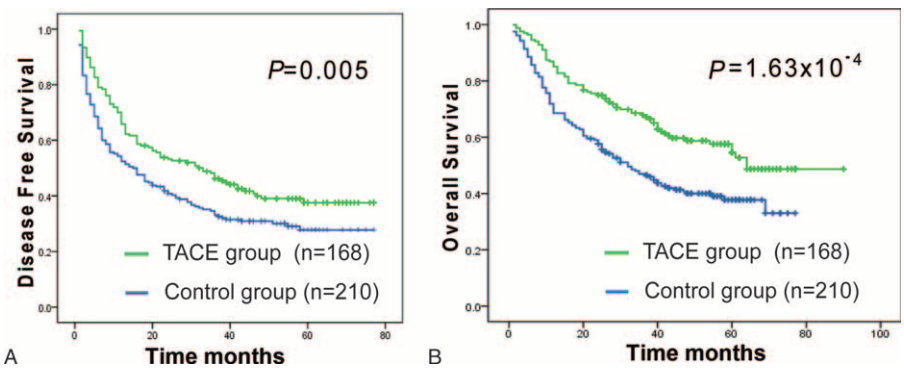


FIGURE 1. Survival curves of patients in control group and adjuvant TACE group. The disease-free survival (DFS) and overall survival (OS) of 378 HCC patients were compared between the control group and adjuvant TACE group.

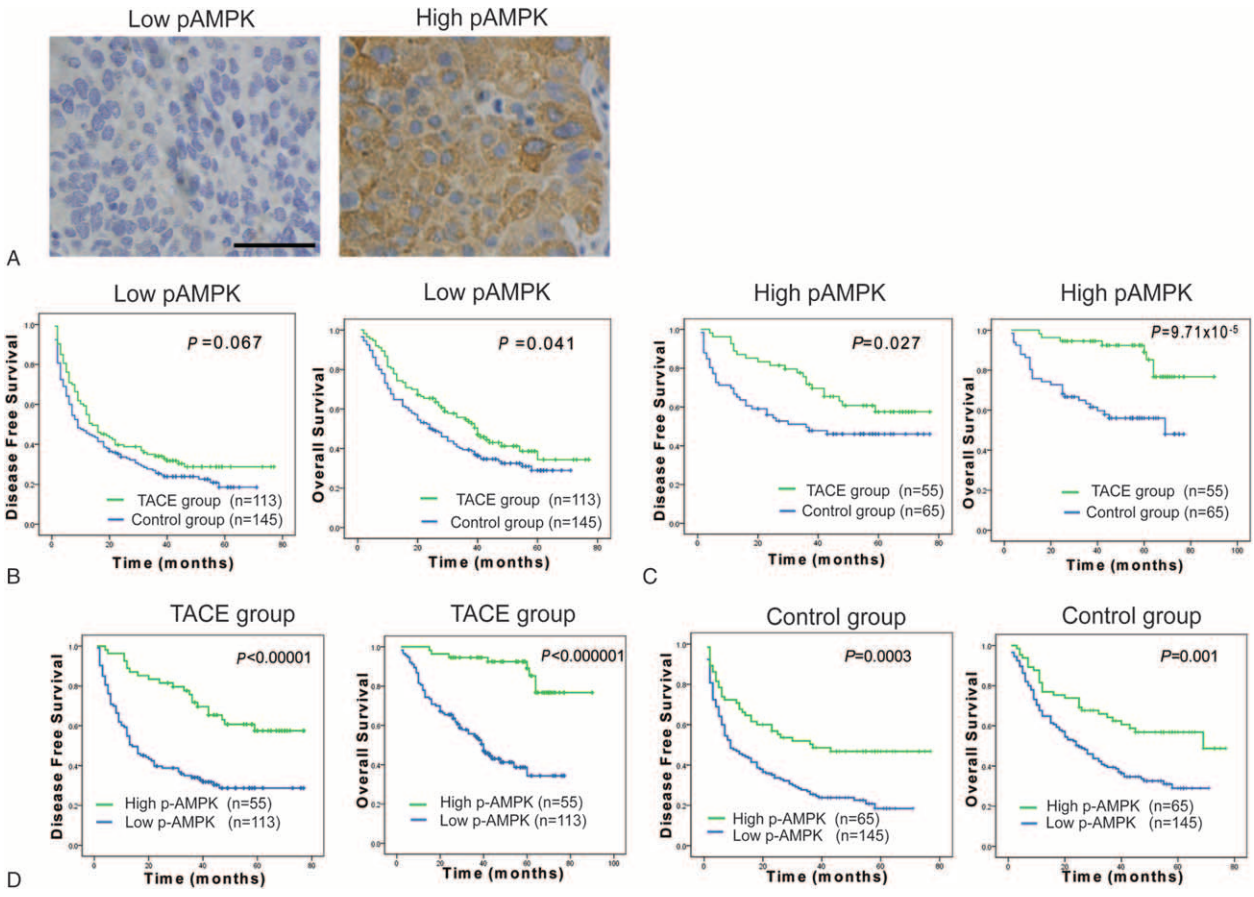
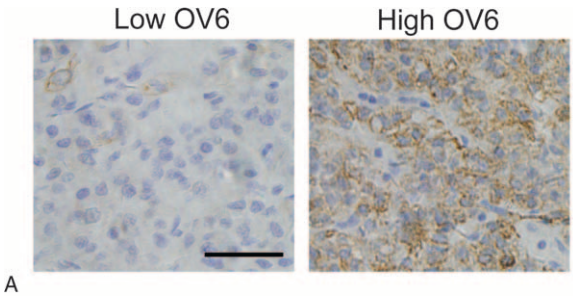


FIGURE 2. p-AMPK expression in the tumor was associated with the response of patients to adjuvant TACE therapy. Representative immunohistochemical staining of p-AMPK in HCC specimens (scale bar = 50 μm). The disease-free survival (DFS) and overall survival (OS) rates of 258 patients with low p-AMPK expression were compared between 2 groups. The disease-free survival (DFS) and overall survival (OS) rates of 120 patients with high p-AMPK expression were compared between 2 groups. The disease-free survival (DFS) and overall survival (OS) of HCC patients received adjuvant TACE treatment (TACE group, n = 168) or no adjuvant therapy (control group, n = 210) were compared between the low and high p-AMPK groups.

TABLE 2. Univariate and Multivariate Cox Regression Analysis of Risk Factors for Shorter Overall Survival in Patients With Adjuvant TACE Treatment

	Univariate Analysis		Multivariable Analysis	
Variable	**Hazard Ratio (95% CI)**	***P***	**Hazard Ratio (95% CI)**	***P***
Age (≥ 50 vs. <50)	0.798 (0.496–1.809)	0.866		
Gender (male vs. female)	0.866 (0.415–1.280)	0.410		
HBV infection (absent vs. present)	1.115 (0.56–1.674)	0.800		
AFP (ng/ml) (<200 vs. ≥200)	1.527 (0.949–2.459)	0.081		
Tumor multiplicity (single vs. multiple)	1.138 (0.644–2.013)	0.656		
Maximal tumor size (cm) (<5 vs. ≥5)	1.562 (0.943–2.587)	0.083		
Tumor encapsulation (present vs. absent)	2.248 (1.359–3.720)	0.0016	2.132 (1.282–3.546)	0.0035
Edmondson grade (I/II vs. III/IV)	1.412 (0.723–2.759)	0.313		
Pathologic TNM stage (I–II vs. III)	2.043 (1.281–3.257)	0.0027	2.007 (1.253–3.214)	0.0037
pAMPK level (low vs. high)	0.512 (0.352–0.745)	<0.0001	0.135 (0.063–0.29)	<0.0001

AFP = alpha-fetal protein, AMPK = AMP-activated protein kinase, HBV = hepatitis B virus, TACE = transcatheter arterial chemoembolization.



A

	Low pAMPK (No. of Cases)	High pAMPK (No. of Cases)	*P* value
Percentage of OV6+ tumor cells			
<30%	40	33	0.00254
≥30%	73	22	

B

FIGURE 3. Low p-AMPK expression was correlated with expansion of OV6^{+} tumor-initiating cells in HCC tissues. Representative immunohistochemical staining of OV6 in HCC specimens (scale bar = 50 μm). Summary of the expression of p-AMPK, OV6 in human HCC specimens.

TRIM28 axis was also reported to ubiquitinate and degrade the AMPKα1.[8] MAGE-A3/6 proteins are testis-specific E3 ubiquitin ligase components whose expression is highly elevated in many cancers, including HCC. Therefore, overexpression of MAGE-A3/6 may also contribute to reduced AMPK activity in HCC. Our previous study revealed that low p-AMPK staining is correlated with aggressive clinicopathologic features and poor prognosis in HCC patients.[11] In this study, our analyses further revealed that pAMPKα (Thr172) status may serve as a candidate predictive biomarker of HCC response to TACE.

Many molecular mechanisms are reported to determine the sensitivity of liver tumors to therapy with postoperative TACE. Accumulating evidence suggests that many solid tumors and hematological malignancies developed from a small fraction of cancer cells, termed T-ICs. These T-ICs possess many characteristics associated with normal stem cells, and are responsible for tumor initiation, relapse, metastasis, and chemoresistance. Similarly, recent studies also highlight the importance of T-ICs in HCC. CD133,[24,25] EpCAM,[13] CD13,[26] CD24,[27] and OV6[15] are useful for isolating liver T-ICs with higher tumorigenicity, self-renewal ability, and resistance to conventional chemotherapies.[28] We showed here that, in low p-AMPK expressing HCCs, percentage of patients with high OV6 staining was 63.4%, higher than that in high p-AMPK expressing group. Thus, our results shed new light on the role of AMPK in regulating expansion of liver T-ICs. Indeed, AMPK activation negatively regulates mTOR, IL6-STAT3, NF-κB signaling pathways that play vital roles in regulating normal hepatic progenitors and liver T-ICs.[11,12,29] So it may not be surprising to find that low p-AMPK expression level was correlated with expansion of T-ICs in HCC specimens. These results suggested that the loss of inhibitory effect of AMPK may contribute to expansion of liver T-ICs and resistance to TACE therapy. In addition, expression of cytokeratin 19 (CK19), another hepatic progenitor cell marker, in HCC, has been reported to be associated with more aggressive tumor phenotype and rapid disease spread.[30] In colon and intestine cancer models, CK19 (+) cancer-initiating cells were also shown to be relatively resistant to radiation.[31] Therefore, whether low p-AMPK expression correlated with high CK19 expression in HCC needs to be further explored.

AMPK is a promising target for cancer prevention and treatment.[6] Modulation of AMPK activity can exert antitumor effects both in vitro and in vivo. Exercise, cannabinoids, aspirin, or AMPK activating compounds, such as metformin, phenformin, AICAR, caused AMPK activation and inhibited tumors progression in different animal cancer models.[7,32] Consistent with observation in clinical study, metformin has been reported to improve the chemotherapy response of breast cancer, colon cancer, in an AMPK-dependent manner.[33–35] Previous studies also revealed that metformin increased chemosensitivity of HCC cells in cultured cell lines and animal model.[11,36,37] Current result provide further evidence that AMPK level may determine the response to chemotherapy, and metformin might be considered as a valuable modulator for HCC chemotherapy.

In conclusion, the key contribution of our study is to identify p-AMPK as a valuable prognostic biomarker in predicting the outcome of HCC patients with TACE treatment. High p-AMPK expression can serve as a positive predictive value of survival in HCC patients undergoing TACE. Although further clinical trials are required to evaluate safety and efficacy, metformin may be a clinically effective TACE sensitizer for patients with low p-AMPK in tumors.

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