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Original Article

High expression of AMPD2 and obesity are associated with poor prognosis in colorectal cancer

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Abstract: The protein-coding gene adenosine monophosphate deaminase (AMPD) 2 plays a critical role in energy metabolism by converting adenosine-5-monophosphate (AMP) to iosine inosine-5-monophosphate (IMP). Obesity affects metabolic abnormalities in tumor cells and has been associated with high expression levels of AMPD2 and colorectal cancer (CRC). In this study, we performed immunohistochemical analysis of AMPD2 expression in 158 patients with CRC. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to determine AMPD2 mRNA expression levels, which were validated by The Cancer Genome Atlas (TCGA) datasets. Chi-square test and Fisher's exact test were used to evaluate the correlation between the expression of AMPD2 and clinicopathological parameters of CRC. Overall survival (OS) rates of the CRC patients were calculated using Kaplan-Meier survival analysis and a Cox proportional regression model was performed for univariate and multivariate analysis. A logistic regression model was used to plot the receiver operating characteristic (ROC) curve and to evaluate the predictive effect of multivariate studies on prognosis outcomes of CRC. We found a significant increase in AMPD2 expression in tumor tissue (91.8%, 146/158) compared to adjacent normal tissue (52.5%, 83/158, $P < 0.01$). The positive rate of AMPD2 expression was 72.7% (39/54) in overweight individuals versus 51.9% (54/104) in individuals with a normal weight ($P = 0.014$). AMPD2 mRNA levels as determined by qRT-PCR elevated levels of AMPD2 transcripts were higher in CRC samples compared to adjacent normal tissues ($P < 0.05$). In both the TCGA colon adenocarcinoma and rectal adenocarcinoma dataset, the number of CRC patients with increased levels of AMPD2 in tumor tissues was significantly higher compared to patients with adjacent normal tissue ($P < 0.001$). High expression of AMPD2 was associated with TNM stage, higher histological grade, obesity, and lower OS rates in patients with CRC. Obesity and high expression of AMPD2 in patients are with poor prognosis. Moreover, multivariate analysis indicated that AMPD2 levels and TNM stage were significant independent prognostic factors in CRC patients. The logistic regression predictive effect of the area under the curve (AUC) was 0.821 ($P < 0.001$). In conclusion, high levels of AMPD2 and obesity are associated with poor prognosis in patients with CRC.

Keywords: Colorectal cancer, AMPD2, obesity, immunohistochemistry, survival analysis

Introduction

Colorectal cancer (CRC) is the third most common cancer in the world and the second leading cause of death the world [1]. Globally, CRC is the third most commonly diagnosed cancer in males and the second most commonly diagnosed cancer in females [2]. Obesity/overweight has been shown to be an increased risk factor for CRC, however the relationship between obesity/overweight and a patients' survival is not known. Higher levels of obesity are related to poor survival [3, 4]. In a recent study in cancer patients in which parenteral and enteral nutrition were compared, it was demonstrated that nutrition status may play an important role in the mortality of CRC [5]. In addition, recent metabolic studies in tumor cells determined that changes in nutrient metabolic pathways resulted in aerobic glycolysis (Warburg effect), and increased rates in the synthesis of macromolecule [6]. This effect is particularly evident, given that the intestines are an important source of energy absorption and metabolic organs of the human body. CRC studies have shown that molecular markers of energy metab-

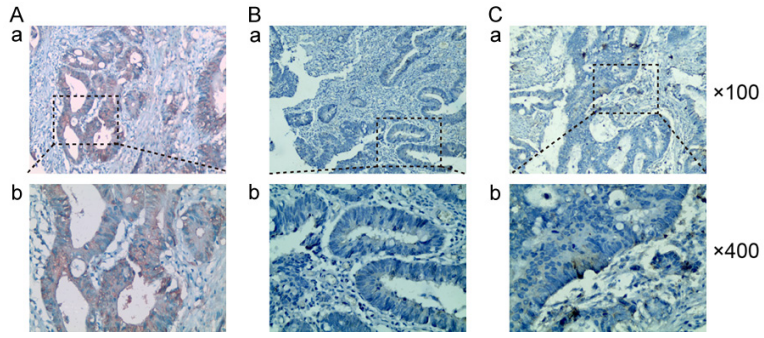

Figure 1. Representative Immunohistochemical results of AMPD2 levels in colorectal cancer patients. Tissue of tumor specimens (Aa, Ab) from the same patient as adjacent normal tissue (Ba, Bb). (A, Ba) Are typical representative images, which showed that AMPD2 expression is elevated in the tumor tissue (T) compared to adjacent normal mucosa (N). (Ca, Cb) In 12 patients, AMPD2 expression could not be observed. The photomicrographs were at 100× and 400× magnification.

olism, such as AMP, appear to be significantly downregulated [7].

The protein-coding gene, adenosine monophosphate deaminase (AMPD), has been shown to play an important role in energy metabolism [8]. AMPD catalyzes the deamination of adenosine-5-monophosphate (AMP) to inosine-5-monophosphate (IMP) and plays a critical role in the purine nucleotide cycle. High levels of tissue AMP-activated protein kinase (AMPK) may be used as a potential prognostic biomarker in this cohort of metastatic colorectal cancer patients [9]. High levels of the AMPD gene allow for increased conversion of AMP to IMP, resulting in down-regulation of AMP. These findings reflect the potential for a correlation between AMPD levels and cancer survival and suggest the presence of high-risk subgroups with upregulated levels of AMPD [10].

In humans, three AMPD are present, including AMPD1 and AMPD3, which are predominant in skeletal muscle and erythrocytes, whereas AMPD2 is the most predominant form in smooth muscle tissue, non-muscle tissue, embryonic muscle tissue, and undifferentiated myoblasts [11]. Colorectal tissue muscles are primarily composed of smooth muscle tissue [12]. Furthermore, to control skeletal muscle confounds the relationship of obesity to CRC survival. AMPD2 expression combined with body mass index (BMI) can verify these phenomena [13]. However, in CRC, the role of AMPD2 in tumor proliferation and invasion remains unclear.

In this study, we investigated the relation between levels of AMPD2, obesity, and clinicopathological parameters of CRC prognosis. In addition, we assessed the prognostic value of high AMPD2 levels and obesity in patients with CRC.

Materials and methods

Patients and samples

This retrospective study was conducted in a Chinese cohort of 158 patients with CRC who received follow up at the Affiliated Hospital of Jiangnan University (Wuxi, China). Formalin-fixed paraffin-embedded tissue from 158 CRCs specimens were retrieved from the Department of Pathology, Affiliated Hospital of Jiangnan University (Wuxi, China). All 158 patients underwent initial surgical resection for CRC between January 2007 and November 2008, and were followed up by telephone or letters up until April 2017. During follow up, general information was collected, including age, sex, clinicopathological parameters (primary tumor location, TNM stage, and differentiation), and nutrition state such as BMI. To calculate the BMI, weight (kg) and height (cm) were measured [14]. Depending on their BMI, patients were classified as undernourished, normal weight, or overweight as determined by Chinese standards [15].

Immunohistochemistry

The expression level of AMPD2 in CRC samples was determined by a standard immunohistochemistry (IHC) approach (**Figure 1**). Briefly, 4-μm slides from 158 formalin-fixed paraffin-embedded specimens were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. The sections were subjected to antigen retrieval and were immersed in sodium citrate buffer (pH 6.0) and 20 min microwaved. Then, sections were incubated in 3% H_2O_2 to block endogenous peroxidase, followed by incubation with anti-goat serum for 40 min at room temperature to block non-specific binding sites. Subsequently, sections were washed with phosphate buffered saline (PBS) and incubated overnight at 4°C with an anti-AMPD2

Table 1. Correlations between clinicopathological characteristics and AMPD2 expressions

Clinical parameters	AMPD2 expression		OR (*95% CI*)	*P* value
	Low, n (%)	High, n (%)		
Gender				0.107
Male (n = 85)	30 (35.3)	55 (64.7)	0.78 (0.57-1.07)	
Female (n = 73)	35 (47.9)	38 (52.1)	1.00	
Age (years)				0.133
< 60 (n = 69)	33 (47.8)	36 (52.2)	1.31 (0.92-1.87)	
≥ 60 (n = 89)	32 (36.0)	57 (64.0)	1.00	
Tumor location				0.725
Colon (n = 80)	34 (42.5)	46 (57.5)	1.06 (0.78-1.44)	
Rectal (n = 78)	31 (39.7)	47 (60.3)	1.00	
TNM stage				0.044
I II stage (n = 94)	44 (47.8)	48 (52.2)	1.96 (1.02-3.80)	
III IV stage (n = 64)	21 (31.8)	45 (68.2)	1.00	
Histological grade				0.543
G1 (n = 24)	11 (45.8)	13 (54.2)	-	
G2 (n = 70)	31 (44.3)	39 (55.7)	-	
G3 (n = 64)	23 (35.9)	41 (64.1)	-	
Tumor grade				0.372
High (n = 108)	47 (43.5)	61 (56.5)	1.10 (0.89-1.36)	
Low (n = 50)	18 (36.0)	32 (64.0)	1.00	
Body mass index				0.014
Normal weight (n = 104)	50 (48.1)	54 (51.9)	1.33 (1.07-1.65)	
Overweight (n = 54)	15 (27.8)	39 (72.2)	1.00	

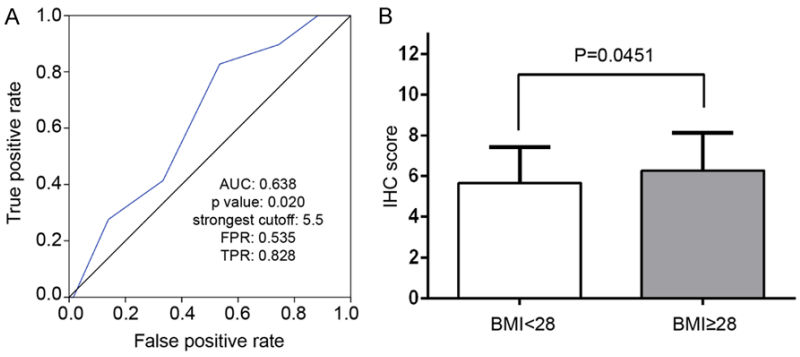

Figure 2. Expression levels of AMPD2 using immunohistochemistry scores may serve as significant prognostic markers in classification of colorectal cancer. A receiver operating characteristic (ROC) curve built on the univariate classification model based on the immunohistochemical scores of AMPD2 expression of 158 patients for predicting the outcome of overall survival (A). The immunohistochemical scores were determined by fractional t-test of obese and non-obese patients (B).

antibody (1:400, Abcam, Hong Kong, China). Sections were washed, incubated with amplification agent and polymerase (reagent A, GTVisionTM III Kit supply, Shanghai, China), stained with 3,3'-diaminobenzidine (DAB, reagent B and C, GTVisionTM III Kit supply, Shanghai, China), and counterstained with hematoxylin for 60 seconds. As negative controls, sections were incubated with PBS only and primary antibodies were omitted.

The staining pattern of each case was scored according to percentage of positively-stained cells in the entire section (0 = no positive staining or ≤ 5%; 1 = 6%-25% positive; 2 = 26%-50% positive; 3 = 51%-75% positive; and 4 = 76%-100% positive). The scoring intensity was as previously described: 0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining [16]. Each case counted four different fields of view under a microscope, and the average of the four field-of-view scores was the final score for the case. The expression was scored by two pathologists who were blinded and unaware of the clinical data. In case of discrepancies, a final score was established by reassessment of the staining using a multi-head microscope.

Quantitative reverse transcription PCR (qRT-PCR)

TRIzol reagent (Invitrogen, San Diego, CA, USA) was used to extract total RNA from fresh tissue of 15 primary CRC tissues and 15 adjacent normal tissues, the distance > 5 cm. cDNA was prepared from 500 ng of total RNA using a cDNA reverse transcription kit (Toyobo, Osaka, Japan). Subsequently, real time PCR was performed using the quantitative SYBR Green PCR kit (TaKaRa Bio, Dalian, China), and according

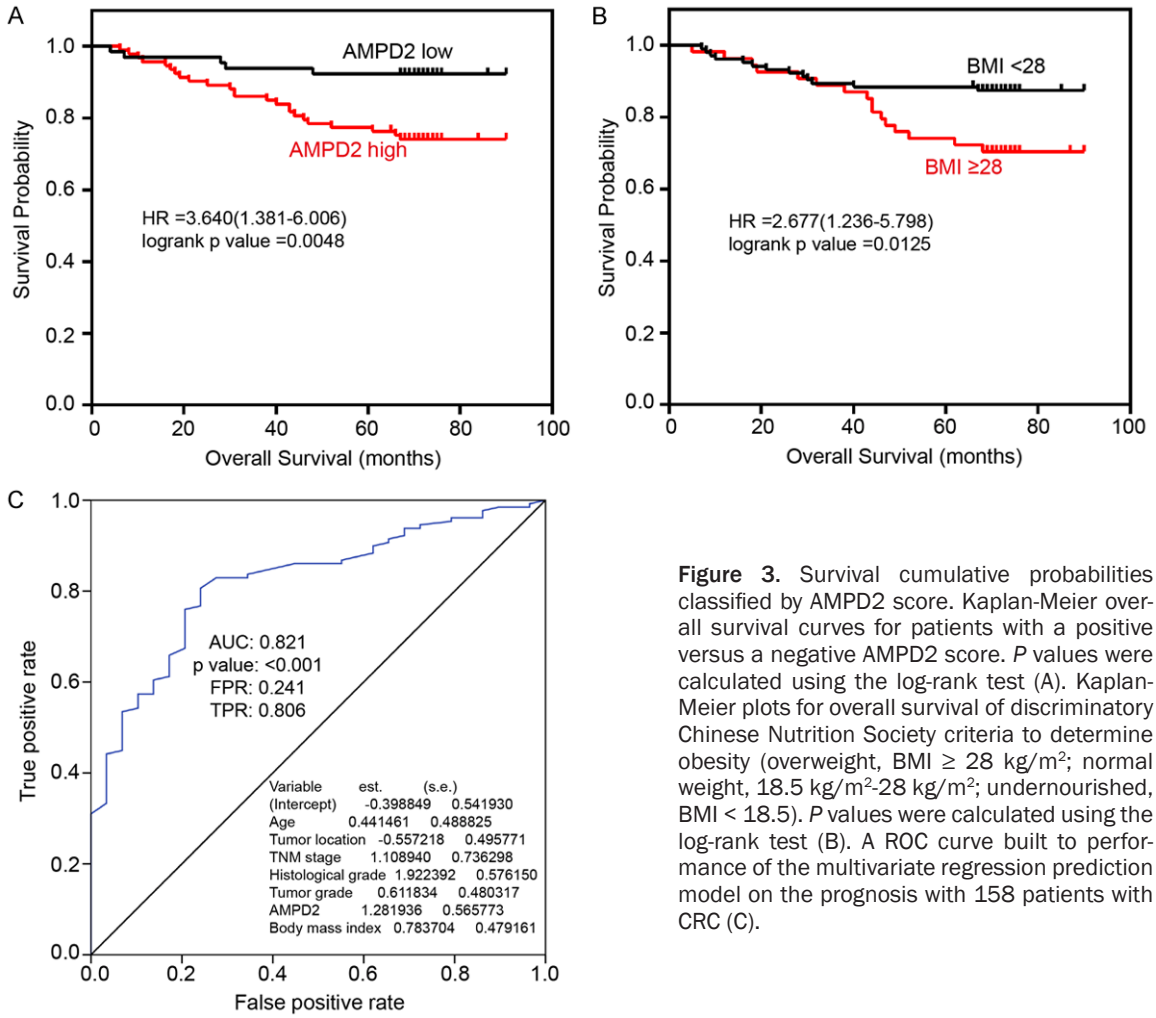

Figure 3. Survival cumulative probabilities classified by AMPD2 score. Kaplan-Meier overall survival curves for patients with a positive versus a negative AMPD2 score. *P* values were calculated using the log-rank test (A). Kaplan-Meier plots for overall survival of discriminatory Chinese Nutrition Society criteria to determine obesity (overweight, BMI ≥ 28 kg/m^2; normal weight, 18.5 kg/m^2-28 kg/m^2; undernourished, BMI < 18.5). *P* values were calculated using the log-rank test (B). A ROC curve built to performance of the multivariate regression prediction model on the prognosis with 158 patients with CRC (C).

to the manufacturer instructions provided with the Bio-Rad sequence detection system. Specific expression of double-stranded DNA was evaluated by the comparative Ct method using 2-ΔΔCt. AMPD2 primers were as follows: 5'-TACAAGGAACAGGGTGAGGG-3' and 5'-ACAGTGCTCATACGGGTGCT-3'. Primers for β-actin, as an internal control, were 5'-CAATGAGCTGCGTGTGGCT-3' and 5'-TAGCACAGCCTGGATAGCAA-3'. Experiments were performed at least in triplicate.

Integrated-signature AMPD2 analysis of the cancer genome atlas

The mRNA expression data integration analysis results were validated in the cancer genome atlas (TCGA) data set (TCGA colon adenocarcinoma and TCGA rectal adenocarcinoma). The mRNA data and clinical data of tumor and adjacent normal tissue of the corresponding patients were downloaded from the TCGA Data portal and expression analysis was performed using BRB arrayTools (version 4.3.2, National Cancer Institute, Bethesda, MD, USA) [17].

Statistical analysis

The statistical significant differences between groups were determined by Student's t test or analysis or variance (ANOVA) tests and the results were expressed as the mean ± SD from at least three independent experiments. The most appropriate cutoff value for AMPD2 score was obtained by generating receiver operating characteristics (ROC) curves. The relationship between AMPD2 expression and clinicopathological parameters was analyzed using the Chi-square analysis and Fisher's exact test. Parameters that were statistical significant on univariate analyses ($P < 0.05$) were included in the multivariate analyses. Multivariate survival

Table 2. Univariate Kaplan-Meier survival analysis

Clinical parameters	Survival, Median (Range)	Log-Rank Chi square	*P* value
TNM stage			0.001
I II stage	85.3 (81.9-88.7)	11.546	
III IV stage	71.5 (64.3-78.7)		
Histological grade			< 0.001
G1	70.5 (65.1-75.9)	16.854	
G2	87.4 (84.6-90.1)		
G3	69.6 (61.9-77.1)		
Tumor grade			0.034
High	82.1 (78.1-86.2)	4.497	
Low	74.0 (66.3-81.7)		
AMPD2			0.005
Low	85.1 (80.6-89.5)	7.945	
High	75.6 (70.2-81.1)		
BMI			0.012
Normal	81.9 (77.6-86.3)	6.241	
Overweight	74.8 (68.0-81.7)		

analysis was performed using the Cox proportional hazard regression method. The Kaplan-Meier method was used to plot OS curve and log-rank test was used to determine statistical difference. A logistic regression model was used to plot the receiver operating characteristic (ROC) curve and to evaluate the predictive effect of multivariate studies on prognosis outcomes of CRC. P < 0.05 was considered statistically significant. Statistical calculation was performed using the R Software (version 2.1.5; http:// www.r-project.org).

Results

Patients' characteristics

Clinicopathological and demographic characteristics are presented in **Table 1**. The study cohort included 158 patients, consisting of 85 males (53.8%) and 73 females (46.2%). The median age was 61.5 years (interquartile range [IQR] 55-69 years). Among the total 158 patients, the median BMI was 24.4 kg/m^2 (interquartile range [IQR], 22-28 kg/m^2), 54 patients (43.2%) were overweight (BMI ≥ 28 kg/m^2), 104 patients (65.8%) presented a normal weight (18.5 kg/m^2-28 kg/m^2), and none of the patients were undernourished. We found that AMPD2 expression was significantly higher in tumor tissue (89.9%, 142/158) compared to adjacent normal tissue (52.5%, 83/158, *P* < 0.01).

Correlation of AMPD2 expression with clinicopathological characteristics

In this study, we determined the correlation between high AMPD2 expression and clinicopathological parameters of colorectal carcinomas. IHC analysis of AMPD2 levels in CRCs cells from 158 patients showed that AMPD2 is predominantly expressed in the cytoplasm (**Figure 1**). The expression index ranged from 0 to 12 (median: 6) (**Figure 1Aa**, **1Ba**, **1Ab**, **1Bb**). Moreover, in 12 patients, AMPD2 expression could not be observed (**Figure 1Ca**, **1Cb**). For the storing of AMPD2 expression, we used an immunohistochemical semi-quantitative score. When the IHC score was equal to 5.5, the Youden index of the survival outcome was predicted by AMPD2 expression levels. Here, we patients were divided into high expression with AMPD2 IHC scores ≥ 5.5 (**Figure 2A**). The positive rates of TNM III and IV stages were 68.2% (45/66), 52.2% (48/92) in TNM I and II stage (*P* = 0.014). The positive rate of AMPD2 was 72.7% (39/54) in overweight patients, but only 51.9% (54/104) in normal weight cases (*P* = 0.014). A significant difference in IHC scores was observed between obese and non-obese patients (*P* < 0.05, **Figure 2B**). However, the expression of AMPD2 was not statistically significant with gender, tumor location, histological grade, and tumor grade (**Table 1**).

High AMPD2 expression combined with obesity is an independent biomarker of poor prognosis in colorectal cancer patients

Kaplan-Meier survival analysis was used to evaluate the ability of each variable to predict mortality. In our study, we found that TNM stage, histological grade, tumor grade, AMPD2 (**Figure 3A**), and BMI (**Figure 3B**) significantly associated with long-term mortality risk (*P* < 0.05, **Table 2**). Univariate Cox proportional hazard regression was performed to verify the predictive value of the proposed scores (**Table 3**). For all variables analyzed, such as TNM stage (*P* < 0.001), histological grade (*P* = 0.012), low tumor grade (*P* = 0.033), AMPD2 high expression (*P* = 0.004), and BMI obesity (*P* = 0.008). Moreover, multivariate Cox regression analysis indicated that TNM stages III and IV associated with a relative hazard of death of 4.35 (95% CI, 1.92-9.96; *P* < 0.01) compared with stages I and II. In addition, AMPD2 overexpression was

Table 3. Univariate and multivariate cox proportional hazard model

Clinical parameters	Univariate		Multivariate	
	HR (*95% CI*)	*P* value	HR (*95% CI*)	*P* value
TNM (III IV stage/I II stage)	3.85 (1.82-8.33)	< 0.001	4.35 (1.92-9.96)	< 0.001
Histological grade (G2 G3/G1)	1.82 (1.12-3.12)	0.012	2.56 (0.74-9.09)	0.138
Tumor grade (Low/High)	2.02 (1.05-3.85)	0.033	1.69 (0.79-3.57)	0.176
AMPD2 (High/Low)	3.34 (1.35-8.33)	0.004	3.33 (1.25-8.33)	0.016
BMI (Overweight/Normal)	2.38 (1.23-4.55)	0.008	1.59 (0.73-3.45)	0.897

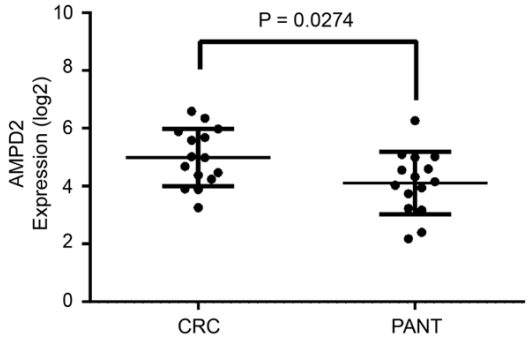

Figure 4. AMPD2 levels are significantly increased in primary colorectal cancer (CRC) tissues compared with precancerous tissue using 15 pairs of fresh CRC/precancerous tissue. Data are expressed as the Mean ± SD. Statistical analysis was conducted using Student's t test.

associated with a relative hazard of death of 3.33 (95% CI, 1.25-8.33; $P < 0.05$). The predictions of the prognosis by logistic regression model results resulted in an area under the curve of 0.821 (95% CI, 0.746-0.896; $P < 0.01$) (**Figure 3C**). We analyzed the AMPD2 mRNA levels of 15 pairs of fresh tumor/normal tissue of by qRT-PCR and confirmed that levels of AMPD2 transcripts were elevated in CRC tissue when compared with adjacent normal tissue (**Figure 4**). We further validated the 5 mRNAs database of carcinoma, including colon carcinoma, rectum carcinoma, breast invasive carcinoma, liver hepatocellular carcinoma, and cholangiocarcinoma using the TCGA. AMPD2 expression levels in colon and rectal cancer were significantly upregulated in tumor tissue compare with normal tissue (**Figure 5A**). In addition, in patients with CRC, AMPD2 provided a high accuracy on tumor tissue classification as estimated by ROC curve analysis. The Kaplan-Meier method was used to evaluate the TCGA data, the OS of a patient's prognosis. The prognosis of patients with high expression of AMPD2 was poorer compared to that of patients with low levels of AMPD2. Although the Log-rank *P* value was 0.0627, the Gehan-Breslow-Wilcoxon *P* value was 0.0373 (**Figure 5B**).

Discussion

The present study allowed us to determine the relation between metabolic parameters in carcinoma and nutrition status with survival [18, 19]. AMPD2 is an important metabolic coding gene, however its role in tumors has not yet been identified. The importance of AMPD2 in nucleic acid metabolism as presented in our previous study in intestinal cancer metabolism shows some potential [20]. In recent years, the AMPK signaling pathway has been one of the best studied subjects of tumor metabolic biomarker research [21, 22]. Obesity-related indicators, especially the BMI, are important indicators of tumor prognosis in metabolic studies [23]. However, BMI does not distinguish between fat and lean mass, which exert different effects on metabolic dysregulation and cancer survival [24]. Several studies have focused on skeletal muscle diagnostic criteria [25], however skeletal muscle diagnostic indexes also lack accuracy. Thus, AMPD2 is mainly present in smooth muscle of the AMPD gene, which is a more realistic and objective biomarker to study the relation between obesity and poor survival.

In our study, we found that AMPD2 was significantly associated with high expression and poor prognosis in obese patients. In addition, in univariate and multivariate regression analysis, we identified AMPD2 as a variable, the prognosis of patients with colorectal cancer has a significance. This is in line with our experimental hypothesis. Fresh tissue qRT-PCR experiments and comprehensive TCGA data analysis have confirmed this. Our study also has some shortcomings. First, our study is relatively small and has a limited sample size [26]. Moreover, the immunohistochemical test method is semi-quantitative and strongly depends on the quality of the pathologist and antibody [27]. We established several control settings to reduce bias [28]. This was a retrospective study, and patients with compliance were well-behaved during follow-up. Therefore, the overall survival rate of our CRC patients may be higher than the

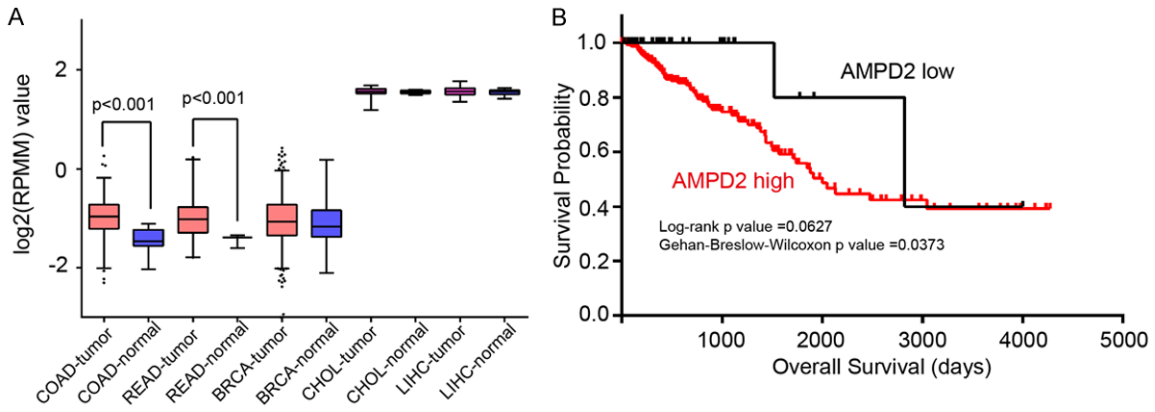

Figure 5. Expression of AMPD2 in colon adenocarcinoma (COAD), rectum adenocarcinoma (READ), breast invasive carcinoma (BRCA), cholangiocarcinoma (CHOL), and liver hepatocellular carcinoma (LIHC) are plotted for both tumor and normal tissues (TCGA dataset). Expression values of mRNAs are log2-transformed (A). Kaplan-Meier plots for overall survival for a discriminatory median AMPD2 expression, from TCGA sequencing data to assess prognostic accuracy. *P* values were calculated using the log-rank test and Gehan-Breslow-Wilcoxon test (B).

real situation [29]. Therefore, large-scale prospective investigation and a more accurate detection approach are warranted.

In conclusion, AMPD2 is frequently upregulated in CRC, suggesting that a combination of high tissue AMPD2 expression and obesity is a prognostic factor for poor prognosis in CRC patients.

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Disclosure of conflict of interest

None.

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