

Original Article

BCAT1 overexpression associates with clinical progression and poor prognosis in patients with hepatocellular carcinoma

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Abstract: Objective: Amino acid biosynthesis is one of the most cardinal events in many carcinogenesis and its progression. In this study, we explored clinicopathological significance of branched-chain amino acid transaminase 1 (BCAT1) transcript and protein expression level in human hepatocellular carcinoma (HCC). Methods: BCAT1 transcript level was detected in 186 cases of paired HCC and adjacent noncancerous liver tissues (ANLT) by qRT-PCR and 12 cases of HCC tissues by semi-quantitative RT-PCR. Immunohistochemistry (IHC) method was utilized to assess BCAT1 protein in 62 HCC tissues. According to the mRNA expression level of BCAT1, we divided all HCC patients into two groups. Cumulative overall survival (OS) and progression-free survival (PFS) curves were estimated using the Kaplan-Meier method and compared by Cox proportional hazard regression model. Results: Compared with the corresponding noncancerous tissues, the BCAT1 expression in HCC tissues, which was remarkably associated with median size ($P = 0.033$), TNM stage ($P = 0.027$), and microvascular invasion ($P = 0.025$), were increased dramatically. The increased BCAT1 predicted worse OS ($P = 0.021$) and PFS ($P = 0.020$) for HCC patients. Multivariate Cox proportional hazards analysis showed that BCAT1 can serve as an independent predictor for OS (hazard ratio [HR] = 1.63, 95% confidence interval [CI] = 1.07-2.76, $P = 0.033$) and PFS (HR = 1.56, 95% CI = 1.05-2.62, $P = 0.040$) of HCC. Conclusions: Increased BCAT1 is associated with malignant characteristics, and predicts advantageous outcomes in HCC patients, which may serve as a prognostic biomarker for HCC survival, and a powerful therapeutic target for HCC therapies.

Keywords: Hepatocellular carcinoma, BCAT1, prognosis

Introduction

As one of the most common pathological type of human liver cancer, hepatocellular carcinoma (HCC) is a kind of cancer with mass mortality worldwide [1]. Surgical resection, transplantation or percutaneous ablation is the three most important approaches for HCC patients to improve long-term survivorship [2, 3]. Despite such remarkable advances were available, patients were almost inevitably experience a recurrence. Moreover, the five-year recurrence rate is about 60% to 80%, which potentially leading to a poor prognosis [4]. Therefore, to explore new biological targets, which can effec-

tively block recurrence and metastasis, and with unique prognostic implications were the main focus of our current study.

Branched chain amino acids (BCAAs), including leucine, isoleucine, and valine, are crucial nutrients for human [5]. Branched chain amino acid transaminase 1 (BCAT1), which located at 12p12.1, is one kind of mammalian BCAT isozymes which catabolizing BCAAs. The typical workflow as follows: BCAT catalyzed BCAAs to α -ketoglutarates (a-KG), and producing glutamates and branched-chain α -ketoacids (BCKAs). BCKAs were further catabolized to acetyl coenzyme A (acetyl-CoA) and succinyl coen-

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zyme A (succinyl-CoA) which participate in tricarboxylic acid (TCA) cycle in order to get adenosinetriphosphate (ATP) [6, 7].

It has been reported that the abnormal expressions of BCAT1, accompanying with BCAAs transamination defect, resulted in hypervolemia and hyperleucine-isoleucinemia, may have great effect on tumor cell proliferation, migration and invasion in some human malignancies [8, 9]. Recent studies found that compared to adjacent non-tumor tissues and L02 hepatic cell line, BCAT1 was significantly higher in HCC tissues and HCC cell lines. BCAT1 knock-down was significantly reduced the invasion and migration of HCC cells [10, 11]. Here we aimed to further investigate the potential role of BCAT1 in HCC, comprehensively analyze the association between BCAT1 expression and clinicopathological elements or survival time of HCC.

Materials and methods

Ethics statement

This study complied with the Helsinki Declaration and was approved by the Clinical Research Ethics Committee of Affiliated Hospital of Guilin Medical University. All patients signed an informed consent for voluntary participation, and that their privacy would be maintained.

Patients and clinical samples

186 pairs of peritumor and tumor tissues with HCC used in our study were obtained from the Affiliated Hospital of Guilin Medical University, Guilin, Guangxi Province, China between November 2003 and April 2010. All diagnoses of HCC were based on pathological evidence. The tumor stage was determined according to the tumor-node-metastasis (TNM) classification system of the International Union Against Cancer, 7th Edition [12]. Patients clinical variables, including of the age, gender, hepatitis B surface antigen (HBsAg), median size, cirrhosis, tumor number, TNM stage, microvascular invasion, alpha-fetoprotein (AFP) and recurrence were shown in **Table 1**.

All HCC patients were followed by monitoring serum AFP levels and abdominal ultrasonography, chest radiography every 2 months for the

first two years, and the same testing every 3-6 months thereafter. Recurrence was diagnosed with new hepatic lesions detected by ultrasonography, dynamic CT or MRI. The mean follow-up period of the 186 HCC patients was 38.6 months (range, 1.0 to 84.0 months). Overall survival (OS) was measured as the time interval from the date of surgery to death or the last follow-up visit. Progression-free survival (PFS) was defined as the time interval between the date of surgery and the date that disease progression or any cause of death was first observed.

Reverse transcription PCR (RT-PCR) and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from tissues using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Prime Script RT Reagent Kit (TaKaRa, Otsu, Japan) was used for cDNA synthesis. Then the first-strand cDNA was stored at -20°C before use.

The mRNA expression levels of BCAT1 in HCC tissues and ANLT were detected with qRT-PCR. The primer sequences were as follow: BCAT1-forward 5'-CAACTATGGAGAATGGTCCTAAGCT-3', BCAT1-reverse 5'-TGTCCAGTCGCTCTCTCTCTCTC-3'; β -actin forward 5'-GAC AGG ATG CAG AAG GAG ATT ACT-3' and β -actin reverse 5'-TGA TCC ACA TCT GCT GGA AGGT-3'. QRT-PCR assays were performed using ABI Prism 7500 Sequence Detector System (Applied Biosystems, Foster City, CA, USA). Gene expression levels were analyzed by using previously described method [13].

Immunohistochemistry (IHC) assay

Two pathologists who were blinded to the patients' clinical and biochemical information scored the results of the staining independently. For the assessment of BCAT1 expression, tissue sections were deparaffinized, rehydrated, and heated in microwave for 3 minutes with citrate antigenic retrieval buffer at pH = 6.0. Then incubated in 3% H₂O₂ for 10 min, and treated with 10% normal goat serum for 30 minutes. Primary antibodies (catalog ab12-5209, Abcam Company, 1:200 dilution) were applied overnight in a moist chamber at 4°C. The next day, after washing, a secondary goat

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Table 1. Correlation between the clinicopathologic variables and BCAT1 in HCC

Clinical character	Clinical variable	No. of patients	BCAT1 mRNA		χ^2	p value
			Low n (%)	High n (%)		
Gender	Female	28	10 (35.7)	18 (64.3)	0.001	0.971
	Male	158	57 (36.1)	101 (63.9)		
Age (years)	≤ 50	95	36 (37.9)	59 (62.1)	0.296	0.587
	> 50	91	31 (34.1)	60 (65.9)		
HBsAg	Negative	33	12 (36.4)	21 (63.6)	0.002	0.964
	Positive	153	55 (35.9)	98 (64.1)		
Median size (range, cm)	≤ 4	47	23 (48.9)	24 (51.1)	4.552	0.033
	> 4	139	44 (31.7)	95 (68.3)		
Cirrhosis	No	18	6 (33.3)	12 (66.7)	0.062	0.803
	Yes	168	61 (36.3)	107 (63.7)		
Tumor number	Single	125	49 (39.2)	76 (60.8)	1.671	0.196
	Multiple	61	18 (29.5)	43 (70.5)		
TNM stage	I	22	8 (36.4)	14 (63.6)	9.202	0.027
	II	58	27 (46.6)	31 (53.4)		
	III	73	17 (23.3)	56 (76.7)		
	IV	33	15 (45.5)	18 (54.5)		
Microvascular invasion	No	154	61 (39.6)	93 (60.4)	5.003	0.025
	Yes	32	6 (18.8)	26 (81.3)		
AFP (ng/mL)	≤ 200	86	29 (33.7)	57 (66.3)	0.367	0.544
	> 200	100	38 (38.0)	62 (62.0)		
Recurrence	No	125	41 (32.8)	84 (67.2)	1.716	0.190
	Yes	61	26 (42.6)	35 (57.4)		

HBsAg, hepatitis B surface antigen; TNM, tumor-node-metastasis; AFP, alpha-fetoprotein.

anti-rabbit antibody conjugated to horseradish peroxidase was applied for 1 hour at room temperature. The reaction was finally developed using 3, 3-diaminobenzidine tetrahydrochloride (DAB) and then lightly counterstained with hematoxylin. Normal rabbit serum was used as negative control. The BCAT1 immunostaining intensities were scored as follows: the percentage of positive cells, grades 0-3 (0, no positive cells; 1, < 25% positive cells; 2, 25%-50% positive cells; 3, > 50% positive cells).

Statistical analysis

Statistical analysis was carried out by using SPSS 13.0 (SPSS Inc, Chicago, IL). The relationship between BCAT1 expression and clinicopathological factors were analyzed by Pearson χ^2 test. Survival curves were calculated using the Kaplan-Meier method and compared by the log-rank test. The Cox proportional hazards regression model was used to identify factors which were independently associated with PFS and OS, and estimat-

ed the hazard ratio (HR) and the 95% confidence interval (CI). For all tests, the significance level for statistical analysis was set at $P < 0.05$.

Results

BCAT1 is highly expressed in HCC tissue samples

To explore the role of BCAT1 in human HCC, we assessed the BCAT1 gene expression in HCC clinical samples using semi-quantitative RT-PCR assay, which showed that the expression levels of BCAT1 in tumor tissues were elevated in 9 of 12 analyzed HCC tissues (75.0%) compared with ANLT (**Figure 1A**). Next we determined BCAT1 expression in HCC specimens using qRT-PCR assays. The results showed mRNA levels of BCAT1 in HCC specimens were noticeably higher than those in matched ANLT ($P < 0.0001$) (**Figure 1B**).

Immunohistochemical analyses of 62 paraffin-embedded HCC specimens also suggested that tumor tissues exhibited stronger BCAT1 immu-

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noreactivity compared with ANLT. Overall, 51 of 62 (82.2.0%) cases exhibited high BCAT1 expression in cancerous tissues, whereas only 26 of 62 (41.9%) cases exhibited positive BCAT1 staining in ANLT (**Figure 1C, 1D**), and the difference was statistically significant ($P < 0.001$).

Relationship between the expression of BCAT1 and clinicopathologic characteristics in HCC

For a better understanding of the clinical relevance of BCAT1 mRNA expression level in HCC, we divided the 186 HCC patients into high-expression group ($n = 119$) and low expression group ($n = 67$). The relationship between BCAT1 expression and various clinical characteristics of HCC patients is presented in **Table 1**. Our results revealed that increased BCAT1 expression was significantly associated with median size ($X^2 = 4.552$; $P = 0.033$), TNM stage ($X^2 = 9.202$; $P = 0.027$), and microvascular invasion ($X^2 = 5.003$; $P = 0.025$), but not with age, gender, HBsAg, cirrhosis, tumor number, AFP or recurrence (all $P > 0.05$, **Table 1**).

High BCAT1 expression with shorter survival in HCC

To further investigate the correlation of the BCAT1 mRNA expression level with survival of HCC patients, Kaplan-Meier analysis and the log-rank test were used to evaluate the effect of BCAT1 expression on survival. As shown on **Figure 1E**, the median OS rates of HCC patients with high BCAT1 expression were 39.19 months (95% CI, 33.33-45.05) compared with 51.42 months (95% CI, 43.12-59.72) for patients with low BCAT1 expression ($P = 0.021$). Additionally, patients with high BCAT1 expression was associated with shorter PFS (mean, 34.14 months) (95% CI, 28.02-40.26) than those in low expression group (mean, 47.75 months) (95% CI, 38.88-56.61) ($P = 0.020$, **Figure 1F**).

Further univariate and multivariate analysis were employed to compare the associations of BCAT1 mRNA expression with traditional clinicopathological variables. In the univariate analysis, the median tumor size > 4 cm ($P < 0.001$), multiple tumors ($P < 0.001$), TNM stage III-IV ($P < 0.001$), microvascular invasion ($P < 0.001$), recurrence ($P < 0.001$) and high BCAT1 expression group ($P = 0.002$) were important predictor for OS in HCC patients. All these factors were enrolled in a multivariable analysis. The median

tumor size > 4 cm (HR, 2.62; 95% CI, 1.46-4.47; $P = 0.001$), multiple tumors (HR, 1.37; 95% CI, 0.92-2.06; $P = 0.116$), TNM stage III-IV (HR, 1.81; 95% CI, 1.12-2.91; $P = 0.015$), microvascular invasion (HR, 1.41; 95% CI, 0.89-2.23; $P = 0.143$), recurrence (HR, 1.29; 95% CI, 0.85-1.95; $P = 0.218$) and high BCAT1 expression group (HR, 1.63; 95% CI, 1.07-2.76; $P = 0.033$) were all identified as independent predict factors for poorer OS of HCC (**Table 2**).

In addition, univariate analysis revealed that median tumor size > 4 cm ($P < 0.001$), multiple tumors ($P < 0.001$), TNM stage III-IV ($P < 0.001$), microvascular invasion ($P < 0.001$), and high BCAT1 expression group ($P = 0.008$) significantly affected the PFS of HCC patients. The multivariate analysis further suggested that median tumor size > 4 cm (HR, 2.60; 95% CI, 1.45-4.36; $P = 0.001$), multiple tumors (HR, 1.41; 95% CI, 0.93-2.10; $P = 0.085$), TNM stage III-IV (HR, 1.79; 95% CI, 1.12-2.83; $P = 0.016$), microvascular invasion (HR, 1.26; 95% CI, 0.84-1.92; $P = 0.231$), and high BCAT1 expression group (HR, 1.56; 95% CI, 1.05-2.62; $P = 0.040$) could independently predict the PFS of HCC patients (**Table 2**).

Discussion

In this study, we investigated the role of BCAT1 in HCC, and found a high expression level of BCAT1 in HCC patients. Furthermore, there was a positive correlation between BCAT1 expression and undesirable clinicopathological features were also demonstrated. BCAT is an evolutionarily highly conserved gene that is crucial for cell growth of yeast homolog [14, 15]. Preview reports suggested that BCAT1 is a target gene of the oncogene c-Myc, which prompt cell proliferation, migration, and invasion in nasopharyngeal carcinoma and HCC [8, 10]. Studies have reviewed that BCAT1 is overexpressed and played an important role in several malignancies, including epithelial ovarian cancer [16], glioma [17], Burkitt lymphoma and breast cancer cell lines [18], nasopharyngeal carcinomas [19], medulloblastoma [20], and non-seminomatous testicular germ cell tumors [21]. The mouse homologue of BCAT1 has been shown to upregulate in a teratocarcinoma cell line [22].

It is well-accepted that complicated genetic or epigenetic alterations may affect tumor devel-

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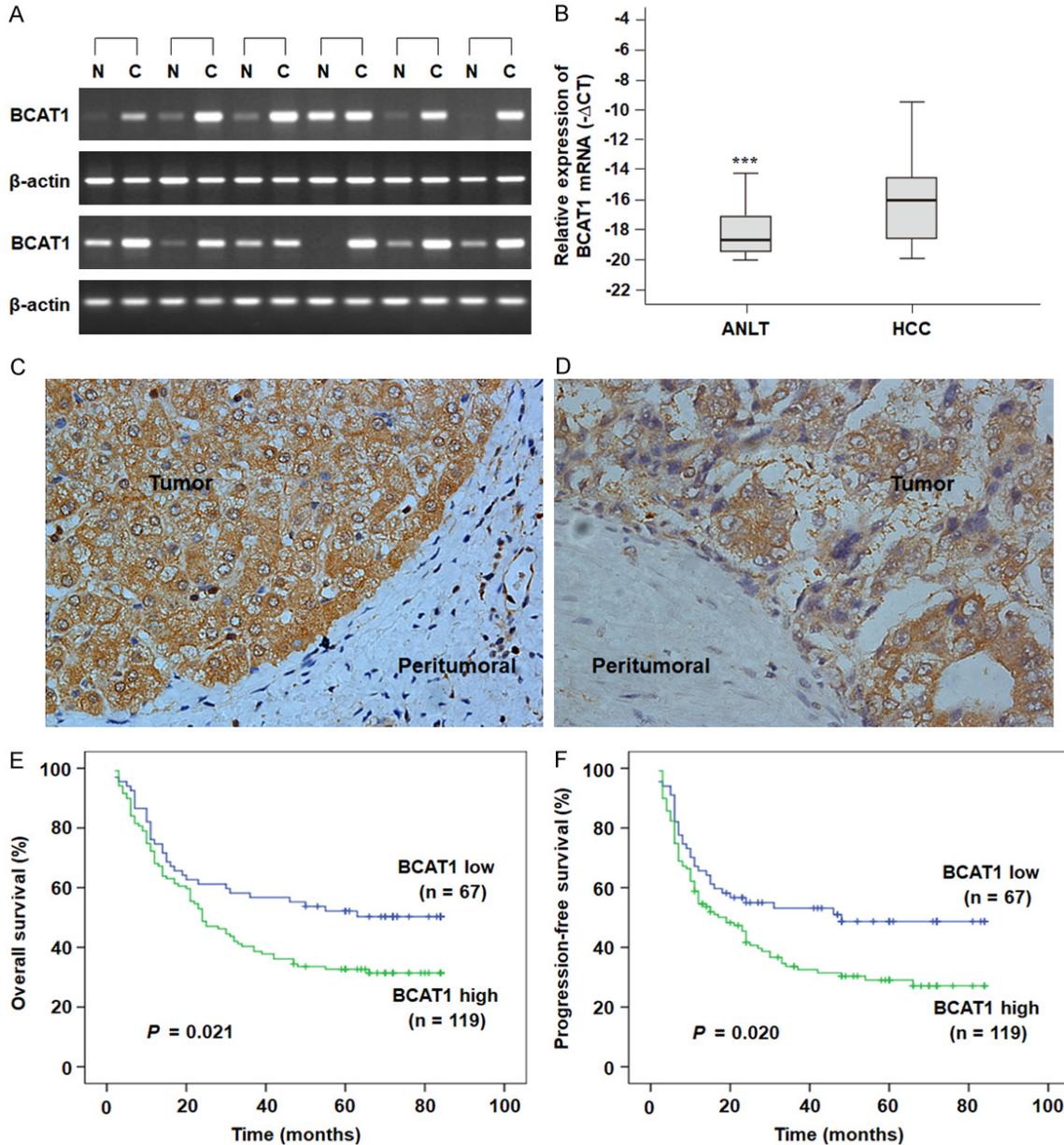


Figure 1. BCAT1 expression is determined and correlated with poor outcome in HCC patients. (A, B) The relative expression of BCAT1 mRNA in 12 paired HCC tissues and matched adjacent noncancerous liver tissues (ANLT) were evaluated by RT-PCR, and in 186 paired HCC tissues and matched ANLT was evaluated by qRT-PCR. The relative mRNA level of BCAT1 was normalized based on that of an internal reference β -actin. (C, D) Representative immunohistochemical expression patterns of BCAT1 in HCC specimen (Tumor) and corresponding ANLT (Peritumoral). The nuclei were counterstained with hematoxylin. Original magnification: $\times 400$. (E, F) The 186 HCC patients were divided into high BCAT1 mRNA expression groups ($n = 119$) and low BCAT1 mRNA expression groups ($n = 67$). Kaplan-Meier analysis was conducted to disclose the relationship between BCAT1 and OS (E) and PFS (F) of all patients according to BCAT1 expression status.

opment and tumor cells growth, migration and invasion, leading to the rapid development of HCC [23-25]. One study showed that the ectopic expression of BCAT1 in BEL-7404 cells enhanced cell proliferation, clone formation, tumorigenic properties, S-G/M phase transi-

tion and chemoresistance to cisplatin, and knockdown BCAT1 expression in HepG2 cells remarkably inhibited cell proliferation, clone formation, and S-G/M phase transition and caused their chemosensitization to cisplatin [11]. Investigating and clarifying the key mole-

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Table 2. Analysis of overall and progression-free survival in HCC patients

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	p value	HR	95% CI	p value
Overall Survival						
Gender (male vs female)	1.51	0.87-2.65	0.146			
Age, year (> 50 vs ≤ 50)	0.95	0.66-1.36	0.770			
HBsAg (positive vs negative)	0.98	0.61-1.58	0.947			
Median size, cm (> 4 vs ≤ 4)	3.12	1.75-4.93	< 0.001	2.62	1.46-4.47	0.001
Cirrhosis (yes vs no)	0.85	0.47-1.54	0.841			
Tumor number (multiple vs single)	2.06	1.39-2.86	< 0.001	1.37	0.92-2.06	0.116
TNM stage (III-IV vs I-II)	2.53	1.72-3.62	< 0.001	1.81	1.12-2.91	0.015
Microvascular invasion (yes vs no)	2.21	1.45-3.26	< 0.001	1.41	0.89-2.23	0.143
AFP, ng/ml (> 200 vs ≤ 200)	1.23	0.85-1.79	0.258			
Recurrence (yes vs no)	2.03	1.35-3.07	< 0.001	1.29	0.85-1.95	0.218
BCAT1 expression (high vs low)	1.89	1.27-2.71	0.002	1.63	1.07-2.76	0.033
Progression-free Survival						
Gender (male vs female)	1.32	0.75-2.31	0.330			
Age, year (> 50 vs ≤ 50)	0.96	0.66-1.39	0.843			
HBsAg (positive vs negative)	0.96	0.59-1.55	0.832			
Median size, cm (> 4 vs ≤ 4)	3.03	1.61-4.11	< 0.001	2.60	1.45-4.36	0.001
Cirrhosis (yes vs no)	0.80	0.44-1.47	0.486			
Tumor number (multiple vs single)	1.92	1.33-2.69	< 0.001	1.41	0.93-2.10	0.085
TNM stage (III-IV vs I-II)	2.45	1.69-3.51	< 0.001	1.79	1.12-2.83	0.016
Microvascular invasion (yes vs no)	2.15	1.41-3.08	< 0.001	1.26	0.84-1.92	0.231
AFP, ng/ml (> 200 vs ≤ 200)	1.19	0.82-1.71	0.353			
BCAT1 expression (high vs low)	1.78	1.25-2.63	0.008	1.56	1.05-2.62	0.040

HR, hazard ratio; CI, confidence interval; HBsAg, hepatitis B surface antigen; TNM, tumor-node-metastasis; AFP, alpha-fetoprotein.

cules involved in the malignant transformation and progression of HCC are vital, which may effectively provide new insights into the mechanism details of HCC, and further supply novel therapeutics for HCC patients.

Our correlation analysis indicates a trend of upregulated BCAT1 mRNA levels was found in subgroups with tumor > 4.0 cm relative to those with tumor ≤ 4.0 cm, or in early (I-II) relative to those in (III-IV) TNM stages, or accompany with microvascular invasion relative to those without microvascular invasion. MYC, a proto-oncogenic transcription factor (TF), which regulates 15% of all genes expression and plays an important role in controlling cell growth, proliferation, apoptosis, and metabolic pathways, as with a complex series of oncogenic events, leading to tumorigenesis [26, 27]. Previous studies have demonstrated that certain elevated MYC by oestrogen increase BCAT1 expression in breast cancer cells MCF-7, which have also been tested in Burkitt's lym-

phomas and nasopharyngeal carcinoma [8, 18]. Another research shows that BCAT1 is a direct target of MYC [28]. Therefore, it is foreseeable that deregulation of MYC signaling, to a certain degree influenced the BCAT1 expression, which would further crimp the rapid progress of a string of malignant events, including larger tumor size, later TNM stages, and microvascular invasion.

In addition, Cox proportional hazards regression model detected that median tumor size > 4 cm, TNM stage III-IV, microvascular invasion can act as an independent prognostic factor for both the PFS and OS of HCC patients, which was consistent with previous studies that, generally, HCC patients with larger tumors had a unfavorable prognosis compared with those with smaller tumors [29]. And, larger HCC neoplasm has the potential to greatly accelerate microscopic vascular invasion and recurrence [30, 31]. All those negative factors combined could motivate the development and progress of HCC.

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More importantly, Kaplan-Meier analysis showed that HCC patients with high BCAT1 expression have significantly reduced OS and PFS than those with low BCAT1 expression. Moreover, both univariate and multivariate analyses demonstrated that BCAT1 was an independent unfavorable predictor of OS and PFS in HCC patients, which are in good agreement with that higher BCAT1 expression was associated with poorer survival rates of HCC patients [10, 11]. Consequently, our findings further validate that BCAT1 can act as an independent adverse prognostic parameter for patients with HCC.

In conclusion, our work identified that BCAT1 was overexpressed in HCC, and involved into the tumorigenesis of HCC. BCAT1, as an independent adverse prognostic parameter, will be a novel therapeutic target for HCC.

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Disclosure of conflict of interest

None.

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