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ORIGINAL ARTICLE

Observational Study

Tissue inhibitor of metalloproteinase-3 expression affects clinicopathological features and prognosis of aflatoxin B1-related hepatocellular carcinoma

Qiu-Ju Liang, Qin-Qin Long, Feng-Qin Tian, Qun-Ying Su, Xiao-Ying Zhu, Xi-Dai Long

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Abstract

BACKGROUND

The dysregulation of tissue inhibitor of metalloproteinase-3 (TIMP3) was positively correlated with the progression of hepatocellular carcinoma (HCC). However, it is not clear whether TIMP3 expression is associated with the clinicopathological features and prognosis of aflatoxin B1 (AFB1)-related HCC (AHCC).

AIM

To assess the effects of TIMP3 expression on the clinicopathological features and prognosis of AHCC.

METHODS

A retrospective study, including 182 patients with AHCC, was conducted to explore the link between TIMP3 expression in cancerous tissues and the clinicopathological characteristics and prognosis of AHCC. TIMP3 expression was detected by immunohistochemistry and its effects on the clinicopathological features and prognosis of AHCC were evaluated by Kaplan-Meier survival analysis and Cox regression survival analysis. Odds ratio, hazard ratio (HR), median overall survival time (MST), median tumor recurrence-free survival time (MRT), and corresponding 95% confidential interval (CI) was calculated to

evaluate the potential of TIMP3 expression in predicting AHCC prognosis.

RESULTS

Kaplan-Meier survival analysis showed that compared with high TIMP3 expression, low *TIMP3* expression in tumor tissues significantly decreased the MST (36.00 mo *vs* 18.00 mo) and MRT (32.00 mo *vs* 16 mo) of patients with AHCC. Multivariate Cox regression survival analysis further proved that decreased expression of TIMP3 increased the risk of death (HR = 2.85, 95%CI: 2.04-4.00) and tumor recurrence (HR = 2.26, 95%CI: 1.57-3.26). Furthermore, decreased expression of TIMP3 protein in tissues with AHCC was significantly correlated with tumor clinicopathological features, such as tumor size, tumor grade and stage, tumor microvessel density, and tumor blood invasion. Additionally, TIMP3 protein expression was also negatively associated with amount of AFB1-DNA adducts in tumor tissues.

CONCLUSION

These findings indicate that the dysregulation of TIMP3 expression is related to AHCC biological behaviors and affects tumor outcome, suggesting that TIMP3 may act as a prognostic biomarker for AHCC.

Key Words: Tissue inhibitor of metalloproteinase-3 expression; Aflatoxin B1; Hepatocellular carcinoma; Clinicopathological feature; Prognosis

Core Tip: This study retrospectively analyzed tissue inhibitor of metalloproteinase-3 (TIMP3) expression and the clinicopathological characteristics and prognosis of aflatoxin B1-related hepatocellular carcinoma (AHCC). It was found that TIMP3 expression was statistically associated with the prognosis of AHCC as an independent risk factor. The dysregulation of TIMP3 expression affected the clinicopathological features and has great value to predict the prognosis of AHCC.

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INTRODUCTION

In China, hepatocellular carcinoma (HCC) is one of the most common malignant tumors and represents a major healthcare challenge. Approximately half of all patients with HCC in the world are in China, making this tumor as the second leading cause of death due to malignant tumors among Chinese people[1]. According to Annals of China Health Statistics Version 2022 (http://www.nhc.gov.cn/mohwsbwstjxxzx/tjtjnj/202305/6ef68aac6bd14c1eb9375e01a0faa1fb.shtml), the death rate of HCC is increasing in the past 50 years. Evidence from epidemiological studies has shown that hepatitis B virus (HBV) infection and aflatoxin B1 (AFB1) exposure are two dominant causes of HCC. Despite significant advances in research on the occurrence and development of HCC induced by different etiological factors over the past few decades[2], the detailed mechanisms underlying the progression of this malignancy remain unclear. Recent studies have shown that HCC patients with different genetic profiles featured different survival duration even though receiving the same treatment[3-6]. Therefore, it is very crucial for patients with HCC to need biomarkers predicting their survival duration.

Tissue inhibitor of metalloproteinase-3 (*TIMP3*), an important member of the TIMP gene family, locates at 22q12.3 in human and consists of four introns and five exons (Gene ID: 7078). This gene encodes a secreted glycoprotein consisting of 221 amino acids with a molecular weight of approximately 21 KD. TIMP3 protein contain two functional domains: An N-terminal domain and a C-terminal domain[7-9]. Functionally, TIMP3 acts as an inhibitor of the matrix metalloproteinases and plays an important role in the degradation of the extracellular matrix (ECM). Recently, increasing evidence has exhibited that the dysregulation of TIMP3 may be involved in the progression of cancers *via* modifying the infiltration and metastasis capacity of cancer cells and remodeling tumor microenvironmental structure[7,9]. However, the roles of TIMP3 expression in AFB1-related HCC (AHCC) prognosis and clinicopathological features have not been investigated. Here, we conducted a hospital-based clinicopathological study to explore whether the dysregulation of TIMP3 expression in cancerous tissues modifies the clinicopathological features and prognosis of AHCC.

MATERIALS AND METHODS

Study population

This is a hospital-based retrospective study. The study was approved by the Ethics Review Committee of Youjiang Medical University for Nationalities (No. AYJM20210708), and informed consent from patients was waived due to the retrospective nature of this study. In this study, AHCC was defined as HCC with AFB1 exposure. The inclusion criteria were as follows: (1) Patients with histopathologically diagnosed HCC; (2) Patients not receiving radiotherapy or chemotherapy before their tumors were resected; (3) Patients with enough available paraffin-embedded tumor samples for TIMP3 protein expression and AFB1 exposure analyses; (4) Patients with available clinicopathological data and survival follow-up information; (5) Patients with AFB1 exposure [AFB1-DNA adducts (ADAs) in tumor tissues: > 1.00 μmol/mol DNA]; and (6) Patients who agreed that their resected tumor tissues can be used for scientific research. The exclusion criteria included: (1) Cases with a history of other tumors such as cholangiocellular carcinoma; and (2) Samples not meeting the needs of immunohistochemistry or enzyme linked immunosorbent assay.

According to the abovementioned inclusion and exclusion criteria, a total of 182 patients with HCC were screened at the Histopathological Sample Library of the Clinicopathological Diagnosis and Research Center (HSL-CDRC), the Affiliated Hospital of Youjiang Medical University for Nationalities between January 2014 and December 2018. Samples and corresponding clinicopathological and survival data for all subjects were obtained from the HSL-CDRC. In this study, tumor grades and stages were elucidated according to the criteria of Edmondson and Steiner (ES) grading system and tumor-node-metastasis staging system, respectively. Tumor grade was divided into two groups: Low grade (ES-I and ES-II) and high grade (ES-III and ES-IV). Survival status and time for all cases were confirmed by telephonic follow-up and medical records. The last follow-up date was October 31, 2023. Overall survival (OS) was defined as the period from the date of tumor surgical resection therapy to the date of death or last follow-up, whereas tumor recurrence-free survival (RFS) was defined as the period from the date of tumor surgical resection therapy to the date of tumor recurrence or last follow-up.

AFB1 exposure data

AFB1 exposure status for all patients with HCC was evaluated through the number of ADAs in cancerous tissues. The number of ADAs was calculated by enzyme-linked immunosorbent assay as previously described[10]. Based on the level of AFB1 exposure, the patients were divided into two groups: Low AFB1-exposure group (ADAs in tumor tissues ≤ 2.00 μmol/mol DNA) and high AFB1-exposure group (ADAs in tumor tissues > 2.00 μmol/mol DNA), according to the average value of the number of ADAs in the cancerous tissues with HCC.

TIMP3 expression assays

The amount of TIMP3 protein expression in HCC cancerous tissues was assayed by immunohistochemistry technique as previously described[11]. Briefly, paraffin-embedded tissues from each patient were fixed on glass slides and were treated with dimethylbenzene. After that, TIMP3 protein was tested using an anti-TIMP3 polyclonal antibody (1:250 dilution; catalog bs-0417R, Beijing Bioss Biology, Inc., Beijing, China) and UltraSensitive™ SP IHC Kit (catalog KIT-9730, Maixin Biotechnology, Inc., Xiamen, China). Positive and negative controls were used. The amount of TIMP3 protein expression was quantified according to the following immunohistochemistry analytic formula of the immunoreactive scoring (IRS) system[12]:

IRS = SI × PP, wherein SI represents staining intensity and PP represents the percentage of positive cells.

Based on the level of TIMP3 protein expression in HCC cancerous tissues, the patients were divided into two groups: Patients with low TIMP3 expression (LTE; IRS < 6) and those with high TIMP3 expression (HTE; IRS ≥ 6).

Tumor microvessel density evaluation

Tumor microvessel density (MVD) in HCC cancerous tissues was analyzed according to our previously reported method [13]. In brief, microvessels were first stained using anti-CD31 antibody [catalog 2011101101, Gene Tech (Shanghai) Company Limited, Shanghai, China] and then counted at × 200 magnification. In this study, MVD was defined as the number of microvessels in the cancerous regions over five fields in each slide. Based on the average MVD, the patients were divided into two groups: patients with low MVD (< 50) and those with high MVD (≥ 50).

Statistical analysis

The χ^2 test was used to analyze the distribution difference of clinicopathological features between LTE and HTE. Risk effects of TIMP3 expression on tumor characteristics, including alpha fetoprotein (AFP), tumor size, tumor grade and stage, MVD, microvessel infiltration (MVI), and portal vein tumor thrombus (PVTT), were estimated by logistic regression analysis, with odds ratio and corresponding 95% confidential interval (CI) calculated. The effects of TIMP3 expression on HCC survival were assessed by Kaplan-Meier survival analysis and Cox proportional hazard regression analysis. For Kaplan-Meier survival analysis, survival curves (with log-rank test) were plotted, and then median OS time (MST) and median RFS time (MRT) were calculated for different dumb variables. Regarding Cox proportional hazard regression analysis, univariable analysis was initially performed for screening potential risk biomarkers, which were then included in multivariable survival analysis (stepwise method with the likelihood ratio test) to identified independent predictors of death and tumor recurrence, with hazard ratios (HRs) and corresponding 95%CIs calculated. Correlation between TIMP3 expression and AFB1 exposure was assessed by Spearman's correlation analysis. In this study, all statistical analyses were performed using the statistical package for social science (SPSS) software (version 29.00) (SPSS Institute, Chicago, IL, United States), and a P value < 0.05 was regarded as statistically significant.

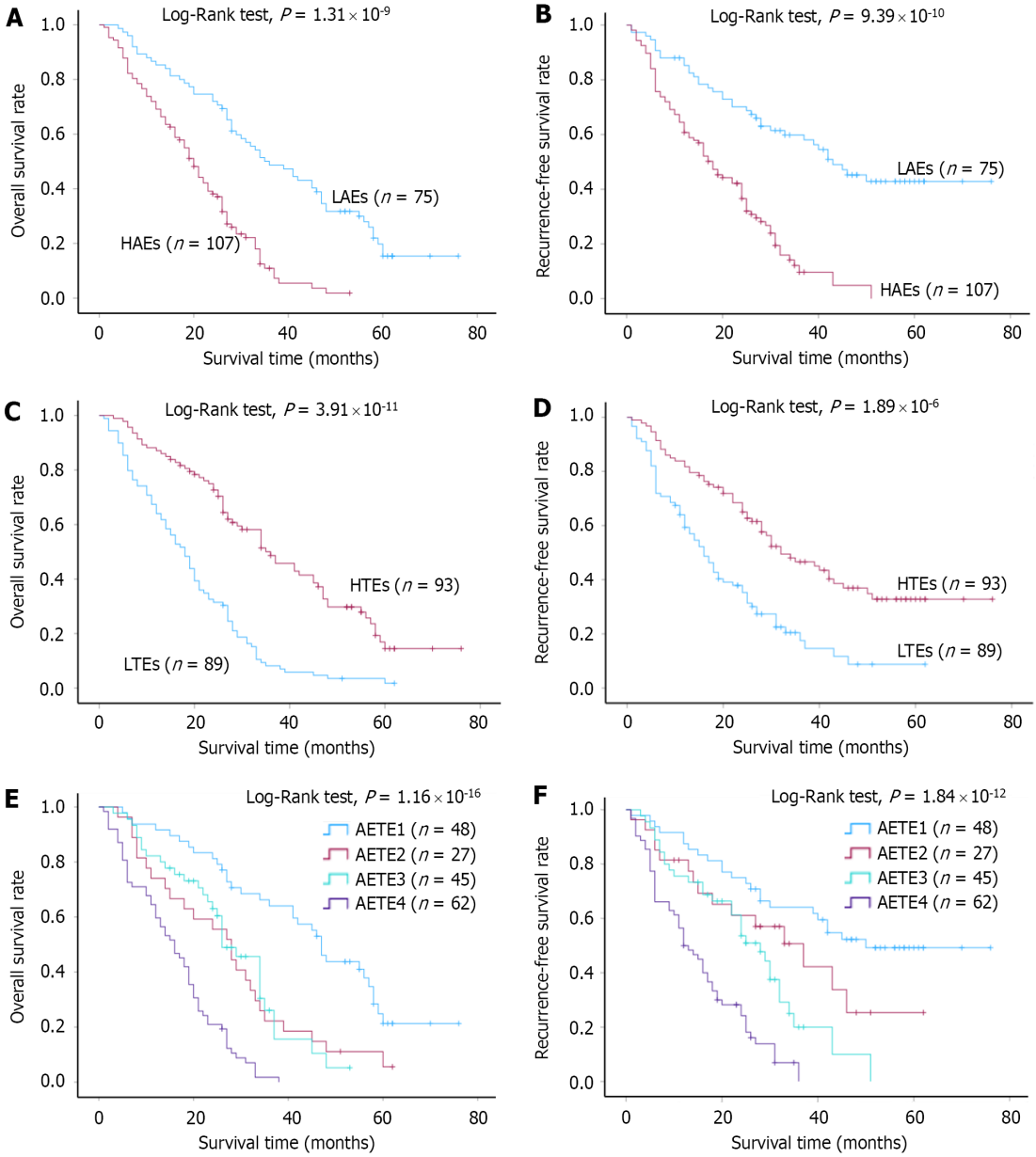

Figure 1 Effects of aflatoxin B1 exposure and tissue inhibitor of metalloproteinase-3 expression on survival of patients with aflatoxin B1-related hepatocellular carcinoma. A and B: Effects of aflatoxin B1 (AFB1) exposure on overall survival (OS) and tumor recurrence-free survival (RFS) of patients with AFB1-related hepatocellular carcinoma (AHCC). The level of AFB1 exposure was assessed using AFB1-DNA adducts in HCC cancerous tissues; C and D: Effects of TIMP3 expression on OS and RFS of patients with AHCC. The level of TIMP3 protein expression in AHCC tissues was tested by immunohistochemistry combined with the immunoreactive scoring system; E and F: Effects of AFB1 exposure combined with TIMP3 expression on OS and RFS of AHCC. Cumulative hazard function was plotted by the Kaplan-Meier method, and *P* value was calculated with two-sided log-rank tests. LAE: Low AFB1-exposure group; HAE: High AFB1-exposure group; LTE: Low TIMP3 expression; HTE: High TIMP3 expression; AETE1: Low aflatoxin B1 exposure plus high TIMP3 expression; AETE2: Low aflatoxin B1 exposure plus low TIMP3 expression; AETE3: High aflatoxin B1 exposure plus high TIMP3 expression; AETE4: High aflatoxin B1 exposure plus low TIMP3 expression.

RESULTS

Clinicopathology information of AHCC patients

A total of 182 patients with AHCC were included in the final analyses (Table 1). Among these patients, about 70% were male and their average age was 49.52 ± 10.79 years old. About 52% of AHCC cases had high differentiation, and 36.3% showed MVI. Kaplan-Meier survival analysis indicated that the MST and MRT of patients with AHCC was 26.00 (23.02-28.98) mo and 25.00 (20.44-29.56) mo, respectively. All patients with AHCC featured positive AFB1 exposure, with an average amount of adducts of 2.00 ± 0.79 μmol/mol DNA. Moreover, patients having higher AFB1 exposure featured a poorer OS (Figure 1A) and RFS (Figure 1B).

Table 1 Clinicopathological features of patients with hepatocellular carcinoma

Variable	*n*	Percentage (%)
Total	182	100.0
Age (years)[1]		
< 49	98	53.8
≥ 49	84	46.2
Gender		
Male	135	74.2
Female	47	25.8
Race		
Han	89	48.9
Others	93	51.1
Smoking		
No	138	75.8
Yes	44	24.2
Drinking		
No	135	74.2
Yes	47	25.8
HBsAg		
Negative	63	34.6
Positive	119	65.4
Anti-HCV		
Negative	149	81.9
Positive	33	18.1
AFP		
Negative	64	35.2
Positive	118	64.8
AFB1 exposure		
Low	75	41.2
High	107	58.8
Tumor size		
≤ 5 cm	56	30.8
> 5 cm	126	69.2
ES grade		
Low	94	51.6
High	88	48.4
TNM stage		
I	30	16.5
II	78	42.9
III	74	40.7
Liver cirrhosis		
No	56	30.8
Yes	126	69.2

MVD		
Low	90	49.5
High	92	50.5
PVTT		
Negative	127	69.8
Positive	55	30.2
MVI		
Negative	116	63.7
Positive	66	36.3

[1]Age was grouped according to the average age of patients with hepatocellular carcinoma (49.52 10.79 years old).
HCV: Hepatitis C virus; AFP: Alpha fetoprotein; AFB1: Aflatoxin B1; ES: Edmondson and Steiner; TNM: Tumor node metastasis; MVD: Microvessel density; PVTT: Portal vein tumor thrombus; MVI: Microvessel infiltration.

Association of TIMP3 expression with clinicopathological features of AHCC

TIMP3 protein expression in AHCC cancerous tissues was assessed by immunohistochemistry technique with the IRS system. According to the average amount of TIMP3 expression, the patients were divided into two groups: Patients with HTE and those with LTE (as described in Materials and Methods). Table 2 summarizes the clinicopathological characteristics of patients with AHCC according to the level of TIMP3 protein expression. Different distributions were found for AFP, tumor size, tumor grade and stage, MVD, MVI, and PVTT, but not other clinicopathological features. To explore the risk effects of TIMP3 expression on clinicopathological characteristics, we accomplished logistic regression analyses (Table 3). The results showed that decreased TIMP3 protein expression in cancerous tissues increased the risk of tumor progression. Compared with patients with HTE, those with LTE featured a higher risk of tumor dedifferentiation (OR = 3.04, 95%CI: 1.65-5.65).

Univariable survival TIMP3 expression and AHCC survival based on univariable survival models

To investigate the effects of TIMP3 protein expression in cancerous tissues on the prognosis of patients with AHCC, we first accomplished Kaplan-Meier survival analyses (Table 4). The results showed that TIMP3 protein expression statistically affected the MST and MRT of patients with AHCC, as well as known clinicopathological variables (including AFP, AFB1 exposure, tumor size, tumor grade and stage, tumor MVD and MVI, and PVTT). Patients with LTE featured a shorter MST or MRT compared to those with HTE (18.00 mo *vs* 36.00 mo for MST; 16.00 mo *vs* 32.00 mo for MRT), which suggested that the downregulation of TIMP3 expression shortened patients' OS and RFS. Figure 1C and D shows this trend. Univariable Cox proportional hazard regression analysis further demonstrated that abnormal expression of TIMP3 significantly modified the death risk of patients with AHCC (HR = 2.92; 95%CI: 2.19-4.09), as well as known risk clinicopathological variables such as AFP, AFB1 exposure, tumor size, tumor grade and stage, tumor MVD and MVI, and PVTT (Table 5). Similar results were also observed in the analyses of RFS.

Given that both TIMP3 expression and some clinicopathological features modified the survival of AHCC, we accomplished stratification analyses of TIMP3 expression based on the risky clinicopathological features of AHCC (including AFP, AFB1 exposure, tumor size, tumor grade and stage, tumor MVD and MVI, and PVTT). The results demonstrated that patients with different TIMP3 expression had a similar MST and MRT among stratified variables AFP, AFB1 exposure, tumor size, tumor grade and stage, tumor MVD and MVI, and PVTT, but not AFB1 exposure (data not shown). Next, AFB1 exposure and TIMP3 was combined to divide the patients into four groups: Low AFB1 exposure plus HTE (AETE1), low AFB1 exposure plus LTE (AETE2), high AFB1 exposure plus HTE (AETE3), and high AFB1 exposure plus LTE (AETE4). Patients with LTE had a shorter OS and RFS in case of high AFB1 exposure compared with low AFB1 exposure (16.00 mo *vs* 28.00 mo for MST and 12.00 mo *vs* 37.00 mo for MRT) (Figure 1E and F, Table 6). Furthermore, these AHCC cases with AETE4 also featured a higher death risk (HR = 7.41; 95%CI: 4.49-12.23) and tumor recurrence risk (HR = 6.38; 95%CI: 3.68-11.05) (Table 6).

Multivariable survival analysis

To explore whether TIMP3 expression is an independent prognostic biomarker for AHCC prognosis, multivariable survival analyses were performed, which showed that the dysregulation of TIMP3 expression in cancerous tissues was an independent biomarker predicting AHCC survival (Table 7). Patients with LTE, compared with those with HTE, featured an increasing death risk (HR = 2.85; 95%CI: 2.04-4.00) and tumor recurrence risk (HR = 2.26; 95%CI: 1.57-3.26). However, multiplicative interactive effects of AFB1 exposure and TIMP3 expression on AHCC survival were not observed.

AFB1 exposure is negatively correlation with TIMP3 expression in HCC tissues

In view that AFB1 exposure is associated with tumor angiogenesis[14] and TIMP3 expression can modify MVD, we explored the correlation between AFB1 exposure and TIMP3 expression by Spearman's correlation analysis (Table 8). The results indicated that AFB1 exposure was negatively correlated with the levels of TIMP3 protein expression in cancerous

Table 2 Clinical pathological features of hepatocellular carcinoma according to tissue inhibitor of metalloproteinase-3 expression

Variable	LTE		HTE		*P* value
	n	Percentage (%)	*n*	Percentage (%)	
Total	89	100.0	93	100.0	
Age (years)					0.78
< 49	47	52.8	51	54.8	
≥ 49	42	47.2	42	45.2	
Gender					0.18
Male	70	78.7	65	69.9	
Female	19	21.3	28	30.1	
Race					0.89
Han	44	49.4	45	48.4	
Others	45	50.6	48	51.6	
Smoking					0.38
No	70	78.7	68	73.1	
Yes	19	21.3	25	26.9	
Drinking					0.50
No	68	76.4	67	72.0	
Yes	21	23.6	26	28.0	
HBsAg					0.13
Negative	26	29.2	37	39.8	
Positive	63	70.8	56	60.2	
Anti-HCV					0.47
Negative	71	79.8	78	83.9	
Positive	18	20.2	15	16.1	
AFP					4.66×10^{-13}
Negative	8	9.0	56	60.2	
Positive	81	91.0	37	39.8	
Tumor size					2.54×10^{-4}
≤ 5 cm	16	18.0	40	43.0	
> 5 cm	73	82.0	53	57.0	
ES grade					3.84×10^{-4}
Low	34	38.2	60	64.5	
High	55	61.8	33	35.4	
TNM stage					1.18×10^{-4}
I	6	6.7	24	25.8	
II	35	39.3	43	46.2	
III	48	53.9	26	28.0	
Liver cirrhosis					0.14
No	32	36.0	24	25.8	
Yes	57	64.0	69	74.2	
MVD					2.97×10^{-3}
Low	34	38.2	56	60.2	

High	55	61.8	37	39.8	
PVTT					2.32×10^{-5}
Negative	49	55.1	78	83.9	
Positive	40	44.9	15	16.1	
MVI					2.99×10^{-4}
Negative	45	50.6	71	76.3	
Positive	44	49.4	22	23.7	

HCV: Hepatitis C virus; AFP: Alpha fetoprotein; ES: Edmondson and Steiner; TNM: Tumor node metastasis; MVD: Microvessel density; PVTT: Portal vein tumor thrombus; MVI: Microvessel infiltration; LTE: Low TIMP3 expression; HTE: High TIMP3 expression.

Table 3 The risk effects of tissue inhibitor of metalloproteinase-3 expression in the cancerous tissues on the clinicopathological features of hepatocellular carcinoma

Risk effects	$OR_{LTE/HTG}$[1]	95%CI	*P* value
Higher AFP	15.32	6.63-35.38	1.65×10^{-10}
Higher tumor size	3.47	1.75-6.87	3.69×10^{-4}
Tumor dedifferentiation	3.04	1.65-5.65	4.31×10^{-4}
Higher TNM stage	2.60	1.64-4.07	4.21×10^{-5}
Higher MVD	2.45	1.35-4.45	3.20×10^{-3}
Higher risk of PVTT	4.38	2.18-8.83	3.52×10^{-4}
Higher risk of MVI	3.24	1.71-6.14	3.20×10^{-4}

[1]$OR_{LTE/HTG}$ represents the risk value of low *TIMP3* expression (compared to high *TIMP3* expression) on the knowledge clinicopathological features of hepatocellular carcinoma.
AFP: Alpha fetoprotein; TNM: Tumor node metastasis; MVD: Microvessel density; PVTT: Portal vein tumor thrombus; MVI: Microvessel infiltration; CI: Confidential interval; OR: Odds ratio.

tissues (r = -0.216; P = 0.003).

DISCUSSION

To our best knowledge, no studies have investigated the linkage between TIMP3 expression and AHCC prognosis. In this study, we explored the effects of TIMP3 protein expression in cancerous tissues on the prognosis of AHCC and found that the downregulation of TIMP3 expression in cancerous tissues shortened the OS and RFS of AHCC patients and increased their risk of death (HR = 2.85; 95%CI: 2.04-4.00) and tumor recurrence (HR = 2.26; 95%CI: 1.57-3.26). These findings imply that the dysregulation of TIMP3 expression may be a potential biomarker predicting AHCC prognosis.

AHCC is mainly caused by the exposure to AFB1 due to the contaminant of nutrients (such as grains, peanuts, soybeans, and corn) in tropic and subtropic regions, including East and South-East Asia and sub-Saharan Africa[15]. Nowadays, AFB1 has been regarded as a type I chemical carcinogen. Mechanically, AFB1 binds with DNA and results in the formation of ADAs and ultimately genomic instability, which is involved in DNA damage (including DNA single strand breaks, double strand breaks, chromosomal aberration damage, unscheduled DNA synthesis, abnormal chromatid exchange, the formation of micronuclei and macronuclei, and oxidation DNA damage), mutagenesis (mainly the mutation from GC to TA), the abnormality of tumor suppressor genes (including *p53*, *bcl2*, *p27*, *p16*, and *p21*) or oncogenes (mainly *c-myc* and *ras*), the interaction between AFB1 and other carcinogens (*e.g.*, HBV), inheritance alterations (*e.g.*, genetic variants in glutathione S-transferase T1), or immunosuppression (*e.g.*, decreasing the expression or secretions of cytokines such as tumor necrosis factor and interleukin)[16,17]. In view of ADAs playing an important role in the AHCC carcinogenesis, they have been regarded as a vital biomarker for HCC patients with exposure to AFB1[18]. In this study, AHCC was defined as HCC with ADAs in tumor tissues > 1.00 mmol/mol DNA mainly because individuals having this amount of AFB1 exposure featured a noticeable death risk and tumor recurrence risk[18]. All patients with AHCC included in the present study were from Guangxi , a high AFB1 exposure area in China, and had a median amount of ADAs of 2.00 ± 0.79 mmol/mol DNA. Similar to our previous studies[14,19], increasing levels of AFB1 exposure significantly shortened the OS and RFS of patients with AHCC.

Table 4 Univariable Kaplan-Meier survival analysis

	OS			RFS		
	De, *n* (%)	MST (95%CI)	*P* value	Re, *n* (%)	MRT (95%CI)	*P* value
Age (years)			0.71			0.97
< 49	79 (80.6)	27.00 (22.12-31.88)		68 (69.4)	27.00 (20.88-33.12)	
≥ 49	71 (84.5)	23.00 (19.04-26.96)		56 (66.7)	24.00 (18.84-29.16)	
Gender			0.68			0.55
Male	113 (83.7)	26.00 (2299-29.01)		91 (67.4)	24.00 (17.96-30.05)	
Female	37 (78.7)	26.00 (21.71-30.29)		33 (70.2)	25.00 (17.96-30.04)	
Race			0.76			0.26
Han	74 (83.1)	27.00 (22.55-31.76)		65 (73.0)	25.00 (19.01-30.99)	
Others	76 (81.7)	26.00 (21.88-30.81)		59 (63.4)	25.00 (15.63-34.37)	
Smoking			0.06			0.01
No	114 (82.6)	24.00 (20.33-27.67)		101 (73.2)	24.00 (18.62-29.38)	
Yes	36 (81.8)	27.00 (19.67-34.43)		23 (52.3)	33.00 (15.27-50.74)	
Drinking			0.10			0.03
No	110 (81.5)	24.00 (20.17-27.83)		97 (71.9)	24.00 (18.44-29.56)	
Yes	40 (85.1)	27.00 (22.53-31.47)		27 (57.4)	30.00 (15.73-44.27)	
HBsAg			0.26			0.05
Negative	44 (69.8)	28.00 (24.38-31.62)		34 (54.0)	29.00 (18.01-40.00)	
Positive	106 (89.1)	24.00 (19.47-28.53)		90 (75.6)	22.00 (16.52-27.48)	
Anti-HCV			0.45			0.88
Negative	123 (82.6)	24.00 (19.69-28.31)		99 (66.4)	24.00 (17.81-30.19)	
Positive	27 (81.8)	28.00 (23.80-32.20)		25 (75.8)	28.00 (20.61-35.39)	
AFP			1.85×10^{-9}			1.03×10^{-6}
Negative	44 (68.7)	41.00 (29.46-52.54)		35 (54.7)	40.00 (30.18-49.82)	
Positive	106 (89.8)	20.00 (17.24-22.77)		89 (75.4)	17.00 (13.10-20.90)	
AFB1 exposure			1.31×10^{-9}			9.39×10^{-10}
Low	58 (77.3)	36.00 (26.75-45.25)		38 (50.7)	43.00 (33.01-52.99)	
High	92 (86.0)	20.00 (17.22-22.78)		86 (80.4)	18.00 (14.25-21.75)	
Tumor size			5.52×10^{-8}			5.3×10^{-6}
≤ 5 cm	37 (66.1)	39.00 (27.27-50.73)		31 (55.4)	40.00 (31.01-48.99)	
> 5 cm	113 (89.7)	19.00 (15.84-22.16)		93 (73.8)	16.00 (12.48-19.52)	
ES grade			3.82×10^{-4}			9.07×10^{-3}
Low	75 (79.8)	33.00 (28.08-37.92)		61 (64.9)	30.00 (25.23-34.77)	
High	75 (85.2)	20.00 (17.07-22.93)		63 (71.6)	19.00 (13.83-24.17)	
TNM stage			7.40×10^{-17}			4.47×10^{-11}
I	24 (80.0)	45.00 (35.63-54.37)		19 (63.3)	37.00 (28.77-45.23)	
II	59 (75.6)	27.00 (23.79-30.21)		47 (60.3)	29.00 (21.89-36.11)	
III	67 (90.5)	12.00 (8.63-15.37)		58 (78.4)	10.00 (6.26-13.75)	
Liver cirrhosis			0.93			0.43
No	45 (80.4)	27.00 (21.49-32.52)		34 (60.7)	27.00 (21.56-32.44)	
Yes	105 (83.3)	25.00 (21.51-28.49)		90 (71.4)	24.00 (19.69-28.31)	

MVD			7.60×10^{-11}			1.07×10^{-11}
Low	68 (75.6)	34.00 (31.65-36.35)		49 (54.4)	36.00 (27.49-44.51)	
High	82 (89.1)	15.00 (11.87-18.13)		75 (81.5)	13.00 (9.00-16.01)	
PVTT			1.50×10^{-6}			9.99×10^{-7}
Negative	103 (81.1)	29.00 (24.61-33.39)		80 (63.0)	30.00 (25.71-34.29)	
Positive	47 (85.5)	12.00 (7.96-16.04)		44 (80.0)	8.00 (5.09-10.91)	
MVI			6.65×10^{-8}			1.55×10^{-7}
Negative	95 (81.9)	29.00 (24.69-33.31)		73 (62.9)	30.00 (25.90-34.10)	
Positive	55 (83.3)	11.00 (6.66-15.34)		51 (77.3)	8.00 (4.38-11.62)	
TIMP3			3.91×10^{-11}			1.89×10^{-6}
LTE	86 (96.6)	18.00 (16.64-21.36)		70 (78.7)	16.00 (12.31-19.69)	
HTE	64 (68.8)	36.00 (28.20-43.81)		54 (58.1)	32.00 (22.79-41.21)	

De: Death patients; Re: Patients with tumor recurrence; OS: Overall survival; RFS: Recurrence-free survival; HCV: Hepatitis C virus; AFP: Alpha fetoprotein; ES: Edmondson and Steiner; TNM: Tumor node metastasis; MVD: Microvessel density; PVTT: Portal vein tumor thrombus; MVI: Microvessel infiltration; LTE: Low *TIMP3* expression; HTE: High *TIMP3* expression; AFB1: Aflatoxin B1; MST: Median overall survival time; MRT: Median tumor reoccurrence-free survival time.

Table 5 Univariable Cox proportional hazard regression survival analysis

	OS		RFS	
Variable	**HR (95%CI)**	***P* value**	**HR (95%CI)**	***P* value**
Age (years), ≥ 49 *vs* < 49	1.06 (0.77-1.47)	0.72	1.01 (0.71-1.44)	0.97
Gender, male *vs* female	0.93 (0.64-1.35)	0.68	0.89 (0.59-1.32)	0.55
Race, Han *vs* others	0.95 (0.69-1.31)	0.76	0.80 (0.58-1.32)	0.27
Smoking, yes *vs* no	0.70 (0.48-1.03)	0.07	0.57 (0.36-0.89)	0.01
Drinking, yes *vs* no	0.74 (0.51-1.07)	0.11	0.63 (0.41-0.96)	0.03
HBV, positive *vs* negative	1.22 (0.86-1.74)	0.27	1.48 (0.99-2.19)	0.05
HCV, positive *vs* negative	0.85 (0.56-1.30)	0.46	0.97 (0.62-1.50)	0.89
AFP, positive *vs* negative	2.93 (2.03-4.24)	1.18×10^{-8}	2.63 (1.75-3.95)	3.04×10^{-6}
AFB1 exposure, high *vs* low	3.00 (2.06-4.36)	8.43×10^{-9}	3.41 (2.25-5.18)	8.67×10^{-9}
Tumor size (cm), > 5 *vs* ≤ 5	2.70 (1.85-3.93)	2.63×10^{-7}	2.50 (1.65-3.77)	1.37×10^{-5}
ES grade, III-IV *vs* I-II	1.79 (1.29-2.49)	5.55×10^{-4}	1.60 (0.79-2.30)	0.01
TNM stage, II *vs* I	1.57 (0.97-2.53)	0.06	1.35 (0.79-2.30)	0.27
TNM stage, III *vs* I	5.95 (3.56-9.94)	1.01×10^{-11}	4.29 (2.48-7.41)	1.92×10^{-7}
Liver cirrhosis, yes *vs* no	1.02 (0.72-1.44)	0.93	1.17 (0.79-1.74)	0.43
MVD, high *vs* low	2.87 (2.06-4.02)	6.84×10^{-10}	3.40 (2.34-4.96)	1.82×10^{-10}
PVTT, positive *vs* negative	2.30 (1.62-3.28)	4.06×10^{-6}	2.43 (1.68-3.53)	2.95×10^{-6}
MVI, positive *vs* negative	2.48 (1.75-3.50)	2.56×10^{-7}	2.53 (1.76-3.64)	5.63×10^{-7}
TIMP3, LTE *vs* HTE	2.92 (2.19-4.09)	4.10×10^{-10}	2.34 (1.63-3.38)	4.80×10^{-6}

OS: Overall survival; HR: Hazard ratio; RFS: Recurrence-free survival; HCV: Hepatitis C virus; HBV: Hepatitis B virus; AFP: Alpha fetoprotein; ES: Edmondson and Steiner; TNM: Tumor node metastasis; MVD: Microvessel density; PVTT: Portal vein tumor thrombus; MVI: Microvessel infiltration; LTE: Low *TIMP3* expression; HTE: High *TIMP3* expression; AFB1: Aflatoxin B1.

Table 6 Effects of aflatoxin B1 exposure and tissue inhibitor of metalloproteinase-3 expression on survival of patients with aflatoxin B1-related hepatocellular carcinoma

	OS		RFS	
Variable	MST (95%CI)	HR (95%CI/*P* value)	MRT (95%CI)	HR (95%CI/*P* value)
AETE1	47.00 (43.20-50.80)	1.00	49.98 (42.03-57.93)[1]	1.00
AETE2	28.00 (21.25-34.75)	2.51 (1.48-4.24/6.44 × 10^{-4})	37.00 (21.45-52.55)	1.84 (0.95-3.57/0.07)
AETE3	26.00 (21.29-30.71)	2.59 (1.53-4.40/4.04 × 10^{-4})	28.00 (21.60-34.40)	2.90 (1.63-5.15/2.86 × 10^{-4})
AETE4	16.00 (11.72-20.28)	7.41 (4.49-12.23/4.77 × 10^{-15})	12.00 (7.65-16.35)	6.38 (3.68-11.05/3.85 × 10^{-11})

[1]Because median tumor reoccurrence-free survival time for AETE1 cannot be calculated using the Kaplan-Meier survival method, it was replaced by the average survival time for AETE1.

AETE1: Low aflatoxin B1 exposure plus high TIMP3 expression; AETE2: Low aflatoxin B1 exposure plus low TIMP3 expression; AETE3: High aflatoxin B1 exposure plus high TIMP3 expression; AETE4: High aflatoxin B1 exposure plus low TIMP3 expression; OS: Overall survival; HR: Hazard ratio; RFS: Recurrence-free survival; CI: Confidential interval.

Table 7 Mulvariable Cox proportional hazard regression analysis for survival

	OS		RFS	
Variable	HR (95%CI)	*P* value	HR (95%CI)	*P* value
AFP, positive *vs* negative	2.85 (1.96-4.15)	5.06 × 10^{-8}	2.48 (1.64-3.73)	1.44 × 10^{-5}
AFB1 exposure, high *vs* low	2.92 (1.99-4.27)	3.71 × 10^{-8}	3.23 (2.12-4.94)	5.78 × 10^{-8}
Tumor size (cm), > 5 *vs* ≤ 5	2.93 (1.99-4.31)	4.53 × 10^{-8}	2.79 (1.83-4.25)	2.00 × 10^{-6}
ES grade, III-IV *vs* I-II	1.81 (1.29-2.55)	6.19 × 10^{-4}	1.63 (1.12-2.37)	0.01
TNM stage, II *vs* I	1.61 (1.02-2.42)	0.03	1.75 (1.04-2.45)	0.02
TNM stage, III *vs* I	6.22 (3.70-10.48)	6.21 × 10^{-12}	4.43 (2.54-7.71)	1.47 × 10^{-7}
MVD, positive *vs* negative	2.88 (2.05-4.04)	1.07 × 10^{-9}	3.34 (2.29-4.89)	4.71 × 10^{-10}
PVTT, positive *vs* negative	2.30 (1.59-3.32)	8.73 × 10^{-6}	2.34 (1.60-3.42)	1.16 × 10^{-5}
MVI, positive *vs* negative	2.48 (1.74-3.54)	5.63 × 10^{-7}	2.44 (1.68-3.53)	2.42 × 10^{-6}
TIMP3, LTE *vs* HTE	2.85 (2.04-4.00)	1.17 × 10^{-9}	2.26 (1.57-3.26)	1.26 × 10^{-5}

OS: Overall survival; HR: Hazard ratio; RFS: Recurrence-free survival; CI: Confidential interval; AFP: Alpha fetoprotein; AFB1: Aflatoxin B1; ES: Edmondson and Steiner; TNM: Tumor node metastasis; MVD: Microvessel density; PVTT: Portal vein tumor thrombus; MVI: Microvessel infiltration; LTE: Low TIMP3 expression; HTE: High TIMP3 expression.

Table 8 Correlation between aflatoxin B1 exposure and tissue inhibitor of metalloproteinase-3 expression in hepatocellular carcinoma

	Low AFB1 exposure		High AFB1 exposure		*r*	*P* value
	n	Percentage (%)	*n*	Percentage (%)		
LTE	27	36.0	62	57.9	-0.216	0.003
HTE	48	64.0	45	42.1		

AFB1: Aflatoxin B1; LTE: Low TIMP3 expression; HTE: High TIMP3 expression.

TIMP3 is a member of *TIMP* gene family (mainly consisting of *TIMP1* to 4) that has been proved to act as a class of negative regulators of matrix metalloproteinases by regulating the degradation of ECM and tissue remodeling. TIMP3 plays an important role not only in stabilizing tissue construction and function, but also in inhibiting the migration of endothelial cells and inducing cell apoptosis[7,9]. In the past decades, increasing evidence has shown that the dysregulation of TIMP3 expression may be involved in the pathogenesis of some human cancers such as liver cancer[20-26], colon cancer[27], esophageal cancer[28], gastric carcinoma[28], renal clear cell carcinoma[29], pancreatic endocrine tumors[30], meningiomas[31], and so on. Most of previous studies showed that TIMP3 expression is downregulated and results in

decreased apoptosis of cancer cells and increased capacity of tumor proliferation, angiogenesis, invasion, and metastasis [7,9]. In this study, we found that the downregulation of TIMP3 protein expression in AHCC cancerous tissues was correlated with an increased risk of tumor proliferation, dedifferentiation, MVD, MVI, and metastasis, and ultimately shortened the OS and RFS of patients with AHCC. This suggest that TIMP3 has tumor suppressing effects in liver cancer. Supporting our findings, several recent studies[20,21,23,25,26,32-34] have demonstrated that the dysregulation of TIMP3, *e.g.*, expression downregulation, genetic variables, and abnormal promoter methylation, results in the progression of hepatocellular carcinogenesis. Also, a previous study has shown that non-viral vectors encoding TIMP3 have the potential to treat HCC[35]. However, RNA *in situ* hybridization implied that *TIMP3* expression is upregulated in head and neck cancer and this upregulation is linked with tumor development and progression[36]. Results from carcinogen-induced HCC mouse model analyses revealed that these mice with TIMP3 loss had a decreased risk of HCC following acute carcinogen treatment, possibly because of the loss of TIMP3 expression, which induces p38 pathway-related cell cycle arrest and hepatic cell differentiation[21]. Taken together, TIMP3 may have dual effects in cancers, which is associated with different signal pathways, such as cell-cycle regulation pathway, cell differentiation pathway, and inflammation pathway[21,37].

Interestingly, our study demonstrated that TIMP3 expression was negatively associated with AFB1 exposure and that the effects of TIMP3 protein expression on AHCC survival were more obvious among patients with high AFB1 exposure. However, we did not find evidence of multiplicative interaction of AFB1 exposure and TIMP3 expression. This implies that TIMP3 and AFB1 may affect the prognosis of AHCC *via* an addictive interaction. Altogether, AHCC patients with both downregulation of TIMP3 expression and high AFB1 exposure should obtain more attention to improve their survival.

CONCLUSION

In summary, our study has demonstrated that the dysregulation of TIMP3 expression is significantly associated with the clinicopathological features of AHCC and affects the survival of AHCC patients. Our findings suggest that decreased TIMP3 protein expression may act as a potential biomarker for predicting the survival of AHCC patients. However, the present study had several limitations. First, because this study was based on a hospital-based retrospective clinicopathological analysis of paraffin-embedded samples, differential analyses of TIMP3 expression between tumor tissues and non-tumor tissues (including normal liver tissues and liver tissues with lesions such as inflammation, cirrhosis, atypical hyperplasia, or adenoma) were not accomplished. Second, despite the levels of TIMP3 protein expression being tested, we did not evaluate the levels of *TIMP3* mRNA expression in cancerous tissues. Finally, although we investigated the effects of TIMP3 expression in AHCC tissues on tumor clinicopathological features and prognosis, functional analyses were not performed. Therefore, further exploration is needed to elucidate the exact molecular mechanisms underlying our findings.

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FOOTNOTES

Author contributions: Liang QJ and Long QQ performed the experiments, formal analysis, and data curation, and they contributed equally to this work and should be considered as co-first authors; Tian FQ, Su QY, and Zhu XY constructed the histopathological sample library and collected the clinicopathological information and following-up data for all patients; Long XD conceptualized and designed the study, received grant support, had full access to the data, and is responsible for the integrity and accuracy of data analysis; all authors contributed to data acquisition and interpretation and reviewed and approved the final version.

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