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Serum thymosin beta 10 level as a potential prognosis prediction of hepatocellular carcinoma and hepatic diseases

Faten M. Zahran[1], Arwa I. EL Ksass[1], and Ashraf A. Tabll[2,3]

[1]Biochemistry Division, Department of Chemistry, Faculty of Science, Zagazig University, Zagazig, Egypt.

[2]Department of Microbial Biotechnology, Biotechnology Research Institute, National Research Center, Giza, Egypt.

[3]Egypt Center for Research and Regenerative Medicine (ECRRM), Cairo, Egypt.

Corresponding author: Arwa I. EL Ksass, Department of Biochemistry Division, Department of Chemistry, Faculty of Science, Zagazig University, Zagazig, Egypt.
Email: arwaelksass@gmail.com.

Abstract

Thymosin Beta 10 (TMSB10) is a thymosin family member that has been identified as being overexpressed in a wide variety of human cancers. This study aimed to determine the expression level of TMSB10 in sera of patients with hepatocellular carcinoma (HCC) and liver cirrhosis. And to reveal the association between TMSB10 and different stages in patients with HCC. We also wanted to know how TMSB10 is predictive in HCC patients and its relation to the Barcelona Clinic's staging system. The study included 41 HCC patients, 15 liver cirrhosis patients, and 15 normal control subjects. The enzyme-linked immunosorbent assay was used to determine serum levels of TMSB10 and alpha-fetoprotein (AFP) in serum of normal control individuals and patients with liver cirrhosis and different stages of HCC, and to evaluate the relationship of AFP with TMSB10. The TMSB10 levels in patients with HCC were statistically different than in the control group and in the different stages of HCC. We found a statistically significant difference in the distribution of TMSB10 between the three study groups ($p< 0.001$). There was an association between TMSB10 concentration and AFP. The level of TMSB10 in the serum of the HCC subgroups was then analyzed. The TMSB10 level increased with the advance of HCC stages. The TMSB10 level in HCC patients did not correlate with levels of liver function tests including aminotransferase, aspartate aminotransferase, albumin, prothrombin, bilirubin, or alkaline phosphatase. In conclusion, serum TMSB10 levels can be used as a potential prognostic marker for clinical stages of HCC.

Keywords: Tumor marker, Thymosin Beta 10, hepatocellular carcinoma, liver cirrhosis.

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Introduction

Hepatocellular carcinoma (HCC) is a highly prevalent disease globally, ranking sixth in terms of frequency of diagnosis. It also stands as the fourth leading cause of cancer-related mortality annually. Over the past decade, there was a notable increase in the incidence of HCC, further emphasizing its status as one of the most lethal forms of cancer.[1] The incidence of HCC was substantially raised in those suffering

from chronic liver disorders. Globally, Egypt exhibits the greatest prevalence of Hepatitis C Virus (HCV) as approximately 14.7% of the general population in the country is afflicted with this infectious disease, according to the study conducted by Omar, et al., 2023.[2] Egypt exhibits a notable incidence of HCV infection, along with a considerable load of nonviral liver ailments like fatty liver disease and autoimmune liver disorders. Additionally, Egypt demonstrates a considerably elevated prevalence of metabolic syndrome.[3]

The utilization of antiviral medications can significantly influence the progression and ultimate result of chronic liver illnesses caused by hepatitis B virus (HBV) and HCV. The treatment goals encompass the sustained inhibition of viral replication over an extended period, eradication of infection (specifically for HCV), and reduction of both liver-related mortality and morbidity.[4] HCC has significantly impacted people's health because of its aggressive invasion, quick progression, and poor prognosis. In the early stages of the condition, patients do not have any distinct symptoms or signs. After being identified, they are already in the late and middle stages.[5] Consequently, it is extremely important to discover a technique for accurate detection to reduce HCC disease mortality and extend the survival period of these patients.

Biomarkers are a class of cell-related indicators, could be used to investigate the disease at the molecular level and provide precise and sensitive assessment of low-level and early damage.[5] The diagnosis of HCC involves the evaluation of various biomarkers. These biomarkers encompass protein markers that are either downregulated or upregulated during the HCC development, as well as circulating nucleic acids or cells, metabolites, and recently discovered biomarkers that show promise. One such promising approach involves the use of quantitative proteomics through the application of isobaric tags for relative and absolute quantitation.[2]

The ideal biomarker possesses several universal characteristics for routine clinical analysis, including sensitivity, specificity, low operator experiences requirements, low cost, high reproducibility, rapid results, correlation with tumor stages, and availability of samples (such as blood or urine) without the need for pretreatment.[6]

There are different methods for classification of different stages of HCC. In this study we used the Barcelona Clinic liver cancer staging system (BCLC).[7] The BCLC staging approach is extensively employed in both North America and Europe. The initial categorization of patients was conducted based on the BCLC classification, which involved the division into four distinct groups labeled A, B, C, and D. Subsequently, an additional stage labeled as stage 0 was introduced to specifically identify patients with extremely early-stage of HCC. The BCLC system incorporates various factors, including patient performance status, tumor load (including quantity, size, vascular invasion, and metastases), and liver function. Individuals diagnosed with BCLC stage 0, also known as an exceedingly early stage, exhibit the presence of a solitary nodule measuring less than 2 cm in diameter. Individuals diagnosed with BCLC stage A exhibit the presence of a solitary tumor of any dimension or up to three tumors, each measuring less than 3 cm in size. Patients classified as BCLC stage B exhibit the presence of multinodular tumors that are larger compared to those observed in BCLC stage A. However, these tumors do not display any signs of vascular invasion or extrahepatic spread. Patients in stage C of the BCLC classification who are diagnosed with advanced HCC exhibit the presence of many nodules in the liver, along with evidence of vascular invasion and/or the spread of cancer cells beyond the liver to other organs or tissues.[7]

Thymosin 10 (TMSB10) belongs to the family of thymosins, which comprise acidic 5-kDa peptides with 40–44 amino acid residues that are highly conserved.[8] TMSB10 is primarily found in the cytoplasm and has 43 amino acid residues. TMSB10 is known to play a role in controlling cell proliferation and motility and overexpressed in the majority of human cancers.[9] TMSB10 initially displayed a high reactivity rate (96%) in tumor tissues.[10] In 2015, TMSB10 was revealed as overexpressed in HCC tissues and associated with the tumor, nodes,

metastasis stage and patients' overall survival.[11] TMSB10 significantly influences breast cancer patients' carcinogenesis and metastasis. The clinical stages of breast cancer are highly correlated with the expression level of TMSB10 in the serum of breast cancer patients.[12] A previous study demonstrated that TMSB10, an oncogene that exhibits increased expression in a wide range of human malignancies, including HCC.[13] In 2019, the study by Song, et al.,[13] presented more evidence on the clinical significance and biological function of TMSB10 in HCC. According to the datasets from the Cancer Genome Atlas, the expression levels of TMSB10 were significantly elevated in HCC tissues in comparison to normal liver tissues.[13] Additionally, there was a significant correlation between elevated TMSB10 expression and advanced tumor stage, large tumor size, distant metastasis, and bad prognosis. Research conducted on the loss-of-function of specific gene or proteins demonstrated that the inhibition of TMSB10 expression resulted in a substantial decrease in HCC cell proliferation, migration, and invasion. TMSB10 exhibits potential as a tumor biomarker for prognostic prediction and as a prospective target for the development of an innovative therapeutic strategy.[13] In the present study, we aimed to measure TMSB10 in the blood of people with HCC, and to determine its predictive value for HCC, and its correlation with HCC stages.

Subjects and Methods

This case-control study included a total of 71 participants, categorized into two groups. A normal control group consisting of 15 subjects, and HCC patients' group consisting of 56 individuals. The patient group comprised newly diagnosed patients with two specific conditions: 41 HCC patients, and 15 liver cirrhosis patients. These participants were selected from the Internal Medicine and Oncology unit at Zagazig University Hospital, during the period August 2022 to October 2022.

The 41 HCC patients were divided into four groups according to the Barcelona Clinic liver cancer staging system (BCLC).[7] Group 1 included 10 HCC patients in the BCLC-0 stage, Group 2, 10 HCC patients in the BCLC-A stage, Group 3, 10 HCC patients in the BCLC-B stage, and Group 4, 11 HCC patients in the BCLC-C stage.

The patients' diagnosis was established using a combination of clinical evaluation, radiographic findings, and histological investigation of tissue biopsies. All these variables were recruited from the hospital data profiles of the patients. Patients with a history of neoplasm treatment that would hinder adequate determination of study variables such as the amount of TMSB10, were excluded from the study.

A fresh venous blood sample (5 ml) was obtained from each liver cirrhosis patient, HCC patient, and the control group. All blood specimens were obtained via venipuncture and divided into two tubes. A plain tube for the separation of serum for estimation of the biochemical parameters, and an EDTA tube both of which were stored at -20 °C until used. All blood samples were used for routine laboratory investigations (liver function tests), AFP, and TMSB10.

Determination of biochemical parameters

The hospital data records of the study patients were based of the following methods. A clinical blood chemistry analyzer (Chemistry Analyzer, semi-auto Photometer 5010, Germany), was used to measure liver function assays, according to the manufacturer's instructions. For these assays, different kits were used from the human diagnostic company (Stegelitzer Straße 339126 Magdeburg, Germany), according to the manufacturer's instructions. For accurate determination of coagulation assays (CoaData 504 device, Semi-automated 1-channel coagulation analyzer) was used to determine prothrombin time (PT). For the PT assay, the kits (Cambridge, UK), were used according to the manufacturer's instructions.

Determination of AFP

To determine AFP, the commercial kits (catalog number L2KAP2, IMMULITE 2000, Siemens Healthcare, GmbH, Germany), were used according to the manufacturer's instructions.

Determination of serum levels of TMSB10

To determine TMSB10 in serum samples, the enzyme-linked immunosorbent assay (ELISA) technique was used. We used competitive ELISA kits (Cat.NO EA0201Hu, Shanghai Korain Biotech company, Shanghai, China), according to the manufacturer's instructions. The absorbance, optical density (OD) reading, of the final ELISA product was measured at a wavelength of 450 nm using an ELISA reader (TECAN; SUNRISE, Austria, GmbH, Germany). The TMSB10 content in a sample was determined by the observed color intensity. Based on standards included in the kits, a standard curve of their OD readings was drawn. Then the concentration values of serum samples from the different study groups were calculated using the Origin Lap 2019 program, as depicted in Figure 1.

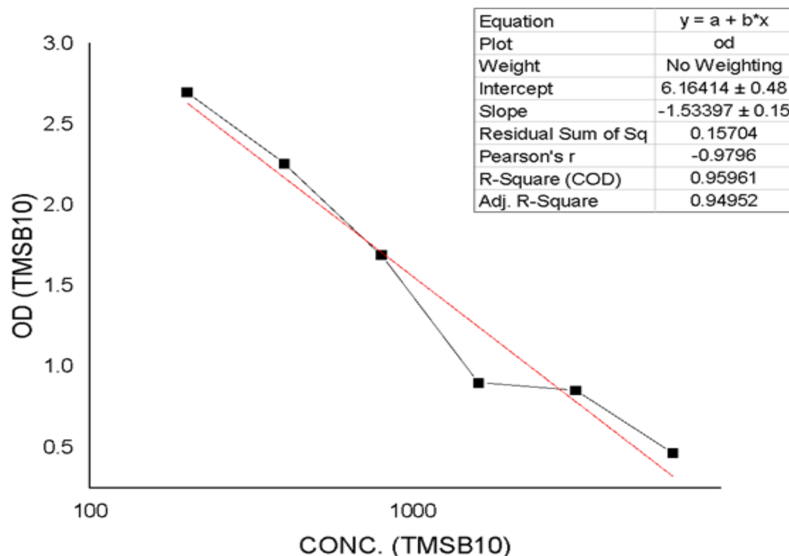

Figure 1. Standard curve of Thymosin Beta 10 (TMSB10) as measured by commercial ELISA.

Statistical Methods

The data were analyzed using the Statistical Package for Social Sciences (SPSS), version 28 (IBM, SPSS Inc., Chicago, IL). The numerical data were characterized using either the median and range or the mean and standard deviation, depending on the context. The qualitative data are presented in terms of the number of occurrences and the corresponding percentage. The Chi-square test (specifically Fisher's exact test) was employed to analyze the association between categorical variables. The normality of numerical variables was assessed by the Kolmogorov-Smirnov test and the Shapiro-Wilk test. Non-parametric tests were used for non-normally distributed variables. Group comparisons were conducted using the Kruskal-Wallis test, followed by the Mann-Whitney U test. The experiments conducted were two-tailed. To account for multiple comparisons, the p-values were adjusted using the Bonferroni correction. The determination of sensitivity, specificity, positive predictive value, negative predictive value, and overall accuracy, and their corresponding 95% confidence intervals, was calculated using the receiver operating characteristics (ROC) curve analysis and a logistic regression model. A *p*-value ≤0.05 was considered statistically significant.

Results

There was no statistically significant variation in sex distribution across the three study groups (p=0.239). However, we observed a statistically significant variation in the age distribution across the three study groups (p< 0.001).

Association between the different groups and liver function tests

The distribution of biochemical parameters including alanine transaminase (ALT), aspartate transaminase (AST), albumin, total bilirubin, direct bilirubin, alkaline phosphatase (ALP), prothrombin time, and prothrombin concentration in the three groups (control, liver cirrhosis, and HCC) is shown in Table 1. There was a statistically significant difference in the distribution of all biochemical parameters between the three groups ($p<0.001$), as shown in Table 1.

Table 1. Association of the biochemical parameters between the different study groups.

		Study Groups			
		Control (n=15)	Cirrhosis (n=15)	HCC (n=41)	*p*-value
Age (years)	Mean ± SD Median (range)	34.73 ±14.15 27.00 (20.00-58.00) (b)	61.40 ±12.45 60.00 (45.00-84.00) (b)	56.90 ±13.18 58.00 (20.00-79.00) (a)	< 0.001
ALT (U/L)	Mean ± SD Median (range)	15.33 ±9.69 12.00 (2.00-34.00) (a)	52.67 ±41.39 54.60 (6.90-124.30) (b)	66.68 ±72.27 39.30 (10.10-421.00) (b)	< 0.001
AST (U/L)	Mean ± SD Median (range)	11.87 ±6.56 12.00 (3.00-23.00) (a)	74.19 ±63.25 63.50 (14.20-205.70) (b)	118.43 ±109.96 73.40 (16.70-436.00) (b)	< 0.001
ALP (U/L	Mean ± SD Median (range)	68.53 ±16.58 65.00 (46.00-91.00) (a)	121.80 ±72.23 90.00 (50.00-275.00) (b)	188.83 ±136.20 148.00 (44.00-784.00) (b)	< 0.001
Albumin (g/dL)	Mean ± SD Median (range)	3.92 ±0.53 3.89 (3.12-5.10) (a)	2.47 ±0.76 2.31 (1.55-4.43) (b)	2.44 ±0.70 2.37 (0.93-4.08) (b)	< 0.001
Total bilirubin (mg/dL)	Mean ± SD Median (range)	0.58 ±0.15 0.57 (0.32-0.85) (a)	2.73 ±3.22 1.30 (0.16-10.00) (b)	5.67 ±4.88 4.14 (0.35-15.80) (b)	< 0.001
direct bilirubin (mg/dL)	Mean ± SD Median (range)	0.20 ±0.06 0.19 (0.12-0.34) (a)	3.21 ±3.55 0.75 (0.05-8.89) (b)	4.59 ±3.96 4.10 (0.14-12.80) (b)	< 0.001
Total protein (g/dL)	Mean ± SD Median (range)	6.94 ±2.77 6.58 (3.24-14.10)	8.58 ±7.30 6.10 (3.83-28.70)	6.06 ±0.70 5.91 (4.50-7.58)	NS
PT (sec.)	Mean ± SD Median (range)	12.18 ±0.69 12.30 (11.00-13.40) (a)	19.42 ±5.34 17.50 (11.90-26.80) (b)	19.63 ±4.90 19.60 (11.00-26.80) (b)	< 0.001
PC %	Mean ± SD Median (range)	101.87 ±5.70 101.00 (95.00-112.30) (a)	50.17 ±18.09 50.70 (28.00-102.90) (b)	52.08 ±22.08 50.30 (29.60-102.90) (b)	< 0.001

Cells that are sharing same letter (a or b) are not statistically significantly different. $p > 0.05$ is not significant (NS).

Comparison of alpha-fetoprotein (AFP) and Thymosin Beta10 (TMSB10) between the study groups

In the HCC group there was considerably higher median levels of AFP than in the control and cirrhotic groups. In contrast, there was an opposing trend in TMSB10 concentration, as shown in Table 3. Moreover, there was a statistically significant inverse association between AFP and TMSB10 concentration (p= 0.006), as shown in Tables 2, 3 and Figure 2.

Table 2. The association between Alpha-fetoprotein (AFP) and Thymosin Beta 10 (TMSB10) among the different study groups.

Group		AFP (ng/ ml)	TMSB10 Concentration (ng/ml)
Control (n=15)	Mean ± SD	10.49 ±10.89	627.74 ±291.46
	Median (range)	6.10 (1.06-34.21) (a)	602.29 (244.72-1204.99) (a)
Cirrhosis (n=15)	Mean ± SD	5476.11 ±14469.59	322.68 ±176.53
	Median (range)	5.80 (1.70-44463.00) (b)	306.97 (73.64-721.16) (b)
HCC (n=41)	Mean ± SD	7783.16 ±12707.41	270.07 ±243.21
	Median (range)	895.00 (1.50-36771.00) (b)	210.61 (40.40-1281.48) (b)
p-value		< 0.001	< 0.001

Cells that are sharing same letters (a or b) are not statistically significantly different. $p \le 0.05$ is significant.

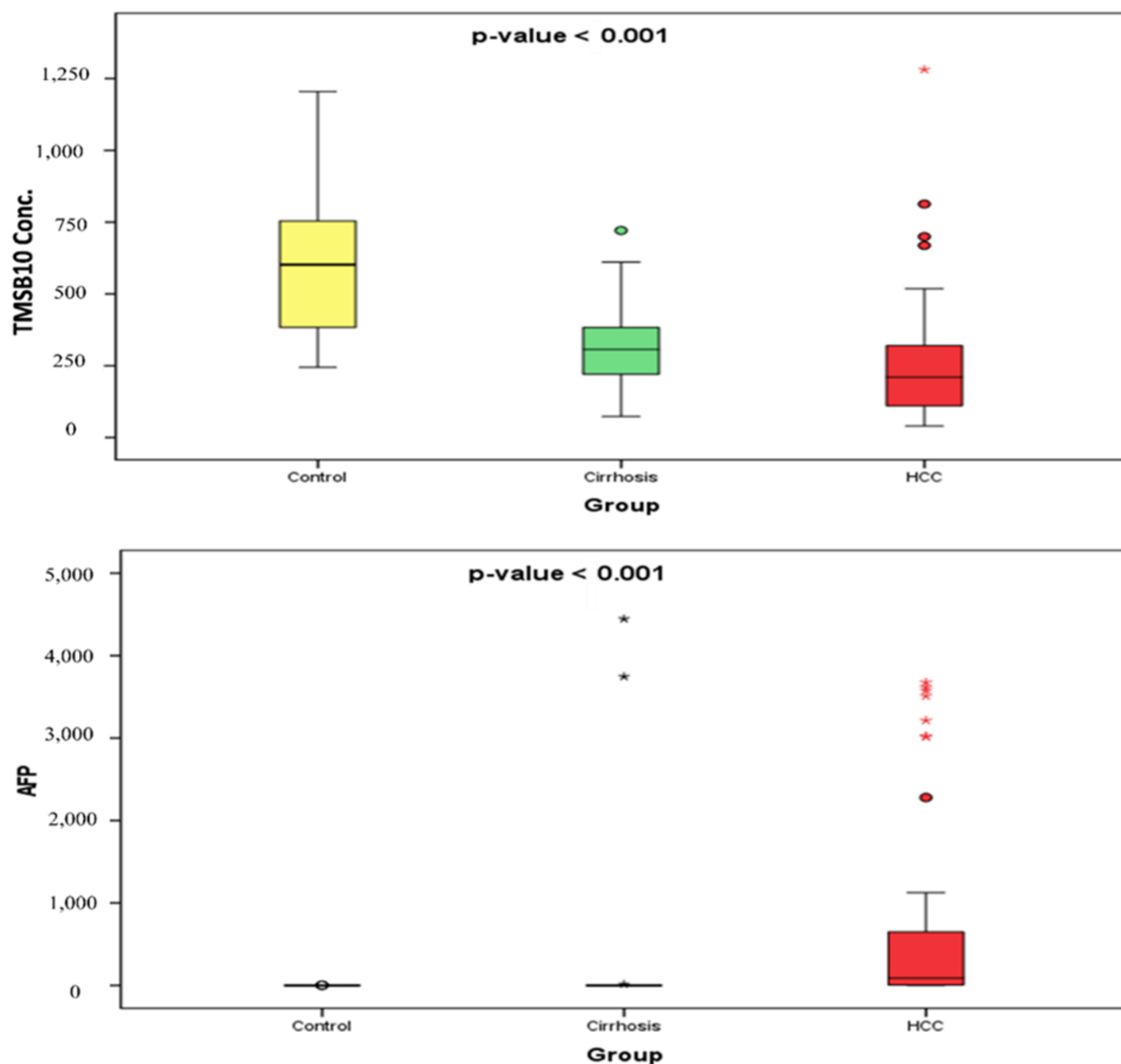

Figure 2. Distribution of alpha-fetoprotein (AFP) and Thymosin Beta 10 (TMSB10) concentrations among all study groups.

Table 3. The association between the concentrations of the alpha-fetoprotein (AFP) and Thymosin Beta 10 (TMSB10).

TMSB10 (ng/ml)		AFP (ng/ ml)		*p*-value
		≤11.10 (n=8)	>11.10 (n=33)	
≤ 170.37	No	0	18	< 0.006
	%	0.0%	54.5%	
> 170.37	No	8	15	
	%	100.0%	45.5%	
	%	25.0%	81.8%	

p ≤ 0.05 is significant.

Table 4. Area Under the Curve for alpha-fetoprotein (AFP) and Thymosin Beta 10 (TMSB10) concentration.

Study Variables	Area Under the Curve	Standard error	*p*-value	95% Confidence Interval	
				Lower Bound	Upper Bound
AFP (ng/ mL)	0.789	0.060	<0.001	0.672	0.906
TMSB10 concentration	0.754	0.059	<0.001	0.638	0.871

p ≤ 0.05 is significant.

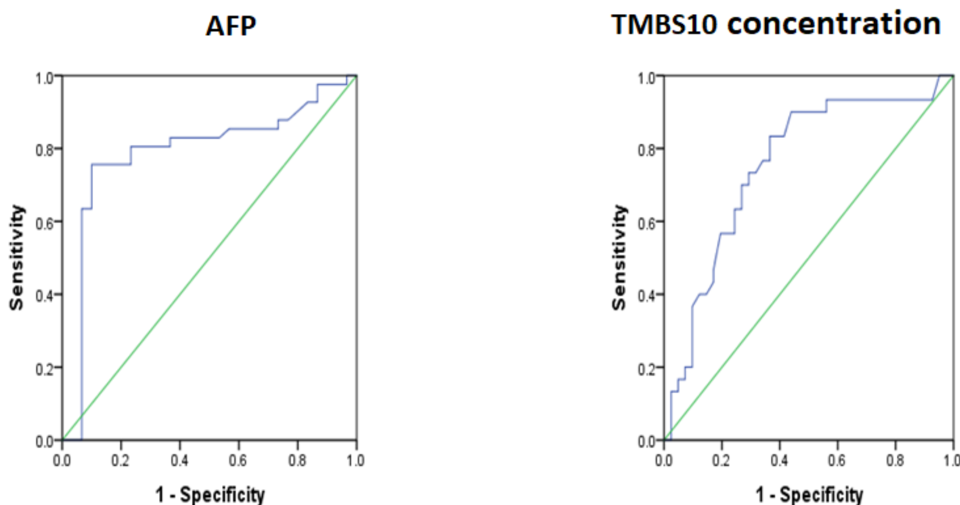

Figure 3. Receiver operating characteristics (ROC) curve of alpha-fetoprotein (AFP) and Thymosin Beta 10 (TMBS10) concentration.

There was a statistically significantly difference of AFP and TMSB10 concentration between all different stages of HCC groups (HCC 0, HCC a, HCC b, and HCC c) (*p*= 0.035 and *p*= 0.016, respectively) Table 5. There was an inverse association between TMSB10 concentration and HCC staging, as TMSB10 concentration decreases with the advance in HCC stages Figure 4.

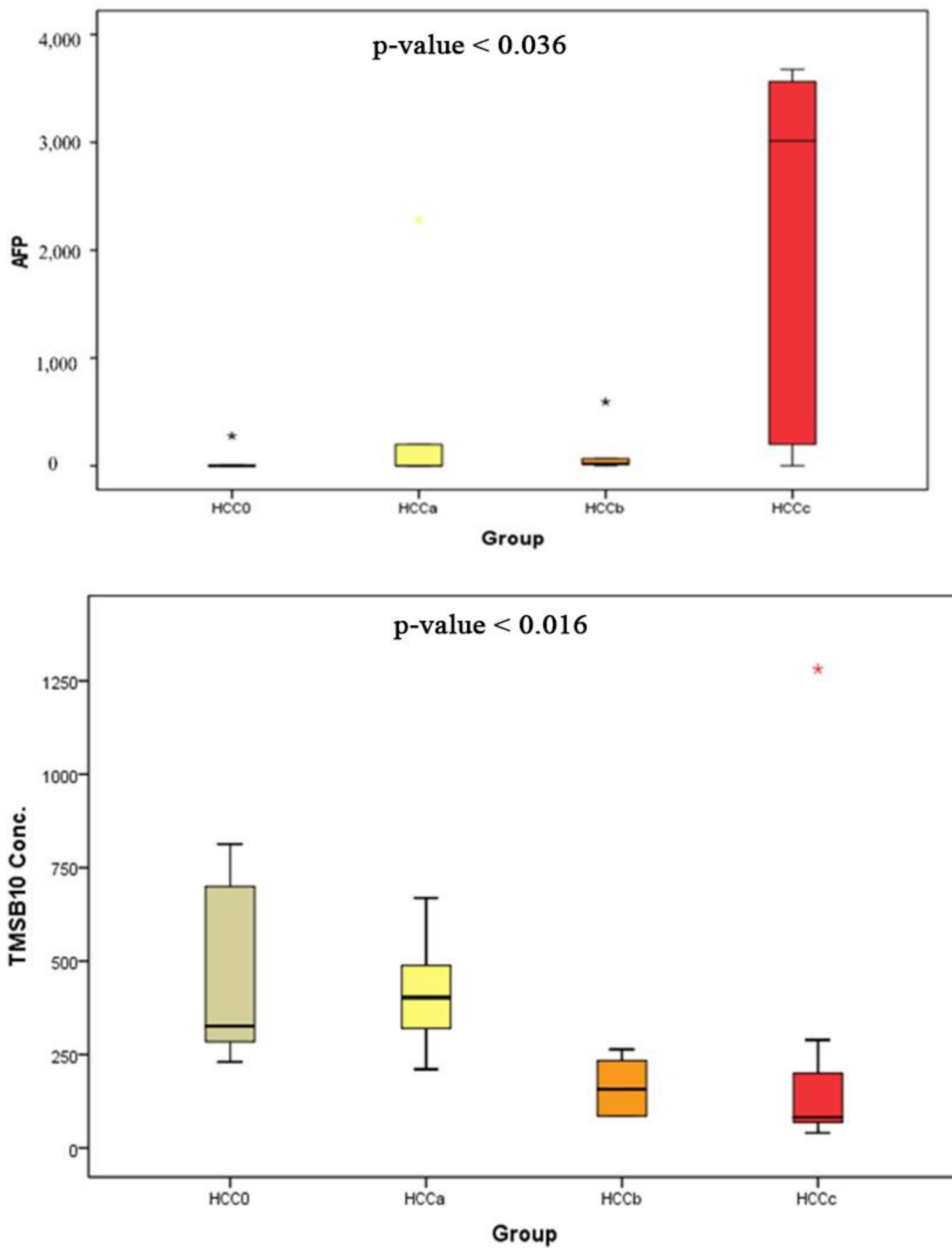

Figure 4. Distribution of Alpha-fetoprotein (AFP)and Thymosin Beta 10 (TMSB10) concentration among the hepatocellular carcinoma (HCC) stage groups.

Table 5. Concentrations of alpha-fetoprotein (AFP) and Thymosin Beta 10 (TMSB10) among the different HCC stages.

HCC stages		Age (years)	AFP	TMSB10 Conc.
HCC 0	Mean ± SD	53.33 ±10.80	477.08 ±1123.53	446.66 ±245.51
	Median (range)	52.50 (39.00-70.00)	11.90 (1.50-2770.00)	326.07 (230.46-813.18)
HCC a	Mean ± SD	57.50 ±12.90	4134.22 ±9179.36	415.64 ±156.64
	Median (range)	58.00 (40.00-79.00)	3.85 (2.30-22801.00)	402.73 (210.61-669.02)
HCC b	Mean ± SD	70.67 ±7.69	1185.08 ±2333.42	163.79 ±78.33
	Median (range)	71.00 (58.00-79.00)	190.00 (2.50-5926.00)	156.93 (85.57-263.79)
HCC c	Mean ± SD	60.00 ±11.70	20320.51 ±17900.62	277.20 ±450.53
	Median (range)	59.00 (40.00-74.00)	30155.00 (7.60-36771.00)	81.80 (40.40-1281.48)
p-value		NS	< 0.035	< 0.016

$p > 0.05$ is not significant (NS).

Discussion

HCC is commonly associated with an unfavorable prognosis due to its tendency to remain asymptomatic during the first stages when curative treatment options are most effective. Consequently, by the time HCC is diagnosed, it has often progressed to an advanced stage.[13] Traditionally, non-invasive criteria are used to diagnose HCC, and the treatment option is determined by the total tumor burden and the degree of underlying liver disease. The BCLC staging technique is widely utilized in North America and Europe. The BCLC system classifies HCC stages according to patient performance status, tumor load (number, size, vascular invasion, and metastases).[7]

In the present study, the TMSB10 expression status was measured in HCC patients' serum and compared with liver cirrhosis patients and the control group. First, we observed that the TMSB10 concentration levels in the serum of patients with HCC and liver cirrhosis were decreased compared with the serum of the normal control group. TMSB10 mRNA and protein levels were also observed to be higher in HCC tissue samples compared to normal adjacent liver tissue samples.[13] Furthermore, it was observed that high TMSB10 expression was significantly linked with advanced tumor stages, large tumor size, distant metastasis, and poor prognosis and that it worked as an independent predictor of poor overall survival in HCC patients.[13]

The process of reducing or suppressing the expression of the TMSB10 gene is referred to as TMSB10 silencing. In HCC, loss-of-function investigations suggested that TMSB10 silencing significantly inhibited cell proliferation, migration, and invasion.[13] When TMSB10 is silenced, it has a notable impact on the behavior of cells in HCC. The study specifically found that blocking TMSB10 expression had a big impact on three important cellular functions in HCC, including cell proliferation, cell migration, and cell invasion. Such investigation is referred to as a "loss-of-function" because it involves disrupting the normal activity of the

TMSB10 gene to observe the consequences on cell behavior in the context of HCC.[14]

AFP has been extensively investigated and is frequently employed as a biomarker for the diagnosis and prognosis of HCC.[15] By the time a child turns one, the expression of AFP, which the fetus's liver primarily produces, has rapidly decreased to a very low level. However, liver disease or cancer can cause a significant increase in blood AFP levels. In a nested case-control study, an increased AFP level may be detected six months before the diagnosis of HCC.[16] The primary complaints leveled about the use of AFP at the moment center on its lackluster sensitivity and specificity for the early identification of HCC when used alone. Additionally, cirrhosis patients with active hepatitis, elevated blood ALT, or non-HCC malignancies may have higher AFP levels. For HCC screening, AFP detection alone is not advised as of yet. Instead of AFP detection, the European Association for the study of the hepatic advises employing liver ultrasound for HCC surveillance.[17] In the current study, we divided all samples into three groups (healthy control, liver cirrhosis, and HCC patients). At first, we estimated all routine laboratory parameters and took all patients' medical histories, such as diagnosis, duration, and pathology, if presented. When we examined liver function index tests (ALT, AST, albumin, prothrombin, bilirubin, and ALP), we found that they were statistically significantly different between all study groups ($p < 0.001$), as shown in Table 1. According to the study by Marrero et al., 2018, AFP is the most extensively researched and often used biomarker for the diagnosis and prognosis of HCC.[15]

However, liver disease or cancer can significantly raise blood AFP levels. A nested case-control study showed that elevated AFP level might be seen six months before the diagnosis of HCC. In the present study, we determined AFP as a tumor marker for all groups. We found that there was a statistically significant difference in the AFP distribution between the three groups, ($p < 0.001$), as shown in Figure 2. The biological role of TMSB10 in HCC cells is not completely known. The ability of HCC cells to proliferate, migrate, and invade was found to be significantly reduced when TMSB10 expression was silenced.[13] In addition, the study by Zhang et al., 2017, showed that suppressing TMSB10 expression inhibited AKT/FOXO signaling, which in turn reduced breast cancer cell proliferation, invasion, and migration in both vitro and in vivo. According to the study by Zhang et al., 2017, the clinical stages of breast cancer are highly correlated with the expression level of TMSB10 in the serum of breast cancer patients.[12] According to the study by Yan et al., 2021,[18] there is a strong correlation between high levels of TMSB10 expression and advanced-stage lymph node metastasis and a bad prognosis in cases of gastric cancer. They demonstrated that increasing TMSB10 promotes gastric cancer cell proliferation and angiogenesis. As a result, the findings of the study by Yan et al., 2021, offer fresh data for gastric cancer prognosis prediction and the development of target therapeutics.[18] The study by Song et al., 2019,[13] showed that TMSB10, an oncogene that is overexpressed in the majority of human cancers, including HCC. The study by Song et al., 2019, provide more proof of the clinical importance and biological role of TMSB10 in HCC. The Cancer Genome Atlas datasets showed that TMSB10 expression levels were higher in HCC tissues compared with normal liver tissues.[13] In addition, they found that HCC tissue samples had higher TMSB10 mRNA and protein levels than nearby normal liver tissue samples. Furthermore, it was observed that elevated TMSB10 expression was independently linked with poor overall survival in HCC patients and was substantially associated with advanced tumor stage, large tumor size, distant metastasis, and poor prognosis. Studies on the loss-of-function of specific genes or proteins found that suppressing TMSB10 expression significantly reduced HCC cell proliferation, migration, and invasion. As a tumor biomarker for prognosis prediction and a possible target for the creation of a novel therapeutic approach, TMSB10 may show promise.[13]

We found that the distribution of TMSB10 concentration between all groups was statistically significantly different ($p < 0.001$), as shown in Figure 2 and Table 2. We studied the

association between AFP and TMSB10 and found that there was an inverse correlation between AFP and TMSB10 concentrations. The ROC curve analysis showed that TMSB10 has high specificity and sensitivity at area under the curve of 0.754, as shown in Figure 3, Table 4.

In the current study, we classified HCC patients into 4 groups: HCC0, HCC a, HCC b, and HCC c, according to the BCLC classification system,[7] and then we studied the relationship between TMSB10 and all HCC subgroups.

It is very interesting to learn that the amount of TMSB10 in the blood of people with HCC was negatively correlated to their different BCLC liver cancer staging system Figure 4, Table 5.

We think that one of the limitations of this study is the low number of included HCC patients, so we encourage further studies with a larger number of study subjects. In conclusion, there is a statistically significant difference in the TMSB10 distribution between the three groups (control, liver cirrhosis, and HCC). Serum TMSB10 may have the potential to be a tumor biomarker for HCC prognosis and prediction.

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Author Contributions

FMZ, contributed to the revision of important intellectual content and supervision. AIE, contributed to the data collection, methodology, and preparation of the manuscript. AAT, contributed to the conception, revision of important intellectual content, supervision, and preparation of the manuscript. All authors equally contributed to editing/reviewing the final manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The protocol of the study was reviewed and approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Zagazig University (reference number Zu-IRB 9416/22-3-2022).

Informed consent

All study participants were informed of the study objectives and verbally agreed to participate in the study.

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