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Original Article

Serum tumor markers and their prognostic value in Turkish hepatocellular cancer patients

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Abstract

Aim: Association between serum tumor markers and hepatocellular cancer (HCC) patients' survival has been investigating for decades. Despite glypican 3 has been reported superior to AFP in predicting the prognosis of HCC patients recently, the prognostic value of glypican 3 not clear. We aimed to investigate the prognostic value of serum glypican 3 and its relationship with the characteristic features in the Turkish HCC patients' cohort.

Material and Methods: A total of 84 HCC patients were enrolled prospectively. Serum glypican 3 levels were analyzed and serum levels of glypican 3 were compared according to many different types of the clinicopathologic features of HCC.

Results: A total of 84 patients, 71 of the patients were male and 13 were female. There were 36 hepatitis B (HBV) and 8 hepatitis C (HCV) infected patients. Forty-eight patients had cirrhosis and 35 patients did not. Serum glypican 3 levels were lower in cirrhotic than non-cirrhotic patients (p =0.6). Difference between overall survival (OS) of patients with serum glypican 3 levels ≥ 2 ng/mL and patient with serum glypican 3 level < 2 ng/mL was not significant, the OS estimates were 7.8 and 6.1 months, respectively (p=0.3). The median OS was 7.38 months. There was a positive correlation between serum AFP level and glypican 3, but not statistically significant (p=0.07).

Conclusion: The study results indicate that serum glypican 3 level is elevated in HCC patients with poorer features. Therefore, despite nonsignificant results, the method that contains the prediction of HCC patients' survival by serum glypican 3 level needs to be clarified with larger trials.

Keywords: Alpha-fetoprotein; hepatitis C; hepatocellular cancer; serum glypican 3; prognostic factor

INTRODUCTION

In the general population, hepatocellular carcinoma (HCC) is the sixth most common cancer, and in adult males is the fifth, and in adult females is the ninth most commonly diagnosed cancer in the world (1, 2). In almost all regions of the world, the incidence rate of HCC is increasing, and this rate is threefold higher in males compared with females (3, 4). The diagnosis of HCC is difficult and generally requires more than one diagnostic modality. Despite not a part of diagnosis criteria, the serum alpha-fetoprotein (AFP) concentration is the most commonly used marker for confirming HCC diagnosis. However, a significant proportion of hepatocellular tumors do not secrete AFP, and the normal serum concentrations are observed in up to 40 percent of small HCC (5). Also, in advanced-stage HCC patients, approximately 15-30% of patients may have normal serum AFP levels. Therefore, investigations to find more reliable serum markers than AFP for the diagnosis and prognosis of HCC have been conducting (6-8). Association between serum tumor markers and the disease survival has been the object of investigations, and serum AFP has appeared to be an independent predictor of survival in various studies (9, 10). Glypican 3, a recently discovered serum and tissue marker, is a cell surface heparan sulfate proteoglycan(11). It is not expressed in normal adult liver, and positive staining of Glypican 3 has 77% of sensitivity and 96% of specificity for the diagnosis of small HCC (12). The soluble fragment of Glypican 3 can be measured from patients' serum because it is released from the cell surface. In this context, in a recent study, the serum AFP has been reported to inferior to glypican 3 in specificity, and accuracy for HCC patients' diagnosis, and worse to predicting the disease prognosis (13). Therefore, serum glypican 3 may have a reliable place as a prognostic marker for HCC. However, the exact value of glypican 3 is not clear in this field. We aimed to examine the prognostic value of serum glypican 3 and its relationship with the characteristic features of the disease in patients with HCC.

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MATERIAL and METHODS

Patient eligibility and selection

Our study was carried out from November 2014 to May 2017 in the cancer institute. The trial was approved by the University Ethics Committee, where the research was conducted. Patients with any stage of HCC diagnosed by either histopathological or radiological modalities were included in the trial after obtained full consent. The eligibility criteria of patients were patients with hepatocellular cancer on the basis of imaging modalities or pathological examinations of tissues; cases with any performance status according to the Eastern Cooperative Oncology Group; aged 18 years or older; all stages of the disease which assessed by the Barcelona Clinic Liver Cancer (BCLC) system; any level of serum α-fetoprotein; any class of the Child-Turcotte-Pugh (CTP) scoring system. Two radiological modalities that used for diagnosing HCC are multidetector CT or dynamic contrast-enhanced MRI. Their pathognomonic findings were accepted as sufficient for the diagnosis of HCC in patients with liver cirrhosis who don't have a tissue sample. In patients with HCC, cirrhotic status was determined according to clinical and laboratory findings which include the presence of ascites and esophageal varices, hepatic encephalopathy, thrombocytopenia, splenomegaly, and laboratory results that reflect the liver function. The Child-Turcotte-Pugh score in HCC patients was calculated according to the serum bilirubin, albumin, and INR values of laboratory parameters, and ascites and hepatic encephalopathy status of clinical parameters. Treatment decisions were discussed in our multidisciplinary tumor boards, and patients' stages determined by the BCLC staging system. All clinical or laboratory parameters were recorded as the data variables. For the data analysis, the Body Mass Index (BMI) of patients was assessed as follows: weight (kg) divided by height (m) squared. Calculated scores of the BMI were categorized into four subgroups of patients according to WHO International Classification like underweight (BMI <18.5), normal weight (BMI ≥18.5 to 24.9), overweight (≥25.0 to 29.9) and obesity (BMI≥30).

Serum Glypican 3 measurement

Venous blood samples were drawn at the time of initial HCC diagnosis or at the time of patient inclusion to study. All serum samples used for the study were collected by centrifugation of blood immediately withdrawn from the patients and stored at -80 degrees until the analysis. Serum levels of glypican 3 were defined by enzyme-linked immunosorbent analysis and analyzed in duplicate using the Human glypican 3 ELISA Kit (Elabscience, catalog no: E-EL-H1712) according to the manufacturer's instructions. In the analysis, the minimum detectable dose of Human glypican 3 was 0.094ng/mL, and the detection range was 0.156-10ng/mL.

Survival analysis method

In our study, two different survival analyzes were performed. The first survival analysis aimed to evaluate the relationship between the serum glypican 3 level and HCC patients' survival, it was done from the date of blood draw to the patient's death, or the date of last communication. The second survival analysis aimed to evaluate the survival of HCC patients that not depended on serum glypican 3 that was done with the time from the date of diagnosis to the time of death, or again the last communication time.

Statistical analysis

The median overall survival (mOS) estimate was defined as the time from diagnosis date to death happen. For patients who didn't have complete follow-up data, their data on survival was censored at the time of the last documented communication. The survival of patients was estimated by the Kaplan-Meier method for all patients and the Log-rank test was used to compare the mOS values. Another analysis was the Mann-Whitney U test for using to compare serum levels of glypican 3 in different groups. Association between serum glypican 3 and other continuous variables were estimated by Spearman's correlation coefficient. Additionally, all categorical variables, number of cases and percentage of patients in each category were provided, and Chi-Square (X^2) or the Fisher's exact test was used to test for statistical differences between the groups. Also, multivariate or univariate analysis using the Cox proportional hazard model was done to assess the relative prognostic significance of serum glypican 3 level and other clinical variables on survival. Statistical significance was taken as $P < 0.05$, and all tests were 2-sided. Hazard ratios (HRs) with 95% confidence intervals (CIs) were calculated. Analyses were performed using SPSS version 22 statistical software (IBM Corporation, Somers, New York, USA).

RESULTS

Baseline Patient Characteristic

Eighty-four patients with HCC were eligible and included in the study during the predetermined period of the time. The median age of the patients was 64 (19-90), 71 (84.5%) of the patients were male and 13 (15.5%) were female. The clinical and demographic characteristics of patients are listed in Table 1. The liver functions of the patients were assessed by the CTP scoring system, 58 (69%) of patients classified as CTP class A, 22 (26.2%) patients CTP class B, and 3 (3.6%) patients were classified as CTP class C. Among our patient population, hepatitis B infected cases were much more common than hepatitis C infected cases. In terms of viral hepatitis and cirrhosis status, there were 36 patients HBV and 8 patients HCV infected, and 35 patients had normal liver, and 48 patients had liver cirrhosis. Survival analysis was performed for two different time intervals as mentioned in the methodology, the first time interval was the time from the blood draw date to death or censorship, and median OS was 7.29 months (95% CI: 4.43 – 10.1months). The second time interval was the time that defined from the date of diagnosis to the date of death or the last follow-up date, and OS was 13.7 months (95% CI: 9.54 – 17.92 months) (Figure 1).

Table 1. Baseline Demographic and Clinical Features of the Patients

		Number of patients	Percentage
All patients		84	100%
Median age		64 (19-90)	100%
Patient gender			
Female		13	15.5%
Male		71	84.5%
Liver cirrhosis			
Not Cirrhotic		35	41.7%
Cirrhotic		48	57.1%
Missing		1	1.2%
Child-Turcotte-Pugh			
A		58	69%
B		22	26.2%
C		3	3.6%
Missing		1	1.2%
The BCLC stage			
Very early stage		1	1.2%
Early stage		18	21.4%
Intermediate stage		13	15.5%
Advanced stage		50	59.5%
Terminal stage		2	2.4%
The largest tumor size			
≤ 5cm		30	35.7%
>5 cm		52	61.9%
Missing		2	2.4%
Portal Vein thrombosis			
No		47	56%
Yes		36	42.9%
Missing		1	1.1%
Serum AFP level			
≤ 400 ng/ml		53	63.1%
> 400 ng/ml		30	35.7%
Missing		1	1.2%
Hepatitis Infection			
Hepatitis B	Positive	36	42.9%
	Negative	48	57.1%
Hepatitis C	Positive	8	9.6%
	Negative	76	90.4%
Patients' weight classes			
Underweight		0	0%
Normal weight		30	35.7%
Overweight		30	35.7%
Obese		15	17.9%
Missing		9	10.7%

AFP, alfa-fetoprotein; the BCLC, the Barcelona Clinic Liver Cancer

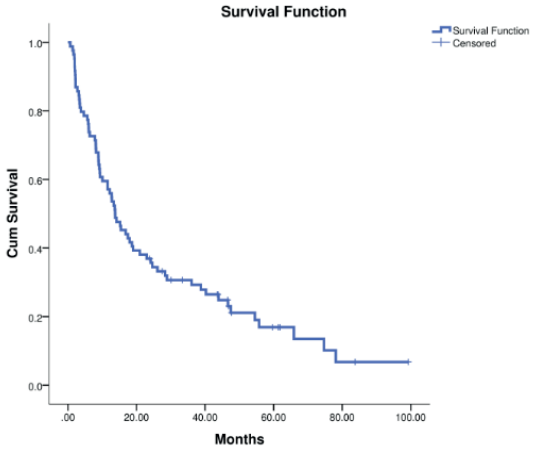


Figure 1. Kaplan–Meier survival curve for OS of all patients with HCC

Table 2. Cox regression analysis of factors for their potential effects on patients' survival

	Hazard Ratio (95% CI)¥	P value
Glypican 3 ≥ 2 vs. Glypican <2	1.31 (0.81-2.11)	0.3
AFP>400 vs. AFP ≤ 400	1.95 (1.19-3.18)	0.008*
The BCLC early stage vs. The BCLC very early stage	0.79(0.10-6.13)	0.8
The BCLC intermediate stage vs. The BCLC very early stage	0.67(0.08-5.38)	0.7
The BCLC advance stage vs. The BCLC very early stage	1.78(0.24-12.99)	0.5
The BCLC terminal stage vs. The BCLC very early stage	24.75(1.94-314.54)	0.01*
CTP class B vs. CTP class A	2.03(1.19-3.45)	0.009*
CTP class C vs. CTP class A	39.1(8.9-170.7)	<0.001*

*,statistically significant; the BCLC, Barcelona clinic liver cancer; the CTP, The Child-Turcotte-Pugh; AFP,alfa-fetoprotein

At the time of the final analysis, among 84 patients, 69 patients of the HCC population had died. Similarly, the median overall survival times of the HCC patients according to the CTP scoring system were calculated, and there was a statistically significant difference in the OS of patients according to CTP classes. The median OS for CTP class A, B, and C was 17.9, 8.8, and 3.4 months, respectively (p=0.001) (Figure 2). There is no standard cut-off value of serum glypican 3, however, in the previous trials, the most used value is 2 ng/mL(14). Therefore, the patients were divided into two groups according to 2 ng/mL cut-off value for serum Glypican 3 level like previous trials. However, the difference between estimated overall survival times of patients with serum Glypican 3 level < 2 ng/mL and patient with serum Glypican 3 levels ≥ 2 ng/mL was not significant (p=0.3) (Figure 3).

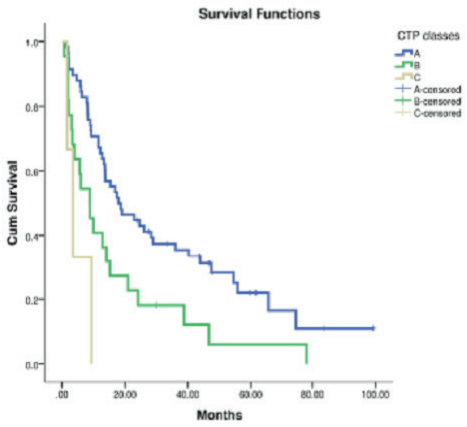


Figure 2. Kaplan–Meier survival curves for OS of HCC patients according to the CTP scoring system

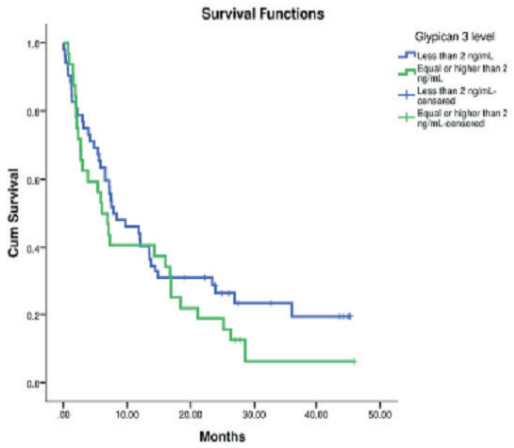


Figure 3. Kaplan–Meier survival curves for OS of HCC patients according to serum Glypican 3 level

Table 3. Serum Glypican 3 level according to different features of patients with HCC

Patients Features	Variables	The mean serum Glypican 3 level ± SD	P value
Age	≤ 60	1.77 ±1.7	0.04*
	>60	3.49 ±5.6	
Gender	Female	2.76±3.2	0.9
	Male	2.90±4.9	
Liver cirrhosis	No	2.67±4.8	0.6
	Yes	3.08±4.6	
The largest tumour size	≤ 5cm	2.33±2.3	0.4
	>5 cm	3.23±5.6	
Portal vein thrombosis	No	2.55±3.9	0.4
	Yes	3.36±5.5	
Serum AFP level	≤ 400	2.01±2.1	0.1
	>400	3.98±6.7	
Viral hepatitis infection (B or C)	Negative	2.66±4.3	0.7
	Positive	3.06±5.1	
Stages according to the BCLC system	Very early stage	7.9	0.4
	Early stage	2.02±1.8	
	Intermediate stage	1.68±1.3	
	Advanced stage	3.46±5.8	
	Terminal stage	1.29±0.6	
Child-Turcotte-Pugh classes	Class A	2.4±4.1	0.2
	Class B	4.2±6.1	
	Class C	0.94±0.7	

*,statistically significant; the BCLC, Barcelona clinic liver cancer; the CTP, The Child-Turcotte-Pugh; AFP,alfa-fetoprotein

The median OS of patients with < 2 ng/mL and ≥ 2 ng/mL were 7.8 and 6.1 months, respectively. Our study median follow-up time was 59.7 months (range 37.9–81.4 months). There was a positive correlation between serum AFP and Glypican 3 level, but not statistically significant (p=0.07). Similarly, although serum Glypican 3 levels were higher in portal vein thrombotic patients than non-thrombotic patients, the relationship was not significant (p=0.06). Tumor diameter is a prognostic factor in HCC patients and 5 cm value is a critical value that's been reported in its association with survival. Therefore, our patients divided into two groups according to this cut-off value (15,16). In terms of tumor size, there was a significant correlation between the largest tumor size and serum AFP level in patients with HCC. The patients whose largest tumor size was ≤ 5 cm had a mean 3.850 ng/mL serum AFP level, and patients whose largest tumor size was > 5 cm had 28.119 ng/mL mean serum AFP level (Rho: 0.27, p=0.013).

The mean serum level of glypican 3 for the patients whose largest tumor size was ≤ 5 and > 5 cm were 2.33 and 3.23 ng/mL, respectively, and the difference was not significant (p=0.4). Despite cirrhotic patients had higher serum Glypican 3 level, the relationship between cirrhotic status and serum Glypican 3 level was not statistically significant. For determining the effect of serum Glypican 3 and AFP levels, the BCLC stages, and the CTP classes on the survival of HCC patients, the univariate Cox regression analysis was conducted (Table 2). Among these parameters, Cox regression analysis revealed significant effects of the serum AFP levels, CTP classes, and the terminal stage of the BCLC on survival. According to patients' serum AFP level, patients were divided into two groups, the first group was AFP ≤ 400ng/ml and the second group was patients with serum AFP level > 400 ng/ml. Since the relationship between 400 ng/ml cutoff values of serum AFP level with survival has been reported, this threshold value was evaluated in our patient group (17). A statistically significant difference between the median OS rates of the first and second groups was found, mOS was 11.8 and 4.1 months, respectively (p=0.007) (Figure 4). Thirty-six patients (42.9%) had portal vein thrombosis, 47 (56%) of patients had normal portal vein and 1 patient couldn't be evaluated. We found a statistically significant difference between OS rates of patients with portal vein thrombosis and without thrombosis, the median OS was 13.5 and 4.1 months, respectively (p=0.002) (Figure 5). According to WHO International Classification for body mass index, 30 of patients were classified as overweight, 15 of patients were classified obese, and 30 of patients were classified as normal weight; there was no significant relation between Glypican 3 and BMI subgroups, and there was no the prognostic role of BMI in HCC patients. The median serum level of Glypican 3 according to clinical and laboratory features of HCC patients were calculated that listed in Table 3.

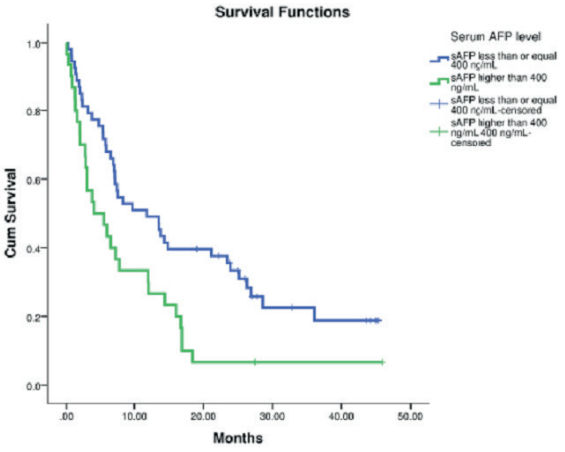


Figure 4. Kaplan–Meier survival curves for OS of HCC patients according to serum AFP level

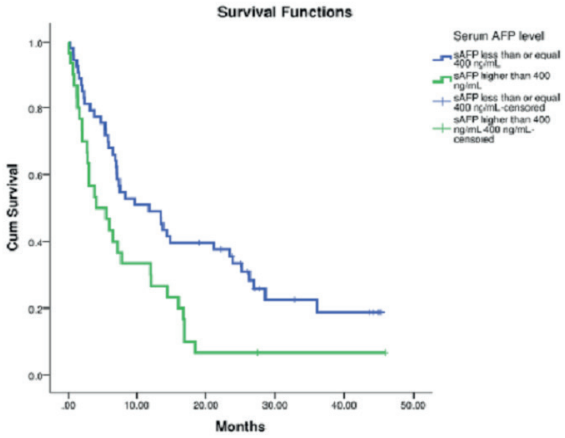


Figure 5. Kaplan–Meier survival curves for OS of HCC patients according to portal vein thrombosis status

DISCUSSION

The methods for predicting cancer-related mortality should be specific, reliable and convenient. Therefore, for covering all these advantages, serum biomarkers have critical roles in HCC management. Currently, there are many investigations that have been conducting to find the most useful prognostic biomarker in clinical trials for the management of HCC (18,19). However, there is no marker that has been found to replace AFP in clinical practice and further studies are needed in this field. Glypican 3, as a promising serum marker has been one of the most often investigated markers in recent years.

In this study, we prospectively examined the prognostic significance of glypican 3 and its relationship with other clinical and pathological features. Unfortunately, in terms of the predictive value of serum glypican 3, we could not obtain a significant positive result from our HCC patient population. However, the effects of other factors such as the largest tumor size, serum AFP level and portal vein thrombosis on HCC patients' survival resulted in compliance with the literature.

The heparan sulfate proteoglycans are a wide family. As an overexpressed marker in almost all HCC tissues, glypican 3 is a member and connects to cell membranes by a glycosylphosphatidylinositol anchor (20). After the immunohistochemical value of glypican 3 expression in tissues has been reported in the diagnosis of HCC patients, numerous studies have been searching for the prognostic significance of glypican 3 expression were conducted in patients with HCC. The immunohistochemical evaluation of glypican 3 reported the correlation between the rate of glypican 3 expression and prognosis of HCC patients, and the patients with high expression of glypican 3 had a poorer prognosis in these trials (21, 22). The soluble fragment of glypican 3 can be measured from patients' serum because it is released from the cell surface. Therefore, assessment of the serum level of glypican 3 has been investigated to predict the prognosis of HCC patients (23). In this study, the significant association between serum preoperative glypican 3 levels and patients' overall survival was observed by investigators, and elevated

serum glypican 3 was found as an independent prognostic marker. Unfortunately in our trial, although patients with low serum glypican levels lived numerically longer, this difference was not statistically significant. However, our study design was different from the previous study, and the main difference of this study was that all patients included in the previous study underwent partial hepatectomy. There are some additional reasons that may explain the incompatibility of our results with previous studies. First, the patients were at different stages and their distribution wasn't homogeneous. Second, a significant proportion of the patients had been diagnosed before serum samples were taken, and a certain proportion of patients had been already exposed to various treatments.

There are several factors that have been proved to affect the survival of HCC patients like portal vein thrombosis (PVT), tumor size, and serum AFP level. Among these factors, PVT means invasion of macrovascular structures of the liver by tumor and is one of the most challenging statuses of the disease (24, 25). Another risk factor tumor size has been demonstrated as an independent risk factor for patients with HCC, and its relationship with patients' survival is more obvious in patients who underwent resection (26-28). Similarly, the relationship between serum AFP level and HCC patients' course has been evaluated by many studies from past to present. The high serum AFP level in HCC has been demonstrated to correlate with poor tumor differentiation, high tumor burden, worse prognosis (9, 29). In our study, the effects of the specified factors PVT, tumor size, and serum AFP level on survival were found similar to previous studies. In particular, the relationship between patients' survival and tumor size or serum AFP level was clearer. Therefore, our study obtains importance in confirming the effects of the mentioned factors.

Basically, the CTP scoring system was developed primarily for the evaluating and grading of the hepatic function status of patients with liver cirrhosis, and this scoring system correlates with survival in patients who undergoing or not surgery. The one-year survival rate in cirrhotic HCC patients decreases dramatically from CTP class A to C. Recently, the CTP scoring system is widely being combined with evaluation systems that developed to estimate the survival of patients with HCC (30-32). The predictive values of the systems that combined with the CTP scoring system seem much higher. Therefore, the CTP has become an essential evaluation system for improved survival estimation in HCC patients. In our study, there was a significant survival difference between the patient groups in the evaluation of our patients according to the CTP scoring system, and these results were consistent with the literature.

This present prospective study has some limitations; there is no validated range of values or a standard method for measuring the serum glypican 3 level in patients with HCC.

Reported serum glypican 3 levels in both HCC and normal cases differ significantly among the results of published studies (33-37). These differences may be due to the different epitopes identified by the antibodies used for measurement in the studies. Therefore, we couldn't confidently compare our serum glypican 3 levels with previous studies results; this may constitute a limitation for our study. Patients with different stages were included in the study and the distribution of patients was not homogeneous according to the stages, this situation may have restricted to determine serum glypican 3 for certain groups in our study. Another limitation of the study could be due to a significant proportion of the patients had been diagnosed before serum samples were taken, and a certain proportion of patients had been already exposed to various treatments. Due to the possible effects of applied treatments on the serum markers level, the clarity of obtained serum glypican 3 results could be limited.

CONCLUSION

In conclusion, with this study, we have confirmed that serum AFP level, tumor size, and portal vein thrombosis are independent prognostic factors and have significant effects on HCC patients' survival, as shown in previous studies. Although our results indicate that serum glypican 3 level is elevated in HCC patients with unfavorable features like portal vein thrombosis, high serum AFP level, and large tumor size, there was no significant difference between compared groups. However, because of the study limitations, that couldn't be possible to reach an exact decision. Therefore, despite nonsignificant results, the method that contains the prediction of HCC patients' survival by serum glypican 3 level needs to be clarified with larger trials that included homogeneous patient populations.

Competing interests: The authors declare that they have no competing interest.

Financial Disclosure: There are no financial supports.

Ethical approval: Ethics Committee Approval was obtained from ethics Committee of the Hacettepe University Faculty of Medicine, Approval date and number: 04.03.2015 and 15/153.

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