Serum sphingosine negatively correlates with albumin predicting the risk of hepatocellular carcinoma

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Background. The roles of sphingosine in various cancers have not been fully investigated. Our aim was to identify the relationship between serum sphingosine and the risk of hepatocellular carcinoma (HCC).

Methods. Serum sphingosine in 34 normal people and 73 HCC patients were reviewed retrospectively. Receiver operating characteristic curve analysis was performed to determine the cut-off values of sphingosine in the serum. Chi-square test, t test and regression analysis were used to test the association between serum sphingosine and individual clinicopathologic parameters.

Results. Serum sphingosine was higher in HCC patients (155.91 ± 331.5 ng/mL) with normal persons as the control (30.92 ± 29.4 ng/mL). The sphingosine threshold according to ROC curve was set at 22.5 ng/mL with a sensitivity of 74%, and a specificity of 55.9%. Meanwhile, sphingosine in HCC patients with abnormal albumin was significantly higher than that in patients with normal albumin ($t=2.452$, $P=0.019$). When HCC patients were divided into two groups serum sphingosine was negatively associated with albumin in HCC patients ($\chi^2=4.469$, $P=0.035$). Moreover, the logistic regression analysis showed that large tumor size ($P=0.018$, $OR=0.13$) and a low albumin ($P=0.005$, $OR=8.856$) were two independent risk factors for serum sphingosine upregulation. High AFP coupled with high serum sphingosine, high sphingosine and high AFP respectively were found in 91.2%, 75.4%, 73% of the HCC patients.

Conclusions. These results suggest that serum sphingosine could be treated as a marker for the risk of HCC. AFP and sphingosine in the serum could be used together for HCC diagnosis.

Key words: serum sphingolipid, HCC, albumin

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a major health problem worldwide, and ranks as the fifth most common cancer and the third most common cause of cancer death¹. In China, the situation threatens the health of large numbers of people². Early diagnosis is very important since therapies for patients in late stages of HCC are not satisfactory. The detection of alpha fetoprotein (AFP) and imageological examination are the principal ways for HCC diagnosis³. However, more and more studies have shown that as a specific marker for HCC serum AFP does not work very well. More interestingly, a low serum albumin (ALB) level has been reported to be consistently associated with a markedly increased risk of HCC (ref.⁴). Moreover, low level of serum ALB is found to be a key determinant of subsequent HCC development in a large prospective cohort of patients with HBV-related chronic liver disease⁵. The high intrahepatic recurrence rate in patients after hepatectomy makes this cancer hard to cure. Of note, patient age, gender, location, status of disease, surgical margins and especially

tumor size are considered as potential risk factors for recurrence⁶.

Sphingosine is generated from the hydrolysis of ceramides through the action of ceramidases and can be further transformed into sphingosine 1-phosphate (S1P) by sphingosine kinase⁷. Sphingosine, ceramides and S1P composing the important part of sphingolipid which are the building block of cell membrane as well as intracellular second messengers are involved in many biological processes⁸. Sphingosine has been shown to mediate growth arrest, differentiation, and apoptosis of mammalian cells in response to various stressful stimuli. Similarly, ceramides participate in cell proliferation, differentiation, autophagy and caspase 9 mediated apoptosis⁹. S1P can regulate cell survival, proliferation, adhesion, and motility by binding to the G-protein-coupled receptors¹⁰. Ceramides are well known as a death promoter for cancer cells. S1P has been implicated in tumor genesis and plays an essential role in the tumor development¹¹. Increased serum S1P is considered to be predictor for many types of cancers¹². However, the potential link between the serum sphingosine and HCC is not yet clear.

In this research, serum sphingosine in 34 normal people and 73 HCC patients were reviewed retrospectively. The result showed that serum sphingosine in HCC patients was significantly higher than that in health donors. Meanwhile serum sphingosine was related to the ALB in HCC patients. Besides, the relationship between serum sphingosine and tumor size, ALB, Cirrhosis or TNM stage was analyzed in this manuscript. Finally, the potential of AFP coupled sphingosine for the HCC diagnosis was evaluated. In conclusion, it was suggested here that serum sphingosine could be treated as a predictor for HCC and AFP combined with sphingosine could be used for HCC diagnosis.

MATERIALS AND METHODS

Study population

73 subjects with HCC were recruited from patients referred to the Affiliated Hospital of Guilin Medical University, Guangxi, China, from 2015 to 2016. No patient had received transhepatic arterial embolization or chemotherapy before surgical resection. Control samples were recruited from 34 people who had a physical examination in the same hospital and were healthy. The multifarious aetiologies of HCC patients contain age, gender, median size, tumor number, alpha-fetoprotein (AFP), cirrhosis, Barcelona Clinic Liver Cancer (BCLC) stage, distant metastasis, alanine transaminase (ALT), ALB and others are listed in table 1. Ethical approval was granted by the Ethics Committee of the Affiliated Hospital of Guilin Medical University, and the written consent was obtained from all examined patients or their guardians prior to surgery.

Chemicals and reagents

D-erythro-sphingosine (C12 base) was from Avanti Polar Lipids (Alabaster, AL, USA). o-Phthalaldehyde (OPA) was from Sigma Chemical Co. (St. Louis, MO, USA). High performance liquid chromatography (HPLC)-grade solvents was from Fisher Scientific (Pittsburgh, PA, USA). All other biochemical reagents were from the Xilong Chemical Co (Guangdong, China).

Serum lipid extraction

Serum samples were first adjusted to a volume of 1 mL with 1 M NaCl, then chloroform/methanol/3 N NaOH (1:1:0.1, v/v) was added and mixed. Proper volume of chloroform/1 M NaCl (1:1, v/v), 3 N NaOH were added. The separated basic aqueous phase was removed. 3 N NaOH in methanol/1 M NaCl (1:1, v/v) was added to the organic phase (containing sphingosine) and then was vigorously mixed. After centrifugation (4000 g) for 10 min, the bottom organic layer which contained sphingosine was transferred to a new glass tube, dried with a Termovap Sample Concentrator in 37 °C water bath, then suspended with 200 µL of methanol.

Sphingosine assay

The lipid extract and OPA buffer (5 mg OPA, 3% boric acid pH 10.05, 0.2 mL ethanol and 5 µL β-mercaptoethanol) of equal volume (200 µL) were mixed in the brown tube for at least 5 min (r.t.). The reaction mixture containing fluorescent sphingosine derivative was diluted with mobile phase (2:5), and centrifuged. The supernatant was then transferred to a glass vial and 20 µL of sample was subjected to an HPLC system LC20AT (Kyoto, Japan) for analysis. The content of sphingosine was quantified using the standard curve generated from the relationship between the concentration of sphingosine standard and the corresponding peak area.

High performance liquid chromatography (HPLC) assay

The HPLC analyses were performed using a Shimadzu (Kyoto, Japan) Model LC20AT pump with C18 column (4.6×150 mm, Kyoto, Japan). The mobile phase was methanol: 5 mM potassium phosphate (pH 7.0, 85:15, v/v), the flow rate was 1 mL/min and the column temperature was 20 °C. The fluorescent sphingosine derivative was monitored using a model RF-20A scanning fluorescent detector (Kyoto, Japan) (excitation 340 nm, emission 455 nm).

Statistical analysis

The statistical analysis was conducted using SPSS software. The chisquare (χ^2) test and the independent t test were used to assess the correlation between sphingosine content and clinicopathological parameters. Receiver operating characteristic (ROC) curve analysis was applied to determine the cutoff value of preoperative sphingosine content by 1, 2-criterion in the serum of the patients with HCC. The statistical significance of the difference was identified when *P* value was less than 0.05. Logistic regression analysis was used to investigate the independent factors that affected serum sphingosine.

RESULTS

Sphingosine is increased in the serum of HCC patients

The serum sphingosine in HCC patients and normal people were measured. Interesting, there was a highly significant increase in the concentration of sphingosine in the HCC patients (median of 155.91 ng/mL for the HCC patients vs 30.92 ng/mL for the control group, respectively; *P*=0.002). The high concentration of sphingosine predicted the risk of HCC (Fig. 1A).

Determination of cut-off value of preoperative serum sphingosine in HCC

To identify an optimal cut-off value of preoperative sphingosine in serum, an ROC curve was generated with SPSS software to validate diagnostic value of this model. It was indicated that the score of 22.5 ng/mL has the maximum sensitivity and specificity for predicting survival status. The area under receiver operating curves (AUC) was 0.705 with a 95% CI of 0.605 to 0.806, a sensitivity of 74%, and a specificity of 55.9% (Fig. 1B).

Table 1. The relationship between sphingosine and clinicopathologic variables of patients with HCC.

		No. of patient	Serum Sphingosine (ng/mL)		χ^2	<i>P</i>
			< 22.5	≥ 22.5		
Age (years)	< 55	35	10 (28.6)	25 (71.4)	0.554	0.457
	≥ 55	38	8 (21.1)	30 (78.9)		
Gender	Male	62	16 (25.8)	46 (74.2)	0.026	0.872
	Female	11	2 (18.2)	9(81.8)		
HBsAg	Negative	19	4 (21.1)	15 (78.9)	0.013	0.909
	Positive	54	14 (25.9)	40 (74.1)		
AFP (ng/mL)	< 200	27	6 (22.2)	21 (77.8)	0.137	0.711
	≥ 200	46	12 (26.1)	34 (73.9)		
Median size (cm)	< 5	15	6 (40)	9 (60)	2.392	0.122
	≥ 5	58	12 (20.7)	46 (79.3)		
Tumor number	Single	34	9 (26.5)	25 (73.5)	0.113	0.737
	Multiple	39	9 (23.1)	30 (76.9)		
Cirrhosis	No	22	3 (13.6)	19 (86.4)	1.297	0.255
	Yes	51	15 (29.4)	36 (70.6)		
TNM stage	I-II	19	5 (26.3)	14 (73.7)	0.038	0.845
	III-IV	54	13 (24.1)	41 (75.9)		
BCLC stage	A-B	40	12 (30)	28 (70)	1.359	0.244
	C-D	33	6 (18.2)	27 (81.8)		
PVTT	No	46	12 (26.1)	34 (73.9)	0.137	0.711
	Yes	27	6 (22.2)	21 (77.3)		
Distant metastasis	No	54	16 (29.6)	38 (70.4)	1.828	0.176
	Yes	19	2 (10.5)	17 (89.5)		
HBV-DNA (copies/mL)	< 1000	37	9 (24.3)	28 (75.7)	0.04	0.947
	≥ 1000	36	9 (25)	27 (75)		
ALT U/L	< 38	48	11 (22.9)	37 (77.1)	0.229	0.633
	≥ 38	25	7 (28)	18 (72)		
AST(U/L)	< 40	26	7 (26.9)	19 (73.1)	0.112	0.738
	≥ 40	47	11 (23.4)	36 (76.6)		
ALB(g/L)	< 35	34	4 (11.8)	30 (88.2)	4.469	0.035
	≥ 35	39	14 (35.6)	25 (64.4)		
CEA (ng/mL)	< 3.4	48	9 (18.8)	39 (81.2)	2.633	0.105
	≥3.4	25	9 (36)	16 (64)		
CA199 (U/mL)	< 39	43	13 (30.2)	30 (69.8)	1.751	0.186
	≥ 39	30	5 (16.7)	25 (83.3)		
CA125 (U/mL)	< 35	37	11 (29.7)	26 (70.3)	1.039	0.308
	≥ 35	36	7 (19.4)	29 (80.6)		

Table 2. Logistic regression result.

Model	β	standard error	<i>P</i>	OR	95% confidence interval	
					lower limit	upper limit
tumor size	-2.272	0.957	0.018	0.13	0.016	0.673
ALB	2.181	0.778	0.005	8.856	1.929	40.65
Cirrhosis	-1.32	0.748	0.077	0.267	0.062	1.156
TNM stage	1.93	1.006	0.055	6.891	0.959	49.509

Fig. 1. Up-regulated serum sphingosine in HCC. (A) Mean serum level of sphingosine detected by HPLC was significantly higher in HCC patients than in health donors. $P=0.002$ (B) ROC analysis was performed to evaluate the diagnosis value of preoperative serum sphingosine. The area under the ROC curve value was 0.705.

Fig. 2. All 73 cases of HCC patients were stratified based on ALB content in serum. ($P=0.019$).

Serum sphingosine in HCC was associated with ALB

A low serum ALB level has been reported to be associated with a markedly increased risk of HCC. HCC patients were divided into two groups according to the serum ALB content and then we explored the relationship between serum sphingosine and serum ALB (Fig. 2). More interestingly, we found that serum sphingosine was significantly higher in HCC patients with lower ALB compared to that in patients with higher ALB $t=2.452$, $P=0.019$ (Fig. 2). The results suggested that the serum ALB negatively correlated to the serum sphingosine.

Association between preoperative sphingosine and clinicopathological features

The relationship between sphingosine and clinicopathologic variables of patients with HCC was investigated after the results obtained from the ROC curve were analyzed. The data showed that sphingosine was associated with ALB ($\chi^2=4.469$, $P=0.035$). Nonetheless, there were no statistical connections between sphingosine and other clinicopathological parameters including age, Gender, HBsAg, AFP, size, Tumor nodule number, cirrhosis, TNM stage, BCLC stage, PVTT, distant metastasis, HBV DNA copies, ALT, AST, CEA, CA199, CA125 (all $P>0.05$, Table 1). Taken together with the fact that ALB is produced in liver cells, it was indicated that serum sphingosine was also an indicator of liver function.

Logic regression analysis between sphingosine and tumor size, ALB, cirrhosis, TNM stage

The above results suggested that sphingosine correlated to ALB, so factors including ALB were analyzed by logistic regression to test the independent risk factors for increased serum sphingosine. According to the regression result, serum sphingosine was positively related to the tumor size ($OR=0.13$, $P=0.018$) and was negatively related to the ALB ($OR=8.856$, $P=0.005$). Therefore, a tumor size and an ALB were identified as two independent factors for sphingosine upregulation (Table 2).

Combination of sphingosine and AFP improved the efficiency of HCC diagnosis

The above assay indicated that increased sphingosine were not completely consistent with increased AFP (≥ 200 ng/mL) in serum from HCC patients. So diagnostic efficiency of sphingosine combined with AFP in HCC were investigated next. An optimal diagnostic cut-off for sphingosine was set at 22.5 ng/mL according to ROC curve. As shown in Fig. 3, both of these markers increment appeared in 46.6% of all HCC patients, only AFP or sphingosine increment were present in 16.4% or 28.8% of all HCC cases. The positive rate of individual sphingosine for HCC diagnosis was 75.4%, while AFP was 73%. Interestingly, positive rate increased to 91.8% when sphingosine and AFP were combined used, which significantly increased efficiency of HCC diagnosis (Fig.3).

DISCUSSION

Our presented study demonstrated that serum sphingosine in HCC patients was higher than that in the control group which has not been reported previously. In addition, it was found that serum sphingosine was negatively related to serum ALB and positively related to tumor size in HCC patients. Besides, combined with AFP serum sphingosine might be a potential predictor for the risk of HCC.

Sphingolipid metabolisms have great impact on cancer signaling and therapy¹¹. The two major bioactive lipids ceramide and SIP, have opposing roles in regulating cancer cell death and survival¹³. However, the role of sphingosine in cancer is currently unknown. In this research, we explored the association between serum sphingosine and the risk of HCC and the results were inspiring.

Serum ALB is one of the important indicators reflecting liver function. Serum ALB synthesized in the liver, is important for physiological function of macromolecules especially drugs in vivo due to its role as the carrier for target molecules¹⁴. It was reported that the risk of HCC was only 3% in patients with an albumin level above 41 g/L, but the incidence reached to 40% in case of an albumin level below 41 g/L (ref.¹⁵). Our results found that serum sphingosine increased in HCC patients and sphingosine in serum was negatively related to ALB which was consistent with the previous reports. Specifically the logistic analysis suggested that the ratio of increased serum sphingosine from HCC patients with abnormal ALB is 8.856 times

greater than that from HCC patients with normal ALB. In some study the reduction in serum ALB level or ALB/GLO ratio was found to be linked to cirrhosis¹⁶. However in this research only minor correlation between serum sphingosine and cirrhosis could be observed. Surprisingly, HCC patients with cirrhosis have the tendency to present low level of serum sphingosine according to the regression analysis which needs to be investigated further. It's needed to be mentioned here that 95% of HCC cases develop in liver cirrhosis patients. However, a considerable number of HCC patients without cirrhosis were included in the study in order to investigate the relationship between sphingosine and cirrhosis in HCC.

It is generally known that the tumor markers of CEA, AFP and CA199 are commonly used in clinical diagnosis of HCC (ref.^{17,18}). However, the diagnostic sensitivity of these tumor markers has been challenged in clinical practice. The specificity of CEA was low and a high level of serum CA199 is frequently seen in normal bile secreted by healthy biliary tract¹⁸. Many factors influenced the diagnosis accuracy of HCC (ref.¹⁹). While, in our research, the serum sphingosine of 73 HCC patients were measured and the concentration were significantly increased. Increased serum sphingosine could be used together with other serum markers to improve the diagnosis of HCC in the future.

Clinical research showed that the serum AFP is negative in 30% of HCC patients. Here we evaluated the potential of AFP coupled with serum sphingosine in HCC diagnosis. Of note, HCC diagnosis could be improved when combined sphingosine and AFP in serum were used. Increased serum sphingosine together with AFP would be a promising diagnosis standard in clinic. It's needed to be mentioned here that serum sphingosine was not significantly related to TMN or BCLC stage (Table 1). Therefore, sphingosine might not reflect the stage of HCC.

Sphingosine was thought to promote cell death or trigger DNA damage^{7,8,20}. Our study revealed that serum sphingosine was upregulated in HCC patients which seems to be irrational. In order to explain this absurd phenomenon, one hypothesis put forward here is that more intracellular sphingosine is excluded from the cancer cells than normal cells contributing to the increased serum sphingosine in patients with HCC. We should mention here that one drawback of the study is the retrospective design.

CONCLUSION

In conclusion, serum sphingosine increased in HCC patients and it was negatively related to ALB and positively correlated to tumor size. This aside, serum sphingosine coupled with AFP present a better positive rate in HCC diagnosis.

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