Significance of the serum brain-derived neurotrophic factor and platelets in hepatocellular carcinoma

ZHEN FAN YANG, DAVID W. HO, CHI KEUNG LAU, KA HO TAM, CHI TAT LAM, WAN CHING YU, RONNIE T.P. POON and SHEUNG TAT FAN

Centre for the Study of Liver Disease and Department of Surgery, The University of Hong Kong, Pokfulam, Hong Kong, P.R. China

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Abstract. The present study investigated the significance of the serum brain-derived neurotrophic factor (BDNF) and platelets in relation to the clinicopathological features of hepatocellular carcinoma (HCC) patients. Localization of the BDNF expression in human HCC tissues was performed by immunohistochemistry. The measurement of soluble BDNF in the serum was performed by enzyme-linked immunosorbent assay. BDNF was expressed in the cytoplasm of the tumor cells. A positive correlation between the tissue and serum levels of BDNF was identified in the HCC patients. The serum levels of BDNF were positively correlated with the platelet counts in the HCC patients. A higher level of serum BDNF was significantly correlated with a tumor size >5 cm, poorly differentiated HCC, the presence of microsatellite tumor nodules, and the absence of cirrhosis in the non-tumorous tissues. A higher level of the serum BDNF/platelet ratio was associated with a poorer disease-free survival after hepatic resection. This study suggested that the tumor cell was a source of serum BDNF in HCC. A higher serum BDNF level was associated with a more advanced tumor status in the HCC patients. The interaction between serum BDNF and platelets might play an important role in HCC tumor progression.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide, with an increasing trend due to the spreading of hepatitis B and C virus infection (1,2). Hepatic resection and liver transplantation are the only two alternatives that may cure HCC. However, the majority of HCC patients have an advanced stage tumor at the time of diagnosis, thus surgical treatment is not applicable (3). Furthermore, the high recurrence rate after hepatectomy and liver transplantation also hinders the long-term survival of patients (4,5). Therefore, exploring the molecular mechanisms of tumor recurrence after hepatic resection or liver transplantation is important in understanding the rapid progression of the cancer and may enhance the efficacy of therapeutic approaches for this disease.

The brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, plays an essential role in maintaining and modulating the physiological functions of neurons, through binding to its receptors, the tyrosine protein kinase receptor B (TrkB) and the p75 nerve growth factor receptor (6,7). In addition, BDNF and TrkB also participate in some pathological conditions (8-10). It has been revealed that BDNF and TrkB could promote the growth, survival and metastasis of a variety of tumor cells (11-13), suggesting the potential roles of the BDNF-TrkB interaction in tumorigenicity and the progression of cancers. BDNF was also involved in the chemoresistance of neuroblastoma cells against cisplatin-induced cytotoxicity (14-16). Furthermore, BDNF was found to favor the survival of endothelial cells under stress conditions, indicating the potential role of this molecule in angiogenesis (17,18).

Our previous study identified BDNF as a novel functional protein that could promote tumor cell growth in a rat HCC model. We also demonstrated that BDNF mRNA was overexpressed in human HCC tumor tissue compared with that in non-tumorous tissue, cirrhotic or normal liver tissues (19). In the present study, we further characterized the protein expression of BDNF in human HCC tumor and non-tumorous tissues. Furthermore, as BDNF is a soluble protein in the circulation, it is of interest to investigate the relationship between the serum BDNF level and the clinicopathological features of HCC patients who have undergone hepatectomy. As a close relationship between BDNF and platelets has been identified in some studies, in which platelets were found to be an important source for BDNF storage (20-22), it is of interest to investigate the potential role of BDNF-platelet interaction in relation to HCC progression.

Correspondence to: Dr Zhen Fan Yang, L9-41, Faculty of Medicine Building, Department of Surgery, 21 Sassoon Road, Hong Kong, P.R. China
E-mail: zfyang@hkucc.hku.hk

Abbreviations: HCC, hepatocellular carcinoma; BDNF, brain-derived neurotrophic factor; TrkB, tyrosine protein kinase receptor B; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; Trk, tyrosine receptor kinase

Key words: brain-derived neurotrophic factor, platelet, hepatocellular carcinoma

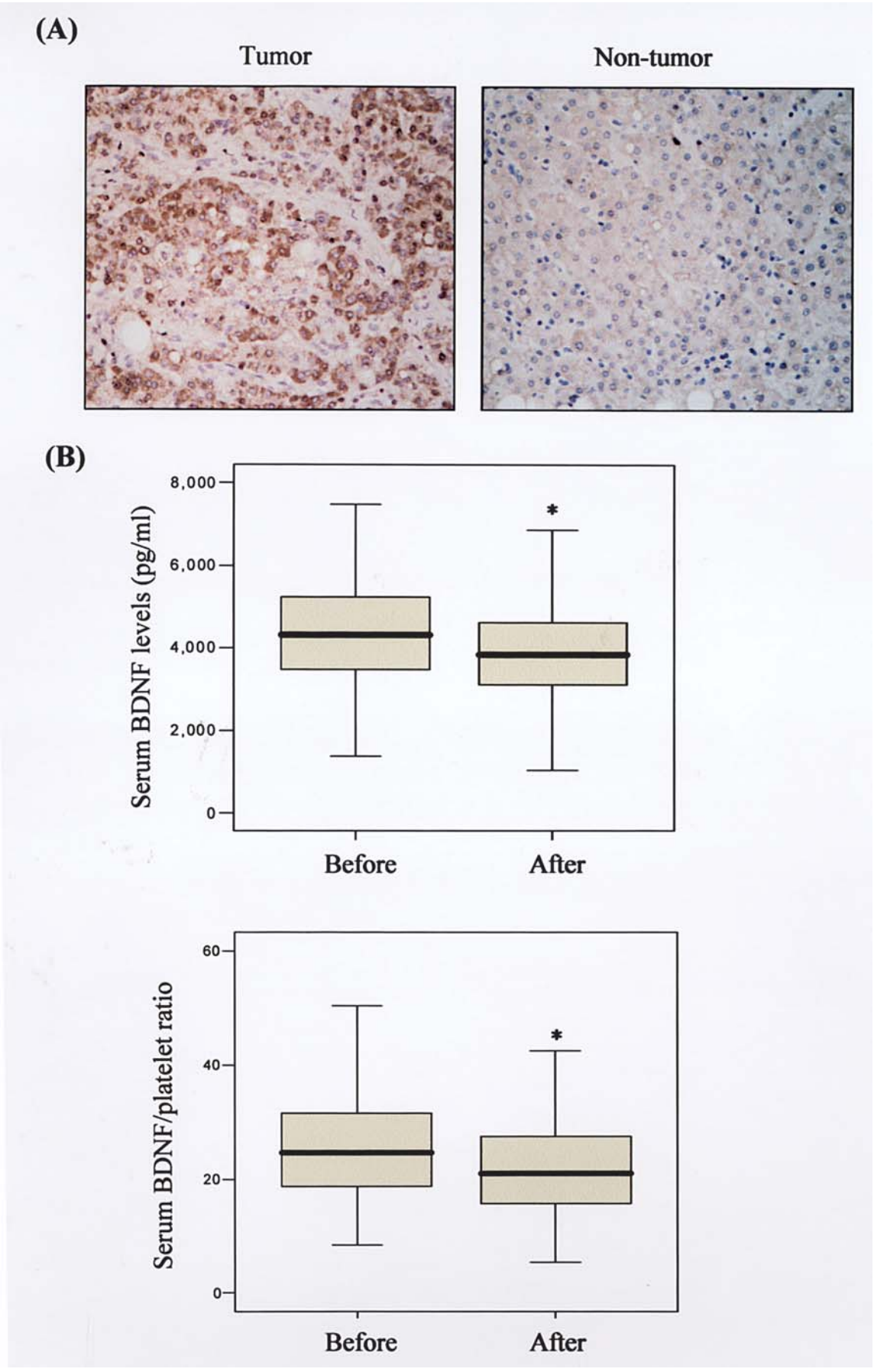

Figure 1. (A) Localization of the brain-derived neurotrophic factor (BDNF) expression in tumor and non-tumorous tissues of hepatocellular carcinoma by immunohistochemistry. Strong positive staining of BDNF was identified in the cytoplasm of the tumor cells, and weak positive staining in some of the hepatocytes in the non-tumorous areas. Magnification, x200. (B) Comparisons of the BDNF levels and the serum BDNF/platelet ratio before and after hepatectomy. Significantly decreased levels of serum BDNF and the serum BDNF/platelet ratio were detected after hepatectomy. *P<0.001, compared with serum BDNF and the serum BDNF/platelet ratio, respectively, before hepatectomy.

Patients and methods

Patients and follow-up data. From August 1995 to August 2003, 137 HCC patients who had undergone curative resection of HCC, which was defined as a complete macroscopic removal of a tumor, were recruited to this study. Seventy-one trans-plantation recipients with cirrhosis alone and 55 healthy liver donors served as the controls. Both the patients with cirrhosis and the liver donors were sex- and age-matched with the HCC patients. The patients were primarily treated by surgical resection if there was no contra-indication. Major contra-indications to surgery included the presence of extrahepatic disease, bilobar disease, and main portal venous or inferior vena cava invasion. The extent of the surgery was determined by the hepatic functional reserve, which was assessed by a combination of Child-Pugh grading, liver biochemistry and the indocyanine green clearance test, as well as the predicted remnant liver volume after resection. Routine laboratory tests, including complete blood cell count, serum alpha-fetoprotein level, and hepatitis viral serology, were performed before and during the follow-up period. Patients with operative mortality (death within 1 month after hepatectomy) were excluded from the present study.

Immunohistochemistry for BDNF expression in tumor and non-tumorous tissues. The resected tumor and non-tumorous tissues from the HCC patients (n=30), cirrhotic liver tissues (n=10), and biopsy tissues from the liver donors (n=3) were fixed in 10% buffered formalin and embedded in paraffin. The paraffin-embedded tissue was cut into 5-μm thick sections for histological studies by H&E staining and immunohistochemical staining of BDNF (rabbit anti-rat BDNF polyclonal antibody, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The sections were incubated with the primary antibody for 2 h at room temperature, followed by 1-h incubation with horseradish peroxidase-conjugated goat anti-rabbit antibody (Zymed Laboratories, Inc., South San Francisco, CA, USA). Color development was performed in substrate solution with diaminobenzidine for 5 min. The slides were then counter-stained with hematoxylin. The expression of BDNF was defined as strong if >30% of the cells were stained positive, and weak if <30% of the cells were stained positive.

Enzyme-linked immunosorbent assay (ELISA) for serum BDNF levels. Peripheral venous blood samples were taken from the patients with HCC (n=137), cirrhosis (n=71) and the liver donors (n=55), put into serum separator tubes, and centrifuged at 3,000 rpm for 7 min. The serum samples were then collected and stored at -80˚C. Serum samples from the HCC patients were collected before and one month after hepatic resection, whereas those from the patients with cirrhosis and the liver donors were collected before surgery.

The serum levels of BDNF were detected by ELISA. The assay exhibited no significant cross-reactivity with other members of the nerve growth factor family. All samples were assayed in duplicate. The procedures were performed according to the manufacturer's instructions (Chemicon, Temecula, CA, USA).

Statistical analysis. Comparisons of the serum BDNF levels and the serum BDNF/platelet ratio in the liver donors, patients with cirrhosis and the patients with HCC were performed using the Mann-Whitney U test. Comparisons of the serum BDNF levels and the serum BDNF/platelet ratio before and after hepatectomy were performed by the paired-sample t-test. The correlation between the serum BDNF levels and the platelet count was performed with linear regression analysis. The correlation between the tissue and serum levels of BDNF was analyzed by the Chi-square test. Confounding pathological variables were entered into a multiple regression analysis to evaluate any independent relationship between each pathological variable and the serum BDNF level. For all statistical analyses, SPSS software (version 10.0 for Windows; SPSS Inc., Chicago, IL, USA) was used. Disease-free survival was analyzed by the log-rank test using the GraphPad Prism software (GraphPad Software Inc, San Diego, CA, USA). A P-value of <0.05 was considered statistically significant.

Results

The expression of BDNF in tumor and non-tumorous tissues. By immunohistochemical staining, the expression of BDNF was found to a variable extent in all the tumors. BDNF was predominantly expressed in the cytoplasm of the tumor cells (Fig. 1A), within which 17 cases demonstrated positive BDNF staining (17/30, 56.7%). Fifteen cases of tumor tissues demonstrated strong positivity. Some of the non-tumorous tissues also demonstrated positive staining of BDNF (10/30, 33.3%). There was weakly positive staining of BDNF in the normal liver tissue in one case, and only two cases of the cirrhotic liver tissues had weakly positive staining of BDNF.

By the paired-sample t-test, we compared the serum levels of BDNF and the serum BDNF/platelet ratios before and after hepatectomy. Significant decreases in the serum BDNF levels and the serum BDNF/platelet ratios were detected after hepatectomy [4,847 (before) vs 4,126 (after) pg/ml, P<0.001; 29.2 (before) vs 23.2 (after), P<0.001] (Fig. 1B).

To evaluate whether there was a correlation between the tissue BDNF expression with the serum BDNF levels, the median level of serum BDNF (in the above 30 HCC cases with tissue immunohistochemical staining for BDNF) was used as a cut-off point to differentiate high and low expressions, and compared with the presence and absence of BDNF staining in the tumor tissues. There was a positive correlation between the tissue and serum levels of BDNF within this group of patients (P=0.025) (Table I).

Serum BDNF levels and correlation with clinicopathological features. The median value of serum BDNF levels in the liver donors was 3,996 (1,313-5,421) pg/ml, and that in the patients with cirrhosis was 1,519 (92-3,220) pg/ml, whereas in the HCC patients, it was 4,847 (1,383-7,379) pg/ml (P=0.001 vs liver donors; P<0.001 vs patients with cirrhosis) (Fig. 2). A significantly positive correlation was detected between serum the BDNF levels and the platelet count in the patients with HCC (r=0.38, P<0.001) (Fig. 3). The patients with cirrhosis presented a significantly lower number of platelets than the liver donors and the patients with cirrhosis

Table I. Correlation between the tissue and serum levels of BDNF.

		Tissue BDNF		Total
		-	+	
Serum BDNF	Low	10	5	15
	High	3	12	15
Total		13	17	30

Chi-square test, P=0.025

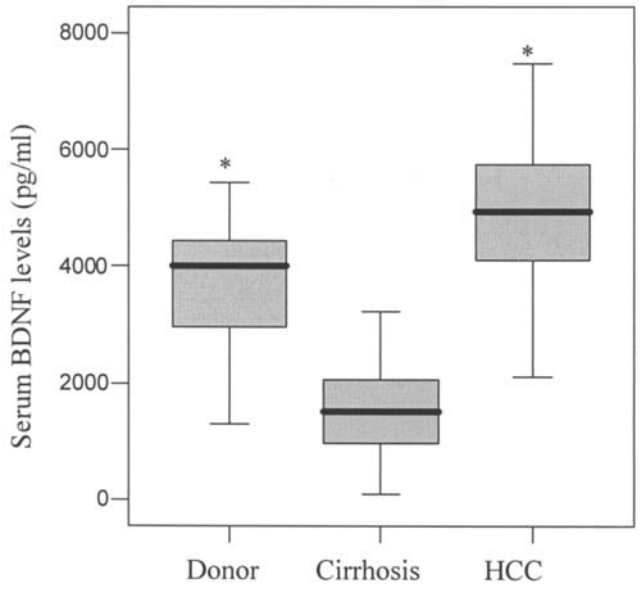

Figure 2. Comparison of the serum brain-derived neurotrophic factor (BDNF) levels among the liver donors, patients with cirrhosis and hepatocellular carcinoma (HCC). The highest level of serum BDNF was detected in the HCC patients, whereas the lowest level of serum BDNF was detected in the patients with cirrhosis (*P<0.005 vs patients with cirrhosis; #P<0.001 vs liver donors and patients with cirrhosis). The comparisons were performed using the Mann-Whitney U test.

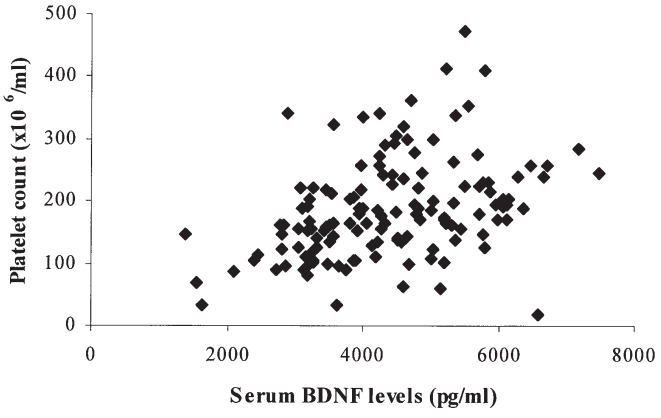

Figure 3. Correlation of the serum brain-derived neurotrophic factor (BDNF) levels and platelet count in hepatocellular carcinoma patients with hepatic resection. A significantly positive correlation of serum BDNF levels and platelet count was identified (r=0.38, P<0.001).

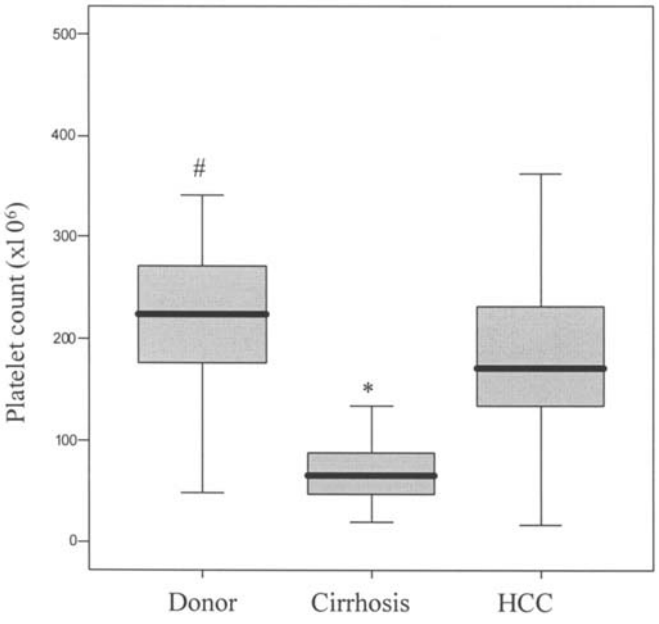

Figure 4. Comparisons of the platelet counts among the different groups. The lowest number of platelets was detected in the patients with cirrhosis (*P<0.001, compared with the platelet count in the patients with cirrhosis). On the contrary, the number of platelets in the liver donors was significantly higher than that in the HCC patients (#P=0.001, compared with the platelet count in the HCC patients).

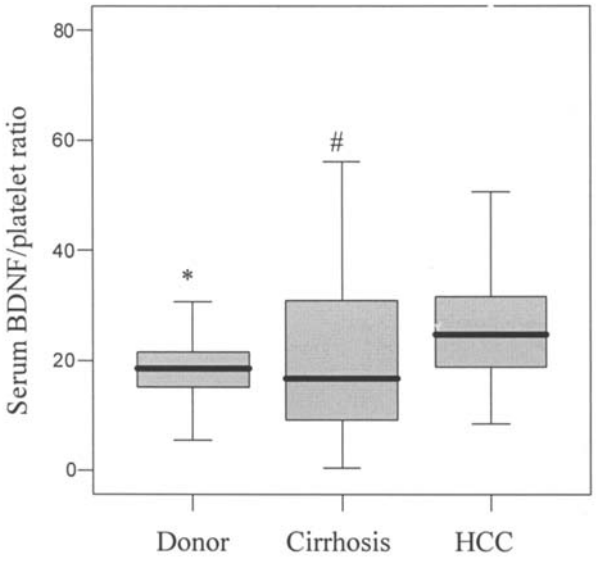

Figure 5. Comparisons of the serum BDNF/platelet ratio among the different groups. The highest serum BDNF/platelet ratio was detected in the HCC patients (P<0.001 and P=0.001, compared with that in the liver donors and the patients with cirrhosis, respectively). However, there was no significant difference in the serum BDNF/platelet ratio between the liver donors and the patients with cirrhosis.

(Fig. 4). The highest value of the serum BDNF/platelet ratio was detected in the HCC group (Fig. 5).

No difference was identified in the serum BDNF levels between gender, age younger or older than 60 years, the serum alpha-fetoprotein level lower or higher than 20 ng/ml, the presence or absence of venous infiltration, and the

Table II. Correlation of the serum BDNF levels with the clinicopathological features of HCC.

Variables	Serum BDNF (pg/ml)	P-value
Sex		
Male (n=110)	4403.2±1210.5	0.577
Female (n=27)	4259.6±1138.2	
Age		
≤60 (n=89)	4420±1222	0.548
>60 (n=48)	4291.1±1147.2	
Serum AFP level		
≤20 ng/ml (n=50)	4387.2±1151.1	0.927
>20 ng/ml (n=87)	4367.8±1224.3	
Tumor size		
<5 cm (n=66)	4011.6±1101.8	<0.001[a]
≥5 cm (n=71)	4712.6±1183.9	
Edmonson grading		
I-II (n=108)	4318.7±1137.5	0.012[a]
III-IV (n=29)	4963±1226.4	
Venous infiltration		
No (n=73)	4400.3±1217.7	0.791
Yes (n=64)	4345.8±1175	
Cirrhosis		
Absent (n=72)	4695.7±1146.9	0.001[a]
Present (n=65)	4019.5±1151.3	
Microsatellite		
Absent (n=102)	4242.6±1220.7	0.026[a]
Present (n=35)	4760.4±1033.8	
AJCC tumor staging		
I-II (n=99)	4313.7±1254.5	0.335
III-IV (n=38)	4534.2±1016.9	

BDNF, brain-derived neurotrophic factor; HCC, hepatocellular carcinoma; AFP, alpha-fetoprotein; AJCC, American Joint Committee on Cancer.

Table III. Correlation of the platelet count with the clinicopathological features of hepatocellular carcinoma.

Variables	Platelet count (x10^6/ml)	P-value
Sex		
Male (n=110)	187.1±78.5	0.993
Female (n=27)	187.6±87.1	
Age		
≤60 (n=89)	194.5±84.4	0.141
>60 (n=48)	173.4±69.6	
Serum AFP level		
≤20 ng/ml (n=50)	191±71.2	0.667
>20 ng/ml (n=87)	184.8±84.8	
Tumor size		
<5 cm (n=66)	146.6±56.9	<0.001[a]
≥5 cm (n=71)	224.7±80.1	
Edmonson grading		
I-II (n=108)	187±83.5	0.912
III-IV (n=29)	188.9±69.9	
Venous infiltration		
No (n=73)	175.6±78.7	0.073
Yes (n=64)	200.2±79.8	
Cirrhosis		
Absent (n=72)	220±78.2	<0.001[a]
Present (n=65)	150.7±65	
Microsatellite		
Absent (n=102)	179.7±81.8	0.064
Present (n=35)	208.6±70.7	
AJCC tumor staging		
I-II (n=99)	176±75.9	0.008[a]
III-IV (n=38)	216.1±83.7	

AFP, alpha-fetoprotein; AJCC, American Joint Committee on Cancer.

American Joint Committee on Cancer tumor-node-metastasis staging. However, a higher level of serum BDNF was significantly correlated with tumor size >5 cm (P<0.001), poorly differentiated HCC (P=0.012), the presence of microsatellite tumor nodules (P=0.026) and the absence of cirrhosis in the non-tumorous tissues (P=0.001) (Table II). When these four factors were put into a multiple regression analysis, only tumor cell differentiation was an independent factor that affected the serum levels of BDNF (tumor size, P=0.052; tumor cell differentiation, P=0.047; microsatellite tumor nodules, P=0.081; cirrhosis in the non-tumorous tissue, P=0.074). A higher value of platelet count was significantly correlated with tumor size >5 cm (P<0.001), the absence of cirrhosis in the non-tumorous tissue (P<0.001) and an advanced tumor stage (P=0.008) (Table III). When the median level of serum BDNF was used as a cut-off point, there was no significant difference of disease-free survival between patients with high and low serum BDNF levels. However, when the median value of the serum BDNF/platelet ratio was used as a cut-off point, a higher level of the serum BDNF/platelet ratio was associated with a shorter disease-free survival (P=0.052) (Fig. 6).

Discussion

In the clinical setting, we characterized that BDNF was expressed in the cytoplasm of HCC tumor cells and hepatocytes in the non-tumorous areas, with a higher level of BDNF expression in the tumor tissue. Corresponding to the findings

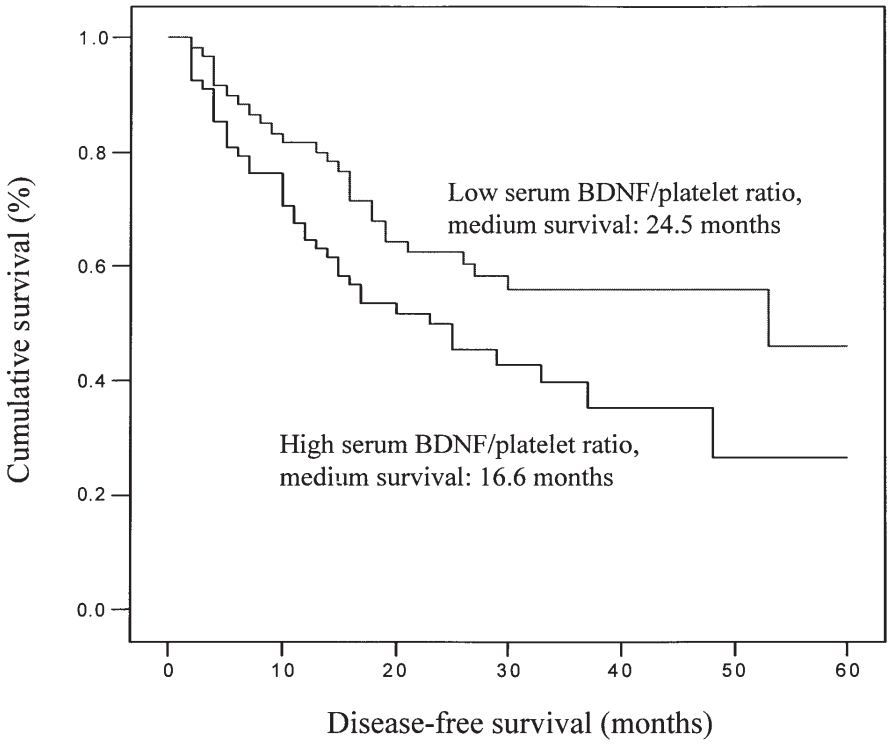

Figure 6. The impact of serum BDNF and the serum BDNF/platelet ratio on disease-free survival of HCC patients. When the median level of serum BDNF was used as a cut-off point, no significant difference in disease-free survival was identified. However, when the median value of the serum BDNF/platelet ratio was used as a cut-off point, a higher level of serum BDNF/platelet ratio was associated with a shorter disease-free survival (higher serum BDNF/platelet ratio, medium survival 14.6 months; lower serum BDNF/platelet ratio, medium survival 24.4 months, P=0.052).

in the tissues, a higher level of serum BDNF was also detected in the HCC patients compared to that in the liver donors and patients with cirrhosis. Cells in the tumor and non-tumorous tissues might both contribute to the higher level of circulating BDNF, but the tumor cells might play a more prominent role. In addition, a decreased level of serum BDNF after the resection of HCC further revealed the importance of tumor cells in producing the circulating BDNF. However, in the normal population, despite weak positive staining of BDNF in the normal liver tissues, we detected a certain level of serum BDNF, indicating that HCC tumor cells were not the only source of serum BDNF.

The positive correlation of the serum BDNF level with the platelet count suggested the importance of platelets in the storage of BDNF. Under normal circumstances, there is an ~200-fold difference between the plasma and serum BDNF levels (20-22). However, it is not clear whether the platelet is a source of BDNF or a place for storage only. The lowest level of serum BDNF measured in the patients with cirrhosis further suggested the close relationship of serum BDNF and platelets, as a significantly lower number of platelets were detected in this group of patients. This was due to thrombocytopenia caused by portal hypertension. Therefore, by correcting the impact of the platelet count using the serum BDNF/platelet ratio, we detected a higher serum BDNF/platelet ratio in the patients with HCC than in the liver donors or patients with cirrhosis, and a higher level of serum BDNF/platelet ratio was associated with a poorer disease-free survival, providing further evidence that the platelets in the HCC patients might store more BDNF than in the normal population or patients with cirrhosis, and that the BDNF release from activated platelets might contribute to the progression of the disease. However, the relationship between the tumor cell, BDNF, and the platelet needs to be further explored.

BDNF is a novel functional molecule recently identified to play a role in cancer progression (12,13). To our knowledge, this is the first study on the clinicopathological significance of serum BDNF in HCC patients. The correlation of serum BDNF levels with tumor size and tumor differentiation supported our previous *in vitro* finding that BDNF was a functional protein that promoted tumor cell growth (19). However, the reason why a higher level of serum BDNF was associated with the absence of cirrhosis in the non-tumorous tissues remains to be determined. One possible reason might be that in the HCC patients with cirrhotic non-tumorous tissue, portal hypertension led to thrombocytopenia and a lower BDNF level. Another reason might be that in the HCC patients with cirrhotic non-tumorous tissue, the tumor size was usually smaller than that in the HCC patients without cirrhosis in the non-tumorous tissue as a result of case selection for hepatic resection, and a larger tumor size was found to be positively associated with a higher level of serum BDNF. We tended not to offer hepatic resection for patients with large HCC associated with significant cirrhosis because of the inadequate liver reserve for major resection. Nonetheless, neither the tumor size nor the presence of cirrhosis was significantly related to serum BDNF in the multivariate analysis. Only poorly differentiated tumors were associated with a higher BDNF level.

In conclusion, this study suggest that HCC tumor cells are an important source of serum BDNF in HCC patients. The serum level of BDNF was positively correlated with the platelet count and the tumor status. The interaction between the serum BDNF and the platelet might contribute to tumor progression and recurrence after hepatic resection.

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