Eur J Nutr (2007) 46:293–299
DOI 10.1007/s00394-007-0665-8

ORIGINAL CONTRIBUTION

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B vitamins deficiency and decreased anti-oxidative state in patients with liver cancer

Received: 28 February 2007
Accepted: 29 May 2007
Published online: 14 June 2007

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Abstract *Background* This study examined the status of oxidative stress and B vitamins in hepatocellular carcinoma (HCC) patients in different tumor-node-metastasis stages. Patients were divided into two groups as I + II (n = 21) and III + IV (n = 19). *Methods* Plasma levels of lipid oxidation, α-tocopherol, β-carotene, vitamin C, glutathione and the activity of antioxidant enzymes (glutathione peroxidase, superoxide dismutase, catalase, and xanthine oxidase) were determined for evaluating oxidative status. Blood B vitamins (B_1, B_2, B_6, B_{12}, and folate) and serum ghrelin were analyzed, and the relationship between serum ghrelin and vitamins B_2 (or B_6) was evaluated. *Results* HCC patients at III + IV stage showed significantly lower ghrelin, higher cholesterol, triglyceride, and uric acid than patients at I + II stage and healthy subjects ($P < 0.05$). Plasma lipid oxidation level in HCC patients was significantly greater than healthy subjects ($P < 0.05$). The activity of glutathione peroxidase, superoxide dismutase or catalase was significantly decreased, but xanthine oxidase activity was significantly elevated in HCC patients ($P < 0.05$). Plasma level of glutathione and vitamin C, not α-tocopherol and β-carotene, in HCC patients was significantly lower ($P < 0.05$). Vitamins B_2 and B_6 levels in red blood cells from these HCC patients were significantly lower ($P < 0.05$). *Conclusion* This study provided novel clinical findings regarding the status of oxidative stress and B vitamins in HCC patients. Plasma glutathione level may be a proper biomarker for evaluating oxidative status for HCC patients. Our data indicate that HCC patients might need B vitamins supplementation. The increased serum level of triglyceride and cholesterol might be a consequence of an impaired hepatic fat metabolism, and might be improved by a lower fat administration to these patients.

Key words hepatocellular carcinoma – oxidative stress – B vitamins – glutathione – ghrelin

Introduction

Liver cancer, also called hepatocellular carcinoma (HCC), is the fifth most common malignancy in the world [1]. In Taiwan, HCC is the leading cause of cancer-related death among men, and is the second among women [2]. Many studies have indicated that cancer associated alternations in host metabolism are

important factors for mortality determination because the disturbance in host metabolism could cause undernutrition, and even cachexia [3, 4]. Thus, any understanding about the nutritional status of cancer patients may give more detailed insight into the metabolic disturbance. So far, little is known about the nutritional status of patients with liver cancer.

It has been proposed that oxidative stress is involved in the etiology and deterioration of cancers, including liver cancer [5, 6]. Those authors thought lipid oxidation products such as malondialdehyde could act as tumor promoters and co-carcinogenic agents via their high cytotoxicity action [6]. Several non-enzymatic antioxidants such as α-tocopherol and ascorbic acid possess antioxidative property, and play important role in the antioxidant protection. However, the clinical information about the status of these non-enzymatic antioxidants in HCC patients is lacked. On the other hand, it remains unclear that these non-enzymatic antioxidants really contribute to anti-oxidative defense for HCC patients.

Ghrelin, a growth hormone-releasing peptide, is involved in metabolic regulation and favors a positive energy balance [7]. The increased plasma ghrelin level in lung cancer patients with cachexia has been observed [8]. However, the variation of ghrelin level in liver cancer patients remains unknown. On the other hand, B vitamins including vitamin B_1 (thiamine), vitamin B_2 (riboflavin), vitamin B_6 (pyridoxine), vitamin B_{12} and folic acid are involved in many important physiological functions such as energy metabolism, protein biosynthesis and cell reproduction. However, besides folic acid, less attention is paid to the variation of these B vitamins in liver cancer progression. Furthermore, less information is available regarding the relationship between B vitamins and ghrelin in liver cancer patients.

The purpose of this study was to examine the status of B vitamins and non-enzymatic antioxidants in HCC patients. The influence of several non-enzymatic antioxidants upon antioxidative protection, and the relationship between B vitamins and serum ghrelin in these HCC patients were also evaluated.

Materials and methods

Patients and healthy subjects

This study protocol was proved by Ethical Committee of the Medicine Faculty at Chung Shan Medical University. Forty patients with cytologically or histologically confirmed liver cancer at Chung Shan Medical University Hospital between January and July, 2006 were included in this study. These patients (25 male and 15 female with age range being 37–80, mean age = 63.7) were newly diagnosed ones and taking no therapy. These HCC patients were classified according to the international tumor-node-metastasis (TNM) staging system: stages I (n = 14), II (n = 7), III (n = 13), and IV (n = 6). In this study, 40 HCC patients were divided into two groups as I + II (n = 21) and III + IV (n = 19). Twenty healthy subjects (10 male and 10 female with age range being 47–82, mean age = 61.4) were recruited in Chung Shan Medical University Hospital between January and July, 2006. These subjects, confirmed by blood and ultrasound examination, did not suffer from HCC or other liver diseases and were included as control group for comparison. The data for body weight and height of patients and healthy subjects were collected.

Dietary record and nutrients analyses

A 3-day dietary record including meal, snack and drink was obtained from each subject. Nutrient composition was calculated by Nutritionist Professional software (E-Kitchen Business Corporation, Taiwan) based on Taiwan Nutrient Databases [9].

Blood sampling

Informed consent for study participation was obtained from 40 HCC patients and 20 healthy control subjects. A peripheral blood sample, 15 ml, from each subject was drawn after an overnight fasting. Plasma or serum was separated from erythrocyte immediately after blood collection.

Biochemical measurements

Serum level of albumin, glucose, cholesterol, triglyceride, creatinine, uric acid and bilirubin was determined by an autoanalyzer (Dr. Lange LP 420, German). Serum alpha fetal protein (AFP) level was measured by a commercial enzyme-linked immunosorbent assay kit (Abbott, North Chicago IL, USA). Serum immunoreactive ghrelin concentration was measured in duplicate using a commercial radioimmunoassay kit (Phoenix Pharmaceuticals, Belmont, California, USA). The intra-assay coefficient of variation (CV) was 8.1%, and the inter-assay CV was 9%. Lactate dehydrogenase (LDH) activity in serum was determined using photometric method by an automated instrument (Shimadzu CL-7300, Japan).

The plasma level of α-tocopherol and β-carotene was quantified by a reverse-phase HPLC method [10]. Vitamin C (ascorbic acid) level was immediately determined right after blood sample was col-

lected by a fluorometric method [11] in order to avoid loss of vitamin C caused by long-term freezing. Glutathione (GSH) concentration in plasma was determined by a commercial colorimetric GSH assay kit (OxisResearch, Portland, OR, USA). The activity of catalase, Cu–Zn superoxide dismutase (SOD) and glutathione peroxidase (GPX) in plasma was determined by catalase, SOD and GPX assay kits, respectively (Calbiochem, EMD Biosciences, Inc. San Diego, CA, USA). Xanthine oxidase (XO) activity was measured spectrophotometrically by the formation of uric acid from xanthine through the increase in absorbance at 293 nm [12]. Lipid oxidation level in plasma was determined by measuring the formation of malondialdehyde (MDA) via an HPLC method [13].

B vitamins analyses

The level of vitamins B_1, B_2, and B_6 in whole blood, plasma, or red blood cells from healthy control and HCC patients was determined by HPLC methods [14–16]. The status of vitamins B_1, B_2, B_6 was determined as thiamine diphosphate, flavin adenine dinucleotide and pyridoxal-5′-phosphate, respectively. Folate and vitamin B_{12} (cobalamin) were analyzed by radioprotein binding assay (Bio-Rad Laboratories, Richmond, CA, USA). For folate determination, folic acid as pteroylglutamic acid was used for calibration, and its ^{125}I-labeled analog was used as the tracer. For cobalamin determination, cyanocobalamin was used for calibration, and its ^{57}Co-labeled analog was the tracer for cobalamin assay.

Statistical analysis

Each measurement was analyzed from 40 HCC patients and 20 healthy subjects. The concentration or activity for each measurement was directly reported. All data presented in this study were mean ± SD. Data were subjected to analysis of variance (ANOVA), and difference with $P < 0.05$ was considered to be significant. Correlation between two variables was calculated by simple regression analysis (Minitab Inc., State College, Philadelphia, USA).

Results

Dietary record was used for nutrient intake analysis. There was no significant difference in the nutrients concerned in this study between cancer patients and healthy control groups ($P > 0.05$, data not shown). The baseline characteristics for HCC patients and healthy controls are presented in Table 1. HCC patients at III + IV stage showed significantly lower BMI and ghrelin, higher cholesterol, triglyceride, uric acid, LDH and AFP than patients at I+II stage and healthy controls ($P < 0.05$). The relationship between serum uric acid level and LDH activity in 40 HCC patients is presented in Fig. 1, and the correlation coefficient was 0.863. Plasma lipid oxidation, determined as MDA

Table 1 Baseline characteristics in patients with liver cancer (HCC) and healthy subjects (control)[a]

Parameters	Control	HCC	
	$n = 20$	Stage I + II $n = 21$	Stage III + IV $n = 19$
Body mass index (kg/m^2)	24.2 ± 2.3	25.2 ± 2.1	21.8 ± 1.5[b]
Albumin (g/dl)	4.35 ± 0.42	3.46 ± 0.47[b]	3.29 ± 0.71[b]
Fasting glucose (mg/dl)	98 ± 14	109 ± 8	92 ± 11
Cholesterol (mg/dl)	163 ± 28	238 ± 31[b]	307 ± 24[b,c]
Triglyceride (mg/dl)	104 ± 20	125 ± 37	219 ± 38[b]
Creatinine (mg/dl)	0.71 ± 0.17	1.16 ± 0.23[b]	1.13 ± 0.25[b]
Uric acid (μmol/l)	218.6 ± 18.7	284.8 ± 20.4[b]	362.4 ± 32.5[b,c]
Bilirubin (mg/dl)	0.51 ± 0.16	1.07 ± 0.24	2.17 ± 0.42[b]
alpha Fetal protein (ng/l)	20.6 ± 3.8	788.4 ± 59.6[b]	5143.2 ± 361[b,c]
Lactate dehydrogenase (U/l)	293 ± 21	394 ± 27[b]	472 ± 33[b,c]
Ghrelin (pmol/l)	133 ± 13	102 ± 18[b]	67 ± 10[b,c]
Associated diseases			
Hepatitis B	0	4	5
Hepatitis C	0	3	4
Diabetes	1	2	2
Renal insufficiency	0	2	1
Hypertension	1	4	3

[a] Values are means ± SD
[b] $P < 0.05$ versus controls
[c] $P < 0.05$ versus patients at stage I + II

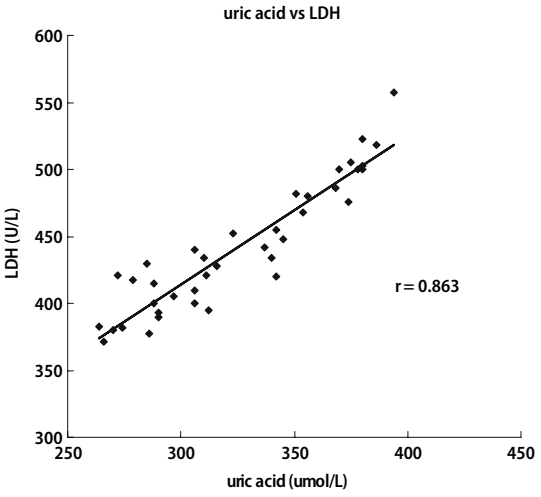

Fig. 1 The relationship between serum uric acid (μmol/l) and lactate dehydrogenase (LDH) activity (U/l) in patients with liver cancer

Table 2 Plasma level of malondialdehyde (MDA), non-enzymatic antioxidants (vitamin C, α-tocopherol, β-carotene, glutathione), and activity of enzymes (glutathione peroxidase, GPX; superoxide dismutase, SOD; catalase, CAT; xanthine oxidase, XO) in patients with liver cancer (HCC) and healthy subjects (control)[a]

Parameters	Control	HCC	
	$n = 20$	Stage I + II $n = 21$	Stage III + IV $n = 19$
MDA (nmol/ml)	1.10 ± 0.23	3.26 ± 0.46[b]	5.83 ± 0.68[b,c]
α-tocopherol (μmol/l)	19.2 ± 2.8	22.8 ± 3.4[b]	24.1 ± 2.7[b]
β-carotene (μmol/l)	0.56 ± 0.14	0.74 ± 0.23	1.05 ± 0.33[b]
Vitamin C (μmol/l)	31.7 ± 3.6	24.8 ± 2.1[b]	17.9 ± 5.3[b,c]
Glutathione (μmol/l)	12.8 ± 1.5	8.3 ± 2.3[b]	5.5 ± 3.0[b]
GPX (U/l)	266 ± 21	154 ± 18[b]	82 ± 16[b,c]
SOD (U/ml)	17.8 ± 0.14	13.5 ± 0.23[b]	10.7 ± 0.56[b]
CAT (U/ml)	11.1 ± 0.32	8.26 ± 0.25[b]	5.07 ± 0.37[b,c]
XO (U/l)	1.2 ± 0.4	4.4 ± 0.6[b]	7.5 ± 1.3[b,c]

[a] Values are means ± SD
[b] $P < 0.05$ versus controls
[c] $P < 0.05$ versus patients at stage I + II

level, in HCC patients was significantly increased ($P < 0.05$, Table 2). When comparing with healthy controls, the plasma level of α-tocopherol and β-carotene was significantly increased in HCC patients ($P < 0.05$), but the level of ascorbic acid and glutathione was significantly decreased ($P < 0.05$). GPX, SOD and CAT activities were significantly reduced in these HCC patients, but XO activity in these patients was significantly elevated ($P < 0.05$, Table 2). Blood levels of B vitamins are shown in Table 3. The RBC level of vitamin B_2, vitamin B_6, and plasma level of B_{12} and folate in HCC patients were significantly lower than healthy controls ($P < 0.05$). When compared with patients at I + II stage, the patients at III + IV stage had, significantly lower vitamin B_1 level in whole blood or RBC ($P < 0.05$). The relationships between serum ghrelin level and RBC vitamin B_2 (and B_6) in 40 HCC patients are presented in Fig. 2. Both RBC vitamins B_2 and B_6 were positively correlated with serum ghrelin, and the correlation coefficients were 0.784 and 0.872, respectively.

Table 3 B vitamins in whole blood, plasma or red blood cell (RBC) from patients with liver cancer (HCC) and healthy subjects (control)[a]

Parameters	Control	HCC	
	$n = 20$	Stage I + II $n = 21$	Stage III + IV $n = 19$
Whole blood vitamin B_1 (nmol/l)	114 ± 15	97 ± 13	67 ± 22[b]
RBC vitamin B_1 (ng/g Hb)	420 ± 23	384 ± 21	327 ± 25[b]
Plasma vitamin B_2 (nmol/l)	64.6 ± 2.7	57.1 ± 2.1[b]	40.7 ± 3.2[b,c]
RBC vitamin B_2 (nmol/g Hb)	2.73 ± 0.40	1.82 ± 0.19[b]	1.23 ± 0.34[b, c]
Plasma vitamin B_6 (nmol/l)	19.8 ± 1.2	17.2 ± 1.1	12.3 ± 2.0[b,c]
RBC vitamin B_6 (pmol/g Hb)	304 ± 15	269 ± 18[b]	218 ± 23[b,c]
Plasma vitamin B_{12} (pmol/l)	334 ± 28	290 ± 21[b]	287 ± 25[b]
Plasmal folate (nmol/l)	31.2 ± 3.2	22.7 ± 2.3[b]	18.5 ± 1.6[b,c]

[a] Values are means ± SD
[b] $P < 0.05$ versus controls
[c] $P < 0.05$ versus patients at stage I + II

Discussion

Several studies have reported that cancer patients suffer from undernutrition, weight loss and even severe catabolic status such as cachexia [7, 8]. Although all subjects participated in our present study did not develop marked cachexia; however, based on the reducing BMI, lower ghrelin level and increasing LDH activity, these HCC patients might be under the risk of malnutrition when compared with healthy controls. HCC patients with high serum LDH activity or uric acid level have been reported [17–19]. Those authors indicated that elevated LDH activity represented rapidly growing malignant tumors; thus, serum uric acid level and LDH activity could be used as biomarkers for the tumor prognostic evaluation. Our present study found both serum uric acid level and LDH activity markedly increased from lower to higher stages in these HCC patients; thus, we agreed that uric acid level or LDH activity could be used as a biomarker for evaluating HCC progression. Furthermore, we found that XO activity in plasma from HCC patients was elevated from lower stage to higher stage. It is known that XO is the rate-limiting enzyme in nucleic acid degradation, and responsible for the conversion of xanthine and hypoxanthine to uric acid. Apparently, the elevated XO activity in these HCC patients could favor purine catabolism, probably in normal cells. This finding not only partially explained the increased production of uric acid in these HCC

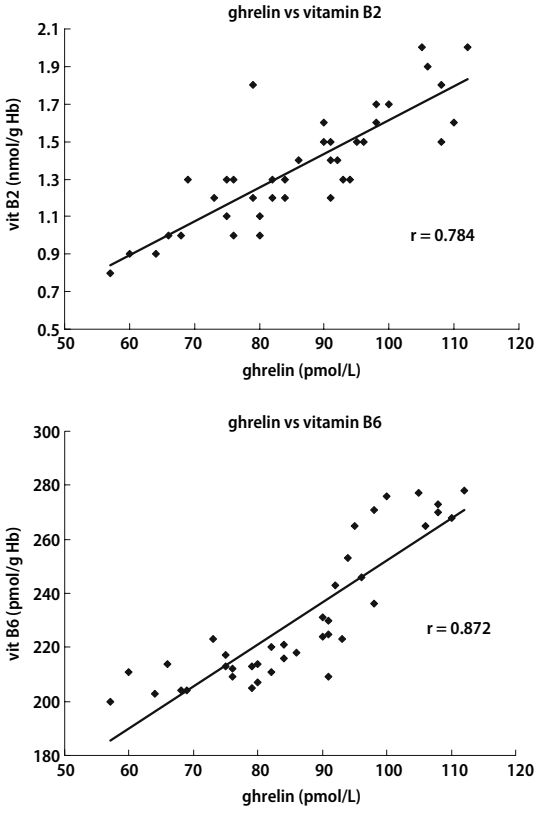

Fig. 2 The relationship between serum ghrelin (pmol/l) and red blood cell vitamin B_2 (nmol/g Hb), vitamin B_6 (pmol/g Hb) in patients with liver cancer

patients, but also strongly suggested that these HCC patients were under catabolic status.

In the study of Alsabti [20] and our present study, increased plasma triglyceride and total cholesterol levels were observed in HCC patients. However, Ahaneku et al. [21], Cicognani et al. [22] and Jiang et al. [23] reported that HCC patients showed lower plasma triglyceride and total cholesterol levels. Racial disparity and dietary discrepancy may be possible causes responsible for the difference in lipid metabolism between those previous studies and our present study. On the other hand, most HCC patients in Chinese population are strongly correlated with HBV and/or HCV infection. So far, less information is available regarding the combined effect of virus infection and HCC upon host's lipid metabolism. Although it is possible that lipid metabolism was impaired in those HCC patients, which consequently caused the accumulation of these lipid associated compounds including triglyceride and α-tocopherol, further large-scale study is necessary to evaluate whether dietary lipid should be limited for these patients.

It is well known that oxidative damage plays an important role in the pathogenesis of liver diseases; however, a large-scale clinical evaluation on oxidative status for HCC patients is lacked. Our present study found that oxidative stress was markedly enhanced in HCC patients from lower stage to higher stage, and the activities of GPX, SOD, CAT in plasma from these patients were markedly reduced. The impaired antioxidative system apparently favored the accumulation of free radicals, which partially explained the enhanced lipid oxidation occurred in these HCC patients. Furthermore, we found α-tocopherol and β-carotene levels in these patients were similar or even higher when compared with healthy subjects. Many studies have indicated that the non-enzymatic antioxidants like α-tocopherol could scavenge free radicals and suppress oxidation [24, 25]; thus, cancer patients are encouraged to consume these antioxidant nutrients [26, 27]. However, the results from our present study made us to raise two questions: (1) since these HCC patients still had sufficient α-tocopherol and β-carotene, why not using these antioxidants to alleviate their oxidative stress? (2) whether these HCC patients need to supply more antioxidant nutrients like α-tocopherol? Further studies are necessary to examine the impact of these lipid soluble non-enzymatic antioxidants in patients with liver cancer. It is ensured that both α-tocopherol and β-carotene are not proper biomarkers for evaluating oxidative stress for HCC patients. On the other hand, the reduction of vitamin C and glutathione levels was apparently along with cancer progression in these HCC patients. It might be due to liver with cancer cells fail to store these water-soluble nutrients, or oxidative stress from cancer progression enhance the requirement of these water-soluble nutrients.

The blood levels of vitamins B_2, B_6 and folate were reduced with liver cancer progression from lower stage to higher stage. Vitamin B_2 affects epithelial integrity, rate of prostaglandin biosynthesis, each of which may have implication for carcinogenesis [28]. Vitamin B_6 is a cofactor for enzymes involved in glutathione metabolism [29]. Thus, the rapid energy metabolism and/or tumor growth occurred in HCC patients caused B vitamins depletion, which further down-regulated GSH synthesis. Definitely, the B vitamins depletion and GSH decrease in HCC patients could impair many physiological functions and antioxidant defense, which might further facilitate cancer detrimental development. On the other hand, it has been documented that vitamins B_2 and B_6 could exhibit antioxidant activity via scavenging oxygen radicals and organic radicals [30, 31]. Thus, the decrease in vitamins B_2 and B_6 in these HCC patients also diminished antioxidant defense. Although it has been reported that vitamin B_6 exhibited anti-angiogenic and anti-cancer effects [32, 33] via inhibiting some types of eukaryotic DNA polymerases, further large-scale study is necessary to re-verify the change of these parame-

ters in HCC patients in order to evaluate whether HCC patients need B vitamins supplement.

The function of ghrelin is to increase food intake and causes a positive energy balance by decreasing fat utilization through growth hormone independent mechanisms [34], thus, the decreased production of ghrelin in these liver cancer patients apparently favored a negative energy balance, which might consequently result in the occurrence of undernutrition in these patients. Furthermore, we found the strong positive correlations between serum ghrelin and RBC vitamins B_2 and B_6. Since vitamins B_2 and B_6 are involved in amino acid metabolism, the deficiency of these two vitamins might influence the biosynthesis of ghrelin. Further study is necessary to elucidate the relationship between ghrelin and vitamins B_2 (and B_6). These results from our present study suggested that the assessment of ghrelin, or RBC vitamin B_2 (or vitamin B_6) could be used as an indicator for evaluating the nutritional status of liver cancer patients.

In conclusion, this study provided novel clinical findings regarding the status of oxidative stress and B vitamins in HCC patients. The blood levels of glutathione, vitamins C, B_2 and B_6 were markedly reduced in HCC patients, in which vitamins B_2 and B_6 were positively correlated with serum ghrelin. Plasma glutathione level may be a proper biomarker for evaluating oxidative status for HCC patients. Lipid metabolism was disordered in these HCC patients. The increased serum level of triglyceride and cholesterol might be a consequence of an impaired hepatic fat metabolism, and might be improved by a lower fat administration to these patients.

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