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Predictive biomarkers for diagnosis of minimal hepatic encephalopathy in patients with liver cirrhosis: A preliminary result in a single center study in Japan

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Abstract

Aim: Minimal hepatic encephalopathy (MHE) in liver cirrhosis (LC) is a subclinical abnormality which is only detectable by neuropsychiatric and neurophysiological tests. Reliable predictive biomarkers for diagnosing MHE have not been confirmed. This study examined potential biomarkers to detect MHE in LC patients.

Methods: We divided 35 outpatients with LC into two groups based on the presence of MHE according to the results of computer-aided neuropsychiatric testing using four psychometric tests. Differences in clinical and laboratory data were compared between the two groups, and predictive biomarkers for diagnosing MHE were determined using the logistic regression model.

Results: The prevalence of MHE in LC was 28.6% (10/35). Clinical features involving Child-Pugh classification and the modified end-stage liver disease score showed no differences between the MHE-positive and MHE-negative patients. Among biochemical parameters, elevated plasma ammonia level (P=0.034), increased creatinine (P=0.008), and low estimated glomerular filtration rate (P=0.042) showed greater significance in MHE-positive patients compared with MHE-negative patients. However, univariate and multivariate analysis showed that ammonia level (odds ratio, 1.023 and 1.031, respectively; 95% confidence interval of coefficient estimate, 1.005-1.041 and 1.006-1.058, respectively) was the only significant independent predictor for the detection of MHE.

Conclusion: Plasma ammonia level may be useful as a predictive biomarker to prediagnose the neuropsychiatric test-based diagnosis of MHE. Our results also reinforce previous findings on crucial roles for renal ammonia metabolism and urinary excretion in patients with LC.

Introduction

Hepatic encephalopathy (HE) in liver cirrhosis (LC) is an important and serious complication which affects the prognosis of LC. As HE shows a wide spectrum, from subclinical impairment of memory and concentration to coma, the classification of HE in LC has been discussed during the past decade [1-4].Because minimal HE (MHE) has been defined as a subclinical form of HE, LC patients with MHE have a clinically normal state of consciousness, but show abnormalities in cognition and/or neurophysiological variables [5-12].MHE frequently progresses to overt HE (OHE), often impairing health-related quality of life [13-15].Therefore, it is necessary to precisely diagnose and appropriately manage patients with MHE. To diagnose MHE, several neuropsychiatric (NP) and neurophysiological tests have been recommended [4,5-15]. However, a gold standard test for the diagnosis of MHE has not been established worldwide. In Japan, a simple computer-aided NP-test, consisting of eight tests that can be performed easily using a touch panel, has been recently developed and evaluated [16,17]. The prevalence of MHE in Japanese LC patients as found using this NP-test system ranges between 30.1-57.1%,although the prevalence of MHE is different according to the number of psychiatric tests performed [17-20].

Many factors, such as ammonia, glutamine, manganese, false neurotransmitters, inflammation, neurosteroids and low grade edema, closely contribute to the pathogenesis of HE[21-24]. Although the precise pathophysiology leading to HE remains unclear, the widely accepted pathophysiological mechanism is that endogenous and gut-derived ammonia crosses the blood-brain barrier, altering neurotransmission via glutamatergic, serotonergic, and δ-aminobutyric acid (GABA)-ergic mechanisms[25]. Therefore, dietary protein restrictions are put into place, and disaccharides and non-absorbable antibiotics are administered in order to prevent endogenous and gut-derived ammonia production[25]. Imbalance of plasma free amino acids; namely, decreased branched chain amino acids (BCAAs) and increased aromatic amino acids (AAA) has also been indicated as being a participating factor in the pathogenesis of HE in LC patients[21]. The plasma BCAA to AAA ratio and/or BCAA to tyrosine (Tyr) ratio is significantly decreased in advanced LC, with protein-energy malnutrition (PEM) present regardless of whether the patient has

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HE[26,27]. Therefore, BCAA supplementation (BCAA granules and BCAA-enriched nutrients) has been widely used, not only in the treatment of HE, but also to bring about improvement of PEM [28-31]. Zinc (Zn) is also associated with ammonia metabolism in the liver and muscles, and Zn deficiency is related to the pathogenesis of HE[32-36]. Therefore, Zn supplementation has been considered as useful in the management of HE with hyperammonemia and zinc deficiency [32-36]. Recently, levocarnitine (L-CA) and acetyl-L-carnitine have been reported as effective treatments in LC patients with overt hepatic encephalopathy (OHE) and/or MHE[37-41]. Although these drugs produce effects of ammonia detoxification via the urea cycle in the liver and improvement of energy metabolism in the brain, the indication and the timing for this treatment have not been fully studied[42-50]. Thus, although it is possible to examine many biochemical parameters contributing to HE or MHE in clinical practice, it is still not clear which biochemical test(s) except the NP test and the neurophysiological test may be best as predictive biomarkers for the diagnosis of MHE.

The purpose of the present study was to determinepotential predictive biomarkers for the diagnosis of MHE in LC patients.

Methods

Subjects

Thirty-five outpatients with LC who periodically visited the Department of Gastroenterology and Hepatology at Iwate Medical University between January 2013 and August 2014 were enrolled in this study. We excluded LC patients who showed OHE within 2 weeks before the study. The diagnosis of LC was made based on routine biochemical examination including peripheral blood count, liver function tests and prothrombin time activity (PT) in conjunction with ultrasonography, computed tomography, and/or liver biopsy. In determining the etiologies of LC, hepatitis B virus (HBV) and hepatitis C virus (HCV) infections were diagnosed based on positivity for hepatitis B surface antigen (HBsAg) and positivity for both anti-HCV and HCV-RNA, respectively. Alcoholic liver disease was diagnosed by a history of alcohol intake (ethanol >40 g per day, 10 years). Autoimmune LC involved autoimmune chronic hepatitis (AIH) and primary biliary cirrhosis (PBC). These diseases were diagnosed by the results of serum anti-nuclear antibody and anti-mitochondria antibody testing and liver pathology. LC due to nonalcoholic steatohepatitis was diagnosed in a patient with fatty liver disease without HBV or HCV infections, alcohol abuse, or autoimmune liver diseases. Cryptogenic LC was defined as LC without any of the aforementioned etiologies. The presence of esophageal varices was examined by upper gastroendoscopy. Diabetes mellitus was diagnosed on the basis of elevated fasting plasma glucose level (>126 mg/dL) or elevated HbA1c (>6.5%) according to the Diagnostic Criteria for Diabetes Mellitus in Japan, or by the patient's use of anti-diabetic agents. Hepatocellular carcinoma (HCC) was diagnosed by elevated serum tumor markers (alpha-fetoprotein, AFP; and/or protein induced by vitamin K absence or vitamin K antagonist-II, PIVKA-II) in conjunction with imaging studies such as ultrasonography, computed tomography, and magnetic resonance imaging.

Neuropsychiatric and biochemical methods

The NP test and biochemical examination were performed under a fasting state in the morning at the outpatient clinic. The NP test was conducted first. The NP test comprised four psychometric tests (number connection tests A [NCT-A] and B [NCT-B], the digit symbol test [DST], and the block design test [BDT]). MHE was diagnosed when more than two of the four tests gave abnormal results. Following the NP test, a fasting peripheral blood sample was drawn. Biochemical examination involved peripheral blood count (erythrocytes, leukocytes, and platelets), liver function tests (total bilirubin, T-Bil.; aspartate aminotransferase, AST; alanine aminotransferase, ALT; and albumin, Alb), renal function tests (blood urea nitrogen, BUN; and creatinine, CRN), electrolytes (sodium, potassium, and chloride), prothrombin time activity (PT), plasma glucose (BS), free fatty acid (FFA), and ammonia (NH_3) by standard clinical laboratory methods. The degree of liver dysfunction was graded according to the Child-Pugh classification and the modified end-stage liver disease (MELD) score [50,51]. The estimated glomerular filtration rate (eGFR) (derived from three factors: age, sex, and serum CRN) was calculated according to the criteria of the Japanese Society of Nephropathy [52]. Serum Zn, the total branched-chain amino acids (BCAA) to Tyr ratio, and carnitine (CA) status (total carnitine [T-CA], free carnitine [F-CA] and acylcarnitine [acyl-CA] levels) in sera were measured at Biomedical Laboratories Inc., Tokyo, Japan (Zn; atomic absorption method, BCAA/Tyr ratio; enzymatic method, and CA status; enzymatic cycling method, respectively).

Ethics

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of Iwate Medical University. The study was performed after obtaining written informed consent from each patient.

Statistical analysis

Data are expressed as mean value ± standard deviation or median (range). Nonparametric multiple comparisons were made by the Mann-Whitney U-test. Categorical comparisons were made by Fisher's exact test. The logistic regression model was used for multivariate stepwise analysis. These statistical tests were performed using SPSS 12.0 software (SPSS, Chicago, IL, USA). $P<0.05$ was defined as statistically significant.

Results

Prevalence of MHE in LC patients and patient characteristics

In the present study, the prevalence of MHE in LC patients was 28.6% (10/35). Etiologies, age, sex, the rates of esophageal varices and diabetes mellitus, and the severity of liver damage based on the Child-Pugh classification and the MELD score showed no significant differences between the MHE-positive and -negative patients. However, the administration rate of lactulose or lactitol was significantly higher in MHE-positive patients compared to MHE-negative patients (Table 1). Among the biochemical parameters, plasma NH_3 and CRN were significantly higher in MHE-positive patients than in MHE-negative patients, while eGFR was significantly lower in MHE-positive patients than in MHE-negative patients. Other serum parameters such as the BCAA/Tyr ratio, Zn, BS, FFA and each CA level did not significantly differ between the MHE-positive and MHE-negative patients (Table 2).

Logistic regression analysis

To determine reliable predictive parameters for the diagnosis of MHE, we performed multivariate stepwise analysis. While plasma NH_3, CRN and eGFR were significant in univariate analysis, plasma NH_3 was the only significant predictive biomarker for diagnosis of MHE in multivariate analysis (Table 3).

Table 1. Clinical features in cirrhotic patients with and without minimal hepatic encephalopathy (MHE).

	MHE-positive	MHE-negative	*P* value*
Numbers	10	25	
Mean age (range)	59.9 (47-74)	62.1 (33-77)	n.s.
Gender (male/female)	9/1	17/8	n.s.
Etiologies [+]			n.s.
HBV	0	1	
HCV	2	7	
HCV + Alcoholic	1	0	
Alcoholic	5	10	
Others (AIH/PBC/NASH)	1	2	
Cryptogenic	1	5	
Complications			
Esophageal varices	7	16	n.s.
Diabetes mellitus	5	8	n.s.
Ascites and /or edema	1	4	n.s.
Hepatocellular carcinoma	3	10	n.s.
History of overt HE (yes/no)	1/9	3/22	n.s.
Child-Pugh classification A/B/C	6/3/2	16/8/1	n.s.
MELD score (points)[++] ~9/10~14/15~20	4/4/2	15/6/4	n.s.
Medication			
Diuretics (%)	5 (50)	11 (44)	n.s.
Lactulose or lactitol (%)	9 (90)	7 (28)	0.001
BCAA nutrients (%) [+++]	6 (60)	10 (40)	n.s.
Zinc (%)	1 (10)	1 (4)	n.s.
Sedative/sleeping drug (%)	3 (30)	5 (20)	n.s.

*Statistical analysis was performed using by Fisher's exact test.
[+]HBV, hepatitis B virus; HCV, hepatitis C virus; AIH, autoimmune hepatitis; PBC, primary biliary cirrhosis; NASH, nonalcoholic steatohepatitis.
[++] MELD; modified end-stage liver disease
[+++]include both BCAA (branched-chain amino acids) granules and BCAA-enriched nutrients

Table 2. Differences in biochemical parameters between cirrhotic patients with and without minimal hepatic encephalopathy (MHE).

Parameters[+]	MHE-positive (n=10)	MHE-negative (n=25)	*P* value*
T-Bil (mg/dL)	1.15(0.30-4.70)	1.10(0.40-6.20)	n.s.
Alb (g/dL)	3.60(2.70-4.40)	3.60(2.60-4.80)	n.s.
PT (PT-INR)	1.17(0.90-1.91)	1.11(0.96-1.73)	n.s.
BUN (mg/dL)	17.85(8.5-40.1)	13.95(4.7-52.2)	n.s.
CRN (mg/dL)	0.89(0.72-2.67)	0.74(0.44-2.17)	0.008
eGFR (ml/day/1.73m^2)	70.30(21.10-82.80)	82.60(25.6-90.0)	0.042
FPG (mg/dL)	111.5(100.0-168.0)	103.50(71.0-186.0)	n.s.
FFA (mEq/L)	790.0(190.00-1381.0)	767.00(140.0-1340.0)	n.s.
B-NH_3 (μg/dL)	110.0(57.00-196.0)	55.50(14.0-169.0)	0.034
Zn (μg/dL)	62.00(44.0-135.0)	69.50(49.0-129.0)	n.s.
BTR	3.52(1.89-10.04)	3.97(1.99-12.05)	n.s.
Total carnitine (μmol/L)	80.25(39.70-110.9)	71.85(41.5-131.40)	n.s.
Free-carnitine (μmol/L)	61.20(29.2-84.4)	56.55(29.7-97.60)	n.s.
Acyl-carnitine (μmol/L)	20.20(10.5-36.0)	15.25(7.2-37.3)	n.s.

*Statistical analysis was performed using by Mann-Whitney U-test. Data are presented as median (range). n.s.; not significant.
[+]T-Bil, total bilirubin; Alb, albumin; PT, prothrombin time; BUN, blood urea nitrogen; CRN, creatinine; eGFR; estimated glomerular filtration rate; FPG, fasting plasma glucose; FAA, free fatty acid; B-NH3, blood (plasma) ammonia; Zn, zinc;; BTR; branched chain amino acids to tyrosine ratio.

Discussion

In the present study, we first evaluated the prevalence of MHE using the NP test. In order to diagnose MHE precisely, not only quantitative neuropsychological function testing, but also neuropsychiatric and neurophysiological testing, representing electroencephalography, auditory and visual evoked potentials, and the critical flicker frequency test have been recommended [4-12]. Furthermore, radiological examinations such as magnetic resonance spectrometry and positron emission tomography have also been useful [53-55]. However, a gold standard test for the diagnosis of MHE has not been well established worldwide [11]. In Japan, a simple computer-aided NP test system, consisting of eight tests that can be performed easily using a touch panel, has recently been developed and used for the diagnosis of MHE [16-20]. However, almost 30 min is required for a patient to complete the eight tests of the NP test. Therefore, in the present study, we performed four tests: 1) NCT-A; 2) NCT-B; 3) DST; and 4) BDT to obtain the results of the NP test within 15 min according to the reports by Michitaka, *et al.* [18] and Kato, *et al.* [19] We diagnosed patients as having MHE when the results of more than two of the four tests were abnormal. As shown in the results, the prevalence of MHE in LC patients in the present study using this convenient NP test was 28.6% (10/35). This prevalence was almost similar to a recent report showing a prevalence of 30.1% (32 of 106 LC patients)[19], but lower compared to the results of recent reports by Saito, *et al.*[20] and Taniguchi, *et al.*[56] (37.5% (24 of 64) and 51.9% (14 of 27) LC patients who underwent the NP test, respectively). Furthermore, Michitaka, *et al.* reported that the prevalence of MHE in LC patients might be different according to the abnormal results among the eight tests in the NP test (two, 39%; and three or more, 55.8% in LC patients, respectively) [18]**.** On the other hand, European and US studies reported a prevalence of MHE in the range of 30-84% [13-14]. The recent report by Mina, *et al.* showed a 44% prevalence of MHE in 125 patients [14]. Furthermore, Citro, *et al.* also reported that the prevalence of MHE in LC patients with HCV infection was 59.1% [57]. This wide variation in the prevalence of

Table 3. Predictive markers in liver cirrhosis patients with minimal hepatic encephalopathy.

	Univariate analysis					Multivariate analysis		
Variables[+]	OR	[95%CI]			*P* value	OR	[95%CI]	*P* value
Age	0.983	0.916	-	1.055	0.637			
Male sex	4.235	0.455	-	39.401	0.205			
Varices (+ vs. -)	0.857	0.055	-	13.479	0.913			
Ascites (+ vs. -)	0.583	0.057	-	5.973	0.650			
HCC (+ vs. -)	1.000	0.224	-	4.468	1.000			
DM (+ vs. -)	2.571	0.565	-	11.712	0.222			
T-CA	1.007	0.973	-	1.043	0.685	0.983	0.927-1.043	0.576
F-CA	0.999	0.952	-	1.048	0.955			
Acy-CA	1.050	0.963	-	1.146	0.269			
FFA	1.001	0.999	-	1.003	0.261	1.001	0.998-1.004	0.472
FPG	1.032	0.995	-	1.070	0.091			
NH3	1.023	1.005	-	1.041	0.012	1.031	1.006-1.058	0.017
BTR	0.947	0.691	-	1.296	0.732			
Zinc	0.998	0.968	-	1.029	0.894			
T-Bil	1.304	0.113	-	15.098	0.832			
Alb	0.778	0.211	-	2.876	0.707			
PT	0.805	0.029	-	22.114	0.898			
BUN	1.039	0.977	-	1.106	0.223	0.944	0.771-1.156	0.576
CRN	3.015	0.659	-	13.793	0.155	2.394	0.012-487.199	0.747
eGFR	0.971	0.936	-	1.006	0.106	0.934	0.819-1.065	0.309

[+]HCC, hepatocellular carcinoma; DM, diabetes mellitus; T-CA, total carnitine; F-CA, free carnitine; Acy-CA, acyl carnitine; FFA, free fatty acid; FPG, fasting plasma glucose; BTR, branched-chain amino acid to tyrosine ratio; T-Bil, total bilirubin; Alb, albumin; PT, prothrombin time; BUN, blood urea nitrogen; CRN, creatinine; eGFR; estimated glomerular filtration rate.

MHE could be attributable to the different diagnostic criteria of MHE, the number of psychometric tests conducted, and the devices used to diagnose MHE, such as for electroencephalography and critical flicker frequency. Furthermore, the results could be affected by the following factors: 1) etiology of LC; 2) age and sex of LC patients; 3) severity of liver damage; and 4) administration of disaccharides and BCAA nutrients. Therefore, in future studies these factors should be matched to accurately diagnose MHE and to confirm the prevalence of MHE.

The main purpose of the present study was to search for predictive biomarkers for the diagnosis of MHE in patients with LC. As shown by the results, although plasma NH_3 level, serum CRN level and eGFR were significantly different between MHE-positive and MHE-negative patients with LC, logistic regression analysis showed that plasma NH_3 level was the only significant biomarker in predicting MHE. However, the *P* value and odds ratio of plasma NH_3 level were low. Two reasons for these results are conjectured: first, the number of LC patients in the present study was small; and second, the rate of lactulose or lactitol administration against hyperammonemia was significantly higher in the MHE-positive patients compared to the MHE-negative patients. Further examination in a large number of LC patients is necessary to confirm these results. Hyperammonemia in LC patients has been known as the most important risk factor for development of MHE or OHE, but blood ammonia levels in MHE have not been high in previous studies [18-20,58]. Actually, hyperammonemia is often observed in LC patients without OHE or MHE, because the hepatic urea cycle, which is the main route of ammonia detoxification, is disturbed, and changes occur in the blood stream according to the formation of intra- and extrahepatic portasystemic shunting. In this situation, alternative pathways of ammonia detoxification are activated in extrahepatic tissues (skeletal muscle, brain, lung and kidney), resulting in an increase in glutamine (Gln) synthesis [59-62]. In particular, the kidneys take up Gln from the blood in an amount that equals 10-15% of whole-body Gln flux, and subsequently distribute the ammonia produced from Gln between the urine and renal veins [61]. Furthermore, in severe LC, the ammonia disposal capacity appears to be exceeded and the kidneys increase the release of ammonia to the renal veins [62]. These findings suggest that the plasma NH_3 level may be useful as a predictive biomarker for the diagnosis of MHE in LC patients with renal dysfunction. In the future, it is necessary to examine the relationship between ammonia metabolism and the profiles of plasma free amino acids, including Gln, in LC patients with MHE.

In the present study, we expected that serum CA status might be associated with the prediction of MHE, because we previously reported that the serum CA status, in particular, the serum acyl-CA to total CA ratio, shows a significant positive correlation with serum FFA concentration [63].This indicates starvation status and is higher in LC patients with MHE than in patients without MHE. Moreover, since a recent study indicated that nutritional management might improve MHE in LC patients, starvation will be one factor considered in the pathogenesis of MHE [19,64-66].However, as shown by the results, we could not confirm that serum CA status was not a predictive biomarker for the diagnosis of MHE.

Recently, Saito, *et al*. reported that the serum taurine level before L-CA administration might be an independent factor associated with the amelioration of MHE [20], although the measurement of serum taurine is difficult in routine clinical practice and has poor cost performance. Because we could not conduct a nutritional assessment using anthropometry and intake volume of diary meat in each LC patient, further study is necessary concerning whether serum CA levels may serve as predictive biomarkers of MHE.

The present study showed that the serum CRN and eGFR levels were significantly different between the MHE-positive and -negative patients. However, these biomarkers were not selected as predictors in logistic regression analysis. The serum CRN level is usually an indicator of renal dysfunction in many diseases and is an established factor of the MELD score [51,67-69]. However, the serum CRN level does not reflect the precise status of renal function in LC, because the production of CRN decreases in LC with skeletal muscle atrophy. In order to precisely estimate renal function, the gold standard in clinical practice is to measure GFR using inulin, ^{125}I-iothalamate or ^{99m}Tc-diethylene triamine pentaacetic acid. However, these examinations are laborious and time-intensive, and many involve radiation exposure. Therefore, eGFR based on CRN (but recently, cystatin C also) has been recommended in LC patients [70-71]. Additionally, since renal function has been known to be an important prognostic factor in advanced LC [72], it is necessary in the future to confirm whether renal function may be an independent predictive biomarker for the diagnosis of MHE.

In conclusion, among the numerous biomarkers used in the management of LC patients with OHE or MHE, only plasma NH_3 level may be a candidate as a predictive biomarker for prediagnosing MHE based on the NP test. However, because many biomarkers, including ammonia, are closely associated with renal function, a large-scale study is needed to confirm the useful predictive biomarkers for the true diagnosis of MHE.

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