Stress-induced Biomarkers in Liver with Non-alcohol Fatty Liver Diseases and Non-alcohol Steatohepatitis

Hiromi Ono,[1] Akira Tanaka,[1,2] Kyoumi Nakazato,[3] Yutaka Hasegawa,[4] Ke Ih Kim,[5] Soo Ryang Kim,[5] Katsuyuki Nakajima [2,3] and Takeaki Nagamine [3]

Background & Aims: A comparative study between plasma diagnostic markers and oxidative stress-induced biomarkers localized differently in the liver has not been reported in non-alcohol fatty liver (NAFLD) and non-alcoholic steatohepatitis (NASH). **Methods:** Pathological observations by Hematoxylin and Eosin (HE) staining and immunostaining by specific antibodies against metallothionein (MT)-1/2 and -3, heme oxygenase -1(HO-1), adiponectin using biopsy samples and plasma diagnostic makers were determined in 37 cases. **Results:** The MT-1/2, HO-1 and adiponectin levels were all significantly reduced in the liver with NASH compared with NAFLD and control. MT-1/2 was most strongly stained in hepatocytes in the normal and NAFLD liver, while it was significantly reduced in NASH. Adiponectin was stained significantly less at blood vessels in NASH compared with NAFLD and controls. HO-1 was also stained significantly less in the Kupffer cells in NASH compared with NAFLD and controls. MT-3 was stained similarly among the three groups at blood vessel cells. Those biomarkers trended negatively with plasma liver injury biomarkers. **Conclusions:** The significantly reduced expression of oxidative stress-induced biomarkers in NASH may be associated with the degree of pathological damage. In particular, MT-1/2 appears to exert an important effect in hepatocytes against stress-induced damage in NASH. (Kitakanto Med J 2014；64：13～22)

Key words： adiponectin, heme oxygenase -1(HO-1), metallothionein (MT), NAFLD (non-alcohol fatty liver), NASH (non-alcoholic steatohepatitis)

Introduction

Non-alcohol fatty liver disease (NAFLD) has a specific range, from fatty liver alone to non-alcoholic steatohepatitis (NASH). While NAFLD is widely believed to be a benign condition with little risk of disease progression, patients with NASH sometimes develop progressive liver disease and cirrhosis.[1-8] An aberration in the metabolism of fatty acids and triglycerides may be the common mechanism underlying the hepatic triglyceride accumulation in NAFLD.[2] NASH is histologically similar to alcoholic steatohepatisis, with the presence of macrovesicular steatosis, mixed inflammatory cell infiltration of the lobules, ballooning degeneration and necrosis of hepatocytes, Mallory body formation and perisinuidal fibrosis or cirrhosis.[2-4,9] Although the pathogenesis of NASH remains unclear, the hypothesis that excessive intrahepatic lipid accumulation triggers a local necro-inflammatory response has been suggested.[2,3,10-13] Such necro-inflammation is accompanied by the production of free radicals associated with lipid peroxidation that can result in damage to the cellular membrane and DNA.[14,15]

In this study, we investigated four stress-induced biomarkers in the liver with NAFLD and NASH and

1 Laboratory of Clinical Nutrition and Medicine, Kagawa Nutrition University, 3-9-21 Chiyoda, Sakado, Saitama 350-0288, Japan
2 Nutrition Clinic, Kagawa Nutrition University, 3-24-3 Komagome, Toshima-ku, Tokyo 170-8481, Japan 3 Gunma University Graduate School of Health Sciences, 3-39-22 Showa-machi, Maebashi, Gunma 371-8514, Japan 4 Educational Center for Clinical Pharmacy, Kobe Pharmaceutical University, 4-19-1 Motoyamakitamachi, Higashinada-ku, Kobe, Hyogo 658-8558, Japan
5 Department of Gastroenterology, Kobe Asahi Hospital, 3-5-25 Boh-oh-ji-cho, Nagata-ku, Kobe, Hyogo 653-0801, Japan
Received: September 30, 2013
Address: KYOUMI NAKAZATO Gunma University Graduate School of Health Sciences, 3-39-22 Showa-machi, Maebashi, Gunma 371-8514, Japan

compared the relationship between them and the degree of pathological damage, including that indicated by plasma biomarkers. In particular, we investigated metallothionein (MT)-1/2 and -3 which have apparently not been reported in liver with NASH or NAFLD, although this stress-induced biomarker (MT-1/2) has been well investigated in various other liver diseases.[16] MT-1/2 is one of the major protective biomarkers of oxidative stress in the liver and it is regulated by nuclear factor erythroid 2-related factor 1 (Nrf2).[17] MT is induced in response to free radicals formed in tissues by lipid peroxidation. Increased MT levels in the liver have been found in various chronic liver diseases, including alcoholic liver injury and chronic viral hepatitis.[16] We also studied MT-3 level in liver together with MT-1/2 in NASH and NAFLD, although MT-3 reportedly is not localized in the liver.[18]

Heme oxygenase-1(HO-1) is another stress-induced anti-inflammatory enzyme regulated by Nrf2, and it is similar to superoxide dismutase (SOD).[19] HO-1 ameliorates hepatic steatosis, as well as necro-inflammation in experimental nutritional steatohepatitis in mice as a way of preventing NASH.[20] The induction of HO-1 is an adaptive response against the oxidative damage elicited by lipid peroxidation and it may be critical in ameliorating the progression of NASH.[21]

Adiponectin is known to act as a protective adipokine by inhibiting liver gluconeogenesis and suppressive lipogenesis.[22] Patients with NASH and NAFLD have higher levels of oxidative stress and inflammation, hypoadiponectinemia and higher C-reactive protein (CRP) compared to controls. [23,24] A significantly reduced adiponectin levels in plasma is known to be a distinctive feature of NASH.[24,25]

Oxidative stress may play a major role in the pathogenesis of NASH and NAFLD.[2,3,10-13] However, in terms of the pathological expression of oxidative stress-induced biomarkers and oxidative damage, it remains unclear whether the changes in stress-induced biomarkers are the cause or consequence of liver injury. This prompted us to investigate the expression of four differently localized oxidative stress-induced biomarkers in the human liver with NASH and NAFLD. We also studied the relationship between the biomarker expression patterns in the liver and plasma clinical diagnostic markers in NASH and NAFLD.

Materials and Methods

Samples

Formalin-fixed, paraffin-embedded liver biopsy samples from 37 patients were used in this study. There were 5 that were nearly normal, 14 with NAFLD and 18 with NASH. The histological criteria for inclusion in this study were the following: the presence of macrovesicular steatosis without any necro-inflammatory changes for NAFLD, and the presence of macrovesicular steatosis with lobular inflammation with or without hepatocytes necrosis for NASH. Other causes of liver disease, including alcohol abuse (less than 20g/day and 140g/week) and hepatitis B and C, had been strictly excluded by history, family interview, laboratory data, liver histology and hepatobiliary ultrasound in all of the patients. Laboratory studies included serum liver tests, alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-GTP, alkaline phosphatase (ALP), total bilirubin (T-Bil), albumin and total protein levels. The serum glucose, cholesterol (TC) and triglyceride (TG) levels were also obtained. All these analysis was conducted routinely at Kobe Asahi Hospital. Five histologically normal liver samples were selected from among the biopsy cases. The characteristics of these patients are summarized in Table 1. Written informed consent was obtained from each patient and the study was approved by the local ethics committee of Kobe Hospital and carried out according to the provisions of the Declaration of Helsinki.

Antibodies

An MT-1/2 polyclonal antibody against rat MT-2 was raised in Japanese white Rabbits at our laboratory (Graduate School of Health Sciences, Gunma University).[26-28] The antibody recognized an NH_2-terminal fragment peptide (7 amino acids) with an acetylated methionine residue. This antibody exhibited a high specificity and affinity for both MT-1 and MT-2. A monoclonal MT-3 antibody was raised against human recombinant MT-3 by the Frontier Institute (Hokkaido, Japan). This antibody recognized 17 amino acids from the NH_2-terminal of human MT-3.[29] The HO-1 and adiponectin antibodies were purchased from Enzo Life Sciences (NY, USA) and R&D Systems (MN, USA), respectively.

Immunohistochemistry

Liver sections (3μm-thick) were routinely deparaffinized in xylene and rehydrated through graded ethanol. For the immunostaining of MT-1/2 and -3, OH-1 and adiponectin, endogenous peroxidase activity was quenched by incubation in 0.3% H_2O_2 in methanol for 30 min. The sections were microwaved in 10mM citrate buffer (pH 6.0) for 5 min for antigen retrieval. After washing with phosphate buffered saline (PBS) three times, the sections were reacted with primary antibodies (1 : 50 dilution) for 120 min at

room temperature (RT). After being washed with PBS containing 0.05% Tween 20 three times, the sections were incubated with a secondary antibody (Histofine Simple Stein MAX-PO (Multi); Nichirei, Tokyo, Japan) for 60 min at RT. Peroxidase activity was developed with 0.2mg/mL 3, 30-diaminobenzidine tetrahydrochloride in the presence of 0.003% hydrogen peroxide in 0.05 M Tris-buffered saline at pH 7.6. Finally, the sections were counterstained for nuclei in Mayer's hematoxylin.

Semi-quantitative assessment of the immunostaining

Areas of positive staining were quantified with ImageJ 1.38 x software (Wayne Rasbans, NIH, Bethesda, USA). The positively stained area was calculated from the ratio of the positive staining (the brown color as shown by immunohistochemistry) to the whole liver in digital images. One ocular field (×200 magnification) per specimen was selected randomly from all samples.

Statistical analysis

Statistical analysis was performed using Spearman and Pearson tests and the Mann-Whitney U-test. Statistical significance was defined as $p<0.05$.

Results

The physiological characteristics of NAFLD and NASH

The demographic data (Table 1) showed a higher prevalence of NASH and NAFLD was found in women than in men ($p<0.001$). Also, a higher prevalence of hypertension was found in cases with NASH than NAFLD ($p<0.01$), and the serum ALT, AST and LDH levels were significantly higher in NASH than NAFLD ($p<0.01$). The plasma choline esterase level (ChE), which is an indicator of a fatty liver, was also higher in NASH than NAFLD. These blood parameters indicated that NASH exhibited more severe injury than NAFLD.

Pathological examination of the NASH and NAFLD livers performed by hematoxylin-eosin (HE) staining

HE staining was performed for histological diagnosis (Fig. 2 A (a), B (a), C (a)). Liver histology was evaluated by an experienced pathologist (Dr. Keiji Suzuki, Prof. Emeritus of Gunma University School of Medicine) blinded to the results of immunostaining. In the NASH cases, each section was examined in order to grade the severity of steatosis and necroinflammation. Steatosis was scored from 1 to 4, as

Table 1 Clinical characteristics of patients with control, NAFLD and NASH

		Control		FL		NASH		pvalue		
		Mean	SD	Mean	SD	Mean	SD	normal-FL	normal-NASH	FL-NASH
Age		52.8	13.9	47.6	13.7	57.2	14.8	0.48	0.56	0.05
Gender	(M/F)	2/3		7/12		4/14		<0.01	<0.01	<0.01
Height	cm	157	127	158	11	155	10	0.93	0.71	0.38
Weight	kg	58	10	65	12	66	13	0.20	0.17	0.85
BMI		23.2	1.6	26.2	5.1	27.3	3.9	0.04	0.00	0.46
DM	%	20		10.5		27.8		<0.01	<0.01	0.14
HT	%	20		33.3		50.0		<0.01	<0.01	<0.01
WBC	$\times10^2$/ul	81.0	47.5	68.9	26.9	65.6	15.6	0.61	0.51	0.65
RBC	$\times10^2$/ul	46.1	31.9	45.0	43.3	45.4	51.5	0.54	0.73	0.77
Plt	$\times10^4$/ul	28.0	120	26.3	9.5	21.5	4.7	0.78	0.30	0.06
TP	g/dl	7.0	0.4	7.1	0.6	7.4	0.4	0.83	0.16	0.06
Alb	g/dl	4.1	0.5	4.2	0.4	4.3	0.3	0.93	0.43	0.13
T-Bil	mg/dl	0.6	0.2	0.6	0.2	0.5	0.2	0.93	0.33	0.15
GOT (AST)	IU/I	48.0	26.8	35.8	19.9	62.8	30.5	0.38	0.32	<0.01
GPT (ALT)	IU/I	48.0	24.7	43.0	23.0	94.0	52.5	0.70	0.01	<0.01
ALP	IU/I	345	95.5	275	83.5	249	11.1	0.24	0.14	0.44
LDH	lU/I	205	52.5	178	51.0	230	55.4	0.34	0.40	0.01
7-GTP	IU/I	37.8	6.3	114	26.0	66	53.0	0.22	0.04	0.44
TC	mg/dl	210	23.1	200	23.4	200	368	0.46	0.48	0.95
TG	mg/dl	172	78.3	131	65.6	190	103	0.33	0.69	0.05
HDL-C	mg/dl	57.7	9.5	56.4	14.9	50.7	14.6	0.86	0.35	0.32
BUN	mg/dI	13.1	2.7	12.2	3.0	14.4	4.5	0.55	0.41	0.09
CRE	mg/dl	0.8	0.2	1.2	1.0	0.8	0.2	0.09	0.97	0.08
uA	mg/dl	4.4	0.4	5.4	1.1	5.5	1.2	0.02	0.01	0.95
GLU	mg/dl	90.3	6.4	95.6	11.9	11.1	27.7	0.24	0.01	0.04
ICG15 RR		9.5	2.1	84	4.9	9.3	4.8	0.63	0.93	0.65
Hyaluronic acid	ng/ml	10.4	—	32.8	34.9	41.9	29.2	—	—	0.56
TTT		4.0	2.0	3.1	2.1	3.4	3.1	0.41	0.63	0.72
ZTT		6.7	4.5	7.4	4.1	6.9	4.5	0.78	0.94	0.74
ChE		6027	1290	5323	2166	6662	1014	0.38	0.35	0.02

p value, $p<0.05$ is statistically significant

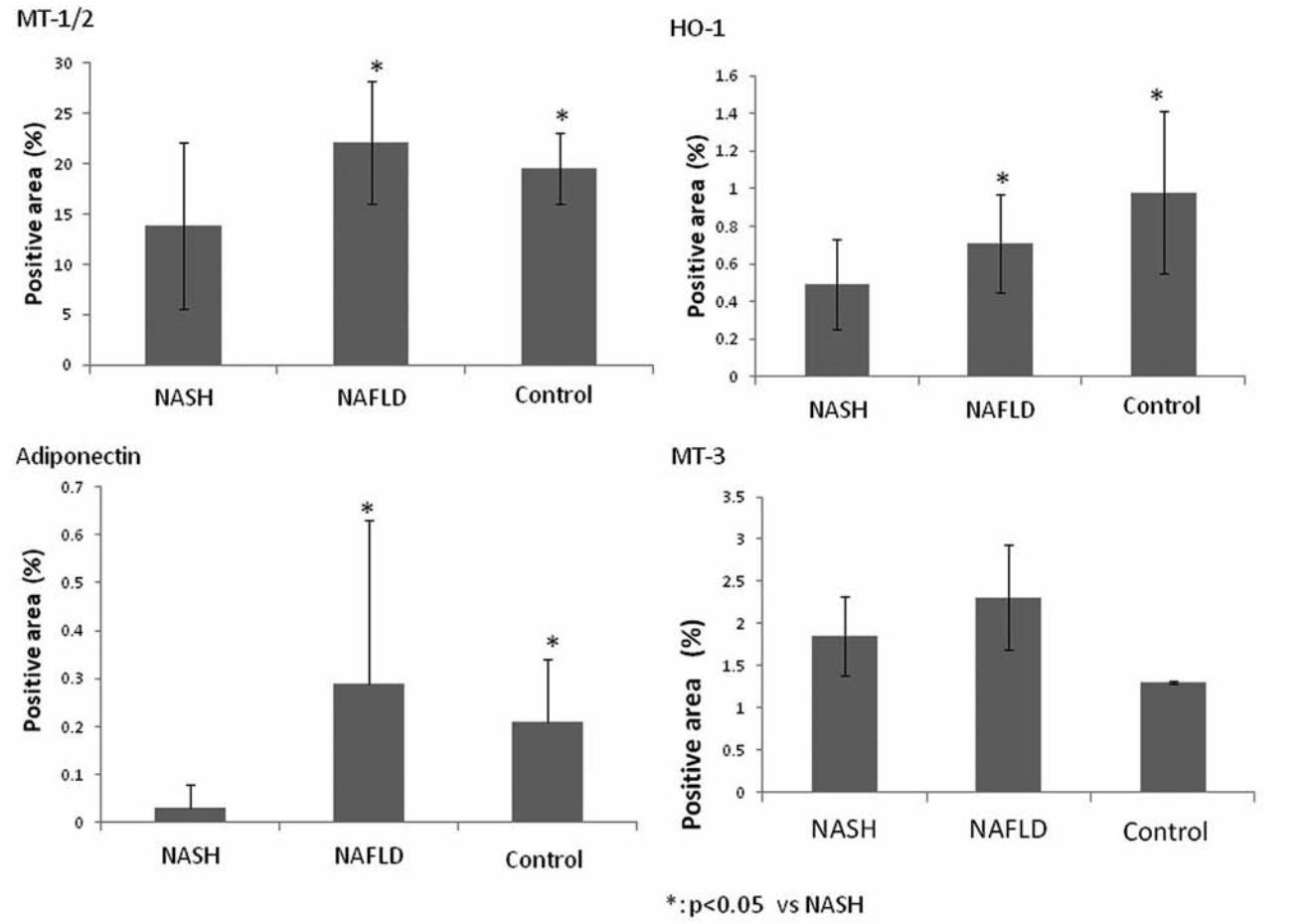


Fig. 1 Expression of MT-1/2, MT-3, HO-1and adiponectin in control, NAFLD and NASH liver. Significantly decreased expressions of MT-1/2, HO-1 and adiponectin in liver were observed in NASH compared to NAFLD and controls by immunostaining ($p<0.05$).

follows. Type 1 was nearly normal. In Type 2, up to 1/3 of the cells contained fat, In Type 3 between 1/3 and 2/3 of the cells contained fat, and in Type 4 fat was present in 2/3 of the hepatocytes. Inflammation and steatosis was evaluated and scored according to the criteria initially developed by Matteoni et al.[30] The NASH cases (Type 4, Fig. 2C (a)) showed more severe liver damage based on the above criteria compared to NAFLD (Type 2 and 3, Fig. 2B (a)).

Immunohistochemical detection of MT-1/2 and -3, OH-1 and adiponectin in the normal, NAFLD and NASH liver

Fig. 1 shows the immunostaining characteristics of the four stress-induced biomarker in controls, NAFLD and NASH in the liver determined by semi-quantitative assessment of the immunostaining. Significantly decreased expression of MT-1/2, HO-1 and adiponectin by immunostaining in liver observed in NASH compared to NAFLD and controls ($p<0.05$). However, MT-3 was not clearly distinguished among the 3 groups because of the very low concentration in the liver in all of the cases.

Fig. 2 shows the immunohistochemical staining of MT-1/2 (b) and MT-3 (c), OH-1 (d) and adiponectin (e) in normal (A), NAFLD (B) and NASH (C) liver. Hepatocyes were most strongly stained by MT-1/2 among the four biomarkers, in particular hepatocytes that were observed as having normal function were stained very strongly by the MT -1/2 antibody (Fig. 2A (b)). MT-1/2 was strongly stained in NAFLD hepatocytes containing a moderate amount of lipid droplets (Fig. 2B (b)), while it was significantly less stained in NASH hepatocytes having a large amount of lipid droplets (Fig. 2C (b)) ($p<0.05$). However, the expression pattern of MT-1/2 was not necessarily in parallel with the number of lipid droplets in hepatocytes. The hepatocytes with strongly stained MT-1/2 in nuclei were often observed in normal and NAFLD cases, while it was very rare in NASH. MT-3 was not stained in hepatocytes as MT-1/2 was, but was stained more clearly at blood vessels (mostly in smooth muscle cells) in the controls (Fig. 2A (c)) than in NAFLD (Fig. 2B (c)) and NASH (Fig. 2C (c)) liver. Comparatively strong staining of lipid membrane or lipofuscin-like particles in he-

A : Controlgroup

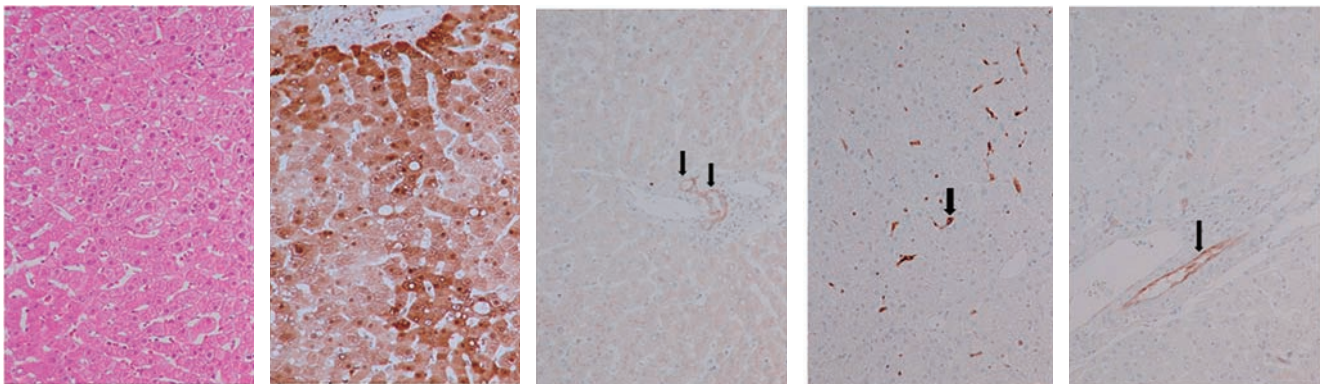
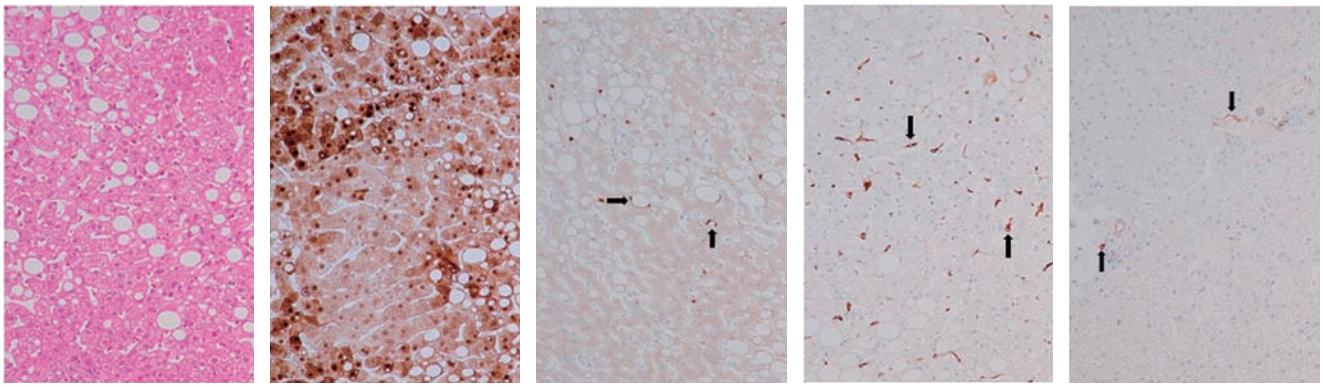


Fig. 2 Representative images of MT-1/2, MT-3, HO-1 and adiponectin expression in the control, NAFLD and NASH liver by HE and immunohistochemical staining.

B : NAFLD group



C : NASH group

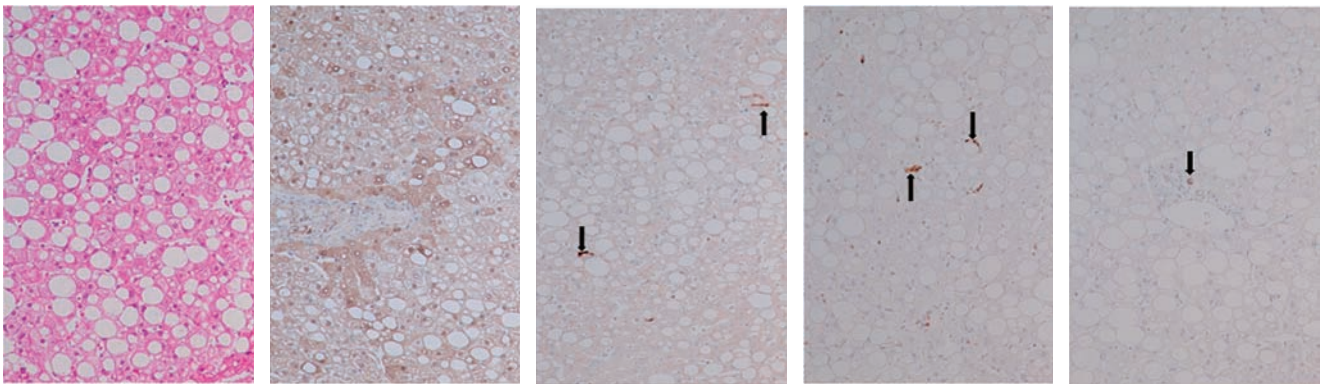


Fig. 2A shows normal controls, **Fig. 2B** shows NAFLD and **Fig. 2C** shows NASH.
Each picture indicates (a) HE staining, (b) MT-1/2 staining, (c) MT-3 staining, (d) HO-1 staining, and (e) adiponectin staining, respectively. The arrows show the localization of MT-3, HO-1 and adiponectin. MT-1/2 is localized in most of the hepatocytes. The quantitative strength of immunostaining was determined by the semi-quantitative assessment method, as shown in Fig. 1. (original magnification ×200)

patocytes was observed in NAFLD (Fig. 2B (c)), but not normal hepatocytes. The number of stained bodies observed in NAFLD was decreased in NASH hepatocytes (Fig. 2C (c)).

HO-1 is constitutively expressed only in Kupffer cells and is not observed in hepatocytes at all. The expression of HO-1 was significantly lower in NASH (Fig. 2C (d)) than controls (Fig. 2A (d)) and NAFLD (Fig. 2B (d)) ($p<0.05$). Although the number of Kupffer cell was increased in NASH, the expression of HO-1 was significantly reduced compared to NAFLD ($p<0.05$).

Adiponectin in the liver was not stained in hepatocytes at all, but was stained in smooth muscle cells and endothelial cells at blood vessels (Fig. 2A (e)). The number of blood vessels in the liver was different between NASH (Fig. 2C (e)) and NAFLD (Fig. 2B (e)), while adiponectin expression at blood vessels in the liver was significantly lower in NASH compared with NAFLD ($p<0.05$).

Correlation among the patterns of MT-1/2 and -3, HO-1 and adiponectin in the liver, and the plasma clinical diagnostic markers associated with liver function in all of the cases

Table 2 and Fig. 3 show that MT-1/2 was positively correlated with T-Bil ($p<0.001$) and negatively correlated with ChE ($p=0.02$), and trended negatively with albumin, ALT and AST. MT-3 was not correlated with any of the parameters in liver and plasma. HO-1 was positively correlated with adiponectin ($p=0.01$) and negatively trended with albumin, ALP, AST and ChE. Adiponectin was negatively correlated with ALT ($p=0.02$) and negatively trended with albumin. These results indicated that MT-1/2, HO-1 and adiponectin were positively correlated with each other and these stress-induced biomarkers trended towards a negative correlation with the plasma liver diagnostic

Table 2 Correlation between stress-induced biomarkers and plasma diagnostic markers

		MT-1/2	MT-3	HO-1	adiponectin	platelet	albumin	ALP	ALT	AST	T-Bil	ChE	Hyeluronete	ICG
MT-1/2	r													
	p													
	N													
MT-3	r	0.33												
	p	0.25												
	N	14												
HO-1	r	0.20	-0.37											
	p	0.25	0.19											
	N	35	14											
adiponectin	r	0.08	0.06	0.44										
	p	0.64	0.84	0.01										
	N	35	14	35										
platelet	r	0.07	-0.41	0.15	0.30									
	p	0.70	0.15	0.40	0.09									
	N	34	14	34	34									
albumin	r	-0.14	0.44	-0.26	-0.01	-0.13								
	p	0.45	0.11	0.15	0.96	0.46								
	N	33	14	33	33	33								
ALP	r	-0.03	-0.33	-0.11	0.27	0.10	-0.09							
	p	0.89	0.24	0.53	0.13	0.57	0.63							
	N	33	14	33	33	33	33							
ALT	r	-0.17	-0.27	-0.15	-0.39	0.06	0.09	0.07						
	p	0.33	0.36	0.38	0.02	0.74	0.62	0.69						
	N	34	14	34	33	34	33	33						
AST	r	-0.28	-0.30	-0.27	-0.20	0.15	-0.10	0.14	0.718					
	p	0.11	0.30	0.13	0.25	0.39	0.57	0.45	0.00					
	N	34	14	34	34	34	33	33	34					
T-Bil	r	0.526	0.10	0.24	0.04	-0.24	0.29	-0.11	0.16	-0.12				
	p	0.00	0.73	0.17	0.83	0.17	0.10	0.54	0.38	0.50				
	N	33	14	33	33	33	33	33	33	33				
ChE	r	-0.39	-0.32	0.12	-0.03	0.22	0.18	-0.12	0.18	0.06	-0.04			
	p	0.02	0.26	0.51	0.85	0.20	0.32	0.51	0.32	0.75	0.82			
	N	33	14	34	24	34	33	33	34	34	33			
Hyaluronate	r	-0.26	0.50	-.645	-0.26	0.18	0.525	0.15	0.19	0.498	-0.24	-0.07		
	p	0.31	0.21	0.01	032	0.50	004	0.58	0.46	0.04	0.38	0.30		
	N	17	8	'17	17	17	16	16	17	17	16	17		
ICG	r	0.00	0.21	-0.11	-0.12	-0.21	0.08	-0.31	0.10	0.02	0.16	-0.14	0.15	
	p	0.99	0.59	0.62	0.58	0.34	0.73	0.15	0.66	093	0.46	0.52	0.62	
	N	23	9	23	23	23	23	23	23	23	23	23	13	

r, Correlation coefficient; p, p value (<0.05 is statistically significant); N, Number of patients

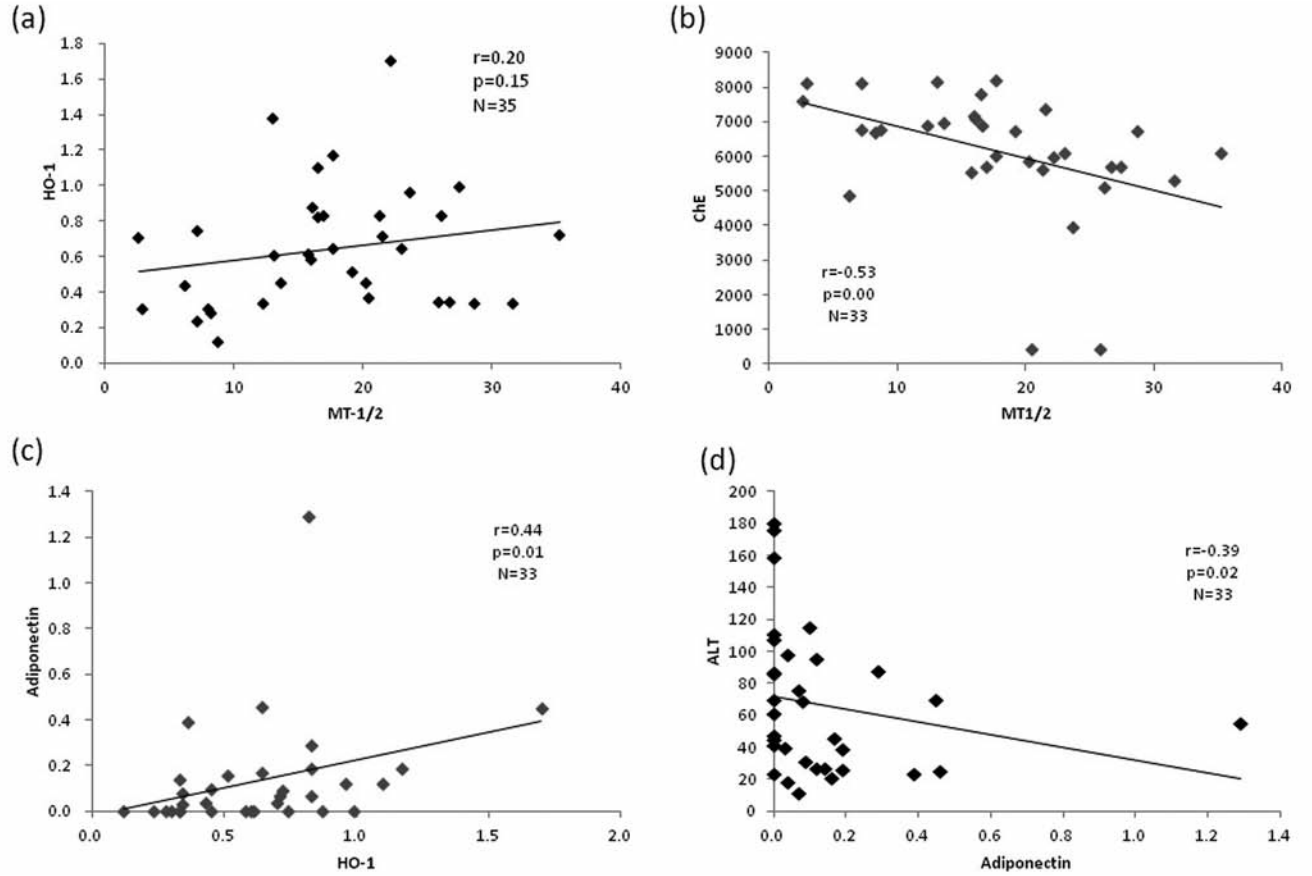


Fig. 3 Correlation among MT-1/2, HO-1 and adiponectin in the liver and diagnostic markers in plasma. MT-1/2 trended positively with HO-1 (a), and negatively correlated with ChE (b). Adiponectin was positively correlated with HO-1 (c) and negatively correlated with ALT (d).

markers.

Comparison between the low MT-1/2 and high MT-1/2 expression groups in NASH, NAFLD and controls, as well as a comparison between other stress-induced biomarkers and serum liver diagnostic markers

We found that approximately half of the NASH cases exhibited significantly lower MT-1/2 expression (8/18) ($p<0.001$), while the rest exhibited a nearly normal (high) expression of MT-1/2 in hepatocytes by semi-quantitative assessment of the immunostaining (Table 3). Therefore we divided the NASH cases into low MT-1/2 and high MT-1/2 expression groups and compared the differences with other biomarkers in NASH. HO-1, adiponectin and the plasma liver diagnostic markers were compared in the low and high MT-1/2 expression groups in NASH, and these parameters were also compared with the cases in NAFLD and normal controls.

Table 3 shows that the low MT-1/2 expression group in NASH was significantly different from the NAFLD cases and controls ($p<0.001$), while the high MT-1/2 expression group in NASH was not different from NAFLD and controls. Positive correlation between MT-1/2 expression and T-Bil in NASH ($p=0.01$) was observed (Fig. 4). Hyaluronic acid levels were higher in the low than high MT-1/2 expression group in NASH, although the difference was not statistically significant. Hyaluronic acid levels were similar between the MT-1/2 high expression group and NAFLD cases (Table 3).

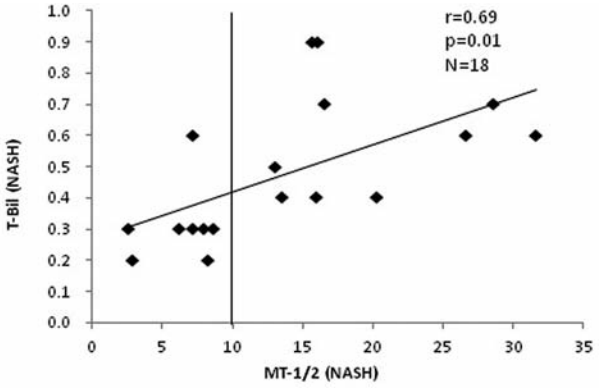


Fig. 4 Correlation between MT-1/2 and T-Bil in NASH cases. The low MT-1/2 group (less than 10 units by semi-quantitative assessment of immunostaining) showed significantly lower levels of T-Bil compared with those of high MT-1/2 group in the NASH cases ($p<0.05$).

HO-1 was decreased in the following order: the

Table 3 Comparison between low MT-1/2 and high MT-1/2 expression group in NASH with NAFLD and with other biomarkers and serum liver injury markers

	NASH				pvalue (AvsB)	NAFLD (C)		Control (D)		pvalue			
	MT1/2 Low(A)		MT1/2 High(B)										
	mean	SD	mean	SD		mean	SD	mean	SD	A vs C	A vs D	B vs C	B vs D
MT1/2	6.4	2.4	19.8	6.7	<0.001	22.1	6.4	19.5	3.8	<0.001	<0.01	NS	NS
HO-1	0.4	0.2	0.6	0.3	NS	0.7	0.3	1.0	0.5	NS	<0.01	NS	NS
Adiponectin	0.03	0.06	0.03	0.05	NS	0.29	0.36	0.21	0.14	NS	NS	NS	<0.05
ALT (IU/L)	80	46	99	51	NS	40	23	48	25	NS	NS	<0.01	NS
AST (IU/L)	73	52	60	25	NS	28	14	48	27	<0.05	NS	NS	NS
T-Bil (g/dL)	0.31	0.12	0.61	0.19	<0.05	0.59	0.24	0.58	0.15	<0.05	NS	NS	NS
Hyaluronate (ng/mL)	57	29	23	17	NS	25	25	—	—	NS	—	NS	—
Platelet ($\times 10^4$/uL)	21	4	22	5	NS	25	8	28	12	NS	NS	NS	NS
ChE ($\times 10^3$IU/L)	6.8	1.2	6.5	0.9	NS	5.3	2.8	6.0	1.3	NS	NS	NS	NS
ICG (%)	6.5	2.4	11.7	5.2	NS	8.0	5.6	9.5	2.1	NS	NS	NS	NS
albumin (g/dL)	4.3	0.4	4.3	0.2	NS	4.4	0.4	4.1	0.5	NS	NS	NS	NS

p value, $p<0.05$ is statistically significant

low MT-1/2 expression group in NASH, the high MT-1/2 expression group in NASH, the NAFLD cases and the controls (Table 3). The HO-1 expression level in the low MT-1/2 expression group in NASH was significantly lower than in NAFLD ($p<0.05$) and controls ($p<0.01$). However, no correlation of the HO-1 expression between the low MT-1/2 and high MT-1/2 in NASH was observed. Adiponectin expression was decreased significantly in NASH compared with NAFLD and controls ($p<0.05$). Adiponectin expression unchanged in the low MT-1/2 and high MT-1/2 expression groups in NASH (Table 3).

Discussion

We investigated the four oxidative stress-induced biomarkers which are located at different cells in the liver and compared the pathological significance of those biomarkers in NASH and NAFLD cases. The biomarkers are located in the hepatocytes (MT-1/2), Kupffer cells (HO-1), smooth muscle and endothelial cells at blood vessels (adiponectin, MT-3). To our knowledge, this is the first report that MT-1/2 expression in hepatocytes is strongly associated with the progression of NASH, as has already been shown with HO-1[20,21] and adiponectin.[24,25] We demonstrated that MT-1/2 was widely and strongly expressed in human hepatocytes, especially in simple fatty liver (NAFLD) and normal controls, while it was expressed significantly weak in NASH. The localization of MT-1/2 was predominantly in the cytoplasm of hepatocytes, a site where the histological damage is mainly observed in NASH.[4,8,9] In addition, we found that MT-1/2 expression significantly correlated with the grade of necro-inflammation. The inverse correlation between the expression of MT-1/2 and the grade of necro-inflammation suggests that lipid peroxidation is involved in the necro-inflammatory reaction in NASH in association with a decreased MT-1/2 concentration. Although it remains to be determined whether lipid peroxidation is the cause or the consequence of the liver injury, MT is known to be a strong anti-chemoattractant for neutrophils and anti-oxidative stress-induced NF-kB activation.[31] Thus, lipid peroxidation associated with a decreased MT-1/2 concentration may plausibly be partly responsible for the pathological features observed in NASH.

HO-1 is constitutively expressed only in Kupffer cells and is not observed in hepatocytes. HO-1 expression was significantly more reduced in NASH than NAFLD, reflecting the severity of the disease in the liver. Increased endogenous HO-1 may suppress liver fibrosis by protecting liver cells, inhibiting inflammatory cell infiltration and/or hepatic stellate cell transformation.[19] A significant correlation was observed between the increased levels of HO-1 and ferritin, and also between the increased levels of HO-1 and lipid peroxidation.[20,21] The induction of HO-1 is an adaptive response against the oxidative damage elicited by lipid peroxidation and may be critical in the modulation of the progression of the disease.[22] These effects were associated with suppressed HO-1 expression and increased TNF-α and IL-6 expression.[19] Many data provided a biochemical, morphological and molecular biological evidence for the protective role of HO-1 in ameliorating hepatic steatosis, necroinflammation in experimental nutritional steatohepatitis.

Visceral obesity is a primary risk factor for NAFLD, and an inappropriate storage of triglycerides in adipocytes and higher concentrations of free fatty acids may add to increased hepatic lipid storage, insulin resistance and progressive liver damage.[23] Most of the adipose tissue-derived proteins are elevated in obesity and may contribute to systemic inflammation and liver damage. Adiponectin is abundant in human serum, but its levels are reduced in obesity and are even lower in patients with NAFLD or NASH.[24,25] Adiponectin antagonizes excess lipid storage in the liver and protects against inflammation and fibrosis.

Adiponectin was found mainly in blood vessels (smooth muscle cells and endothelial cells) in the liver and was significantly reduced in NASH compared with NAFLD and controls. The low adiponectin levels were a feature of NASH independent of age, sex, BMI, insulin resistance and metabolic syndrome. Low levels of adiponectin are reportedly significantly correlated with the degree of hepatic steatosis and necro-inflammation, and thus might contribute to the development of a more advanced form of NAFLD.[23-25]

Subsequent to the immunohistochemical staining studies using stress-induced biomarkers, we have hypothesized a physiological role for MT-1/2 in hepatocytes as a new stress-induced biomarker in NASH. MT-1/2, an anti-oxidative damage protein, is localized with a significantly higher concentration in the liver[26-28] when compared to other biomarkers in this study. The major cell damage in NASH is known to occur in hepatocytes. Therefore, it may be that MT-1/2 plays a more important role in the protection against liver damage in NASH than HO-1[19-22] or adiponectin,[23-25] which are not present in hepatocytes.

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver. Approximately 70-90% of HCC cases are associated with hepatitis B and C infection, and the increasing incidence of NASH is a major factor of HCC in developed countries.[32] It is necessary to determine the risk of developing HCC in patients with NASH, since the factors that increase risk among cohorts with NASH are still unclear. Recently, Park and Yu[33] reported that the loss of nuclear and cytoplasmic expression of MT-1/2 was significantly present in HCCs compared with the adjacent noncancerous liver, and suggested their utility as a prognostic marker in hepatocellular carcinoma. As we found that decreased level of MT-1/2 in NASH hepatpcytes was associated with the severity of the liver damage, MT-1/2 expression in liver may be a marker for the prediction of developing HCC in NASH.

As probucol, a lipid-lowering agent with a potent anti-oxidant effect is a known agent for the treatment of NASH patients,[34-38] we investigated the induction of MT-1/2 by probucol in the liver. A significant induction of MT-1/2 by probucol was found in the rat liver (to be published elsewhere). It has been already reported that probucol induces HO-1,[39,40] adiponectin[37,41] and SOD[42,43] in the liver and other tissues, but there is no report of MT-1/2 induction by probucol. The induction of MT-1/2 in hepatocytes may be partly associated with the efficacy of probucol in NASH patients, affording protection against oxidative stress-induced damage.[34-38]

In conclusion, our immunohistochemical investigation provided evidence that NASH is strongly associated with a lower concentration of oxidative stress-induced biomarkers in liver compared with NAFLD. These results suggest that all these stress-induced biomarkers exert a protective function against oxidative stress in NAFLD. When the anti-oxidative function of these parameters decreased, NAFLD developed as the result of continuous oxidative stress induced by the lipid peroxidation generated at lipid droplets and then proceeded to NASH, resulting the formation of liver fibrosis and possibly, hepatocarcinogenesis. MT-1/2 is a new stress-induced biomarker in NASH and may have potential as a therapeutic target in oxidative stress-induced damage in hepatocytes.

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