Appendix-I

Spatiotemporal expression of matrix metalloproteinase-1 in progression of nonalcoholic steatohepatitis

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Supplementary materials and methods

Materials

We studied three groups of specimens: normal liver tissue, liver tissue from early stage NASH, and liver tissue from advanced stage NASH. Twenty-nine were histologically proven NASH patients (12 patients from the Kitasato University Medical Center (KMC) and 7 patients from the International University of Health and Welfare (IUHW) hospital including three cases with NASH-derived HCC). Liver specimens from four patients with metastatic liver cancer were used as control samples. The study was approved by the ethics committees of both university hospitals. Written consent was obtained from all patients. Diagnosis of NASH was established using a needle liver biopsy specimen or a surgical wedge biopsy tissue. During their annual health check, all NASH patients showed abnormal liver function tests and fatty liver on abdominal ultrasonography and computer tomography with or without hyperglycemia/ dyslipidemia. All patients had at least a six-month history of elevated aminotransferases with no other identifiable cause (viral infection, autoimmune disease, metabolic disorder, or alcohol use <30 g/d for males and <20 g/d for females) or other liver disease detected via serological testing and imaging studies (Supplementary Table 1).

Methods

IHC expression of MMP-1, caveolin-1, αTGF-β, lTGF-β, and type I procollagen

Immunohistochemical analysis was conducted as reported earlier [1, 2] with minor modifications. Serial sections of formalin-fixed, paraffin-embedded tissue sections (4 μm) were deparaffinized and rehydrated by immersion in xylene and graded alcohol concentrations. Endogenous peroxidase activity was quenched by incubation in 0.3% (v/v) H_2O_2 in methanol for 30 min at room temperature (RT). Heat-mediated antigen retrieval was used for autoclave treatment (10mM citrate buffer; pH 6.0) for 15min at 120°C. Sections were blocked with 10% normal goat serum (Vector Laboratories Inc., Burlingame, CA, USA) and incubated for 90–120min with primary

antibody in 2% serum. They were incubated overnight at 4°C with 1:100 dilution of anti-MMP-1, anti-caveolin-1, anti-activated form of TGF-β (aTGF-β), anti-latent form of TGF-β (lTGF-β), or anti-type1 procollagen. The sections were incubated with the N-Histofine® Simple Stain MAX PO (Nichirei Corp., Tokyo, Japan) at RT for 30min. After repeated washing with PBS, the sections were reacted with diaminobenzidine containing 0.01% H_2O_2 and counterstained with hematoxylin for light microscopic study. We further investigated dual staining for immunohistochemical expression of Kupffer cell marker (CD68) or capillary endothelial cell marker (CD34) or hepatic stellate cell marker (vimentin). To avoid background interference, heat-mediated antigen retrieval autoclave treatment was conducted for 5min at 110°C. Treatment with primary antibody with CD68, CD34, or vimentin was done for 30min at room temperature (RT), followed by staining (N-Histofine @ Simple Stain AP, MULTI; Nichirei Corp.). Bound antibody was visualized using a peroxidase reaction in a 3, 3'-diaminobenzoic tetrahydrochloride (DAB, brown, D-8001; Sigma-Aldrich Corp., St. Louis, MO, USA) and H_2O_2 solution, or using an alkaline phosphatase reaction for BCIP**/**NBT Substrate System **(**blue, Dako**)**.

Computer-assisted morphometric analysis

Morphometric variables were determined by labeling: anti-human MMP-1 and hepatic progenitor cells with CK-19; OV-6; anti-human MMP-1 and blood vessels with anti-CD34 antibody; anti-human MMP-1 and macrophages with CD68; anti-human MMP-1 and hepatic stellate cells and macrophages with vimentin. Three periportal regions and regenerative fibrotic areas were assessed separately for each section of NASH liver. Briefly, the immunostained sections were scanned using light microscopy at low magnification (×4.2). Vessels were counted using high magnification (×20; 0.304 mm^2/field) in each region. Stained sections were visualized using a standard light microscope (Olympus Corp., Tokyo, Japan) at ×20 magnification. At least five images representing the entire periportal area were captured using a digital camera (3072 × 2304 size, 32-bit) and saved in a TIFF format. This procedure included conversion of the captured image in points or pixels according to the red tone to assess the MMP-1as HPCs, macrophages, Kupffer cells, hepatic stellate cells, and capillary endothelial cells. Images were captured and digitized using an internal frame grabber board by the soft of Image J 1.48 [2]. Differences in vessel density were analyzed using one-way ANOVA. A Dunn post-hoc analysis was performed.

Western blot analysis

The liver tissue sample was homogenized in 10 volumes of homogenization buffer (20μM Tris–HCl, pH 7.5, 5mM $MgCl_2$ 0.1mM phenylmethanesulfonyl fluoride (PMSF), 20μM pepstatin A, and 20μM leupeptin). The proteins obtained were used for immunoblotting. Proteins (30μM/ml) were separated using sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride membranes (Merck Millipore, Billerica, MA, USA). The blots were blocked with 5% (w/v) dried milk in PBS overnight at 4°C, and incubated with

anti-MMP-1 diluted 1:3000 diluted 1:2000 in 0.1% Tween 20μ in PBS. After washing, the blots were incubated with horseradish peroxidase-conjugated anti-rabbit and anti-mouse immunoglobulin antibody (Jackson ImmunoResearch Inc., West Grove, PA, USA) for 1hr at room temperature. Protein bands were detected using an enhanced chemiluminescence detection system (ECL Plus; Amersham Biosciences Corp., Uppsala, Sweden). The blots were exposed using a CCD analyzer (General Electric Co., Fairfield, CT, USA). The exposed bands were analyzed using anti-β-actin antibody (Merck Millipore Co., Billerica, MA, USA) as a loading control [1, 2]. Densitometric analyses of western blotting were conducted using software (Scion Image ver. Beta 4.0.2; Scion Corp., Frederick, MD, USA). One-way ANOVA with Dunn post-hoc analysis was used to assess differences between individual groups (SuperANOVA; Abacus Concepts Inc., Berkeley, CA, USA). Differences for which $p < 0.05$ were considered significant.

IEM expression of MMP-1, caveolin-1, and type 1 procollagen

To clarify the ultrastructural localization of MMP-1, we conducted an IEM study as described in a previous report [1, 2]. In brief, wedge biopsies of roughly 3cm × 1cm × 1cm or liver biopsy specimens were obtained from the margin of a liver lobe as soon as the operator had access to the control or NASH liver. The tissue was transferred immediately to a container filled with PBS (pH 7.4) at 37°C. Injection perfusion with periodate-lysine-paraformaldehyde (PLP) fixative was done in a Petri dish filled with saline. Each specimen was held at a corner by forceps. Then PLP was injected from multiple sides using a 26G syringe until discoloration and hardening of the tissues was achieved. After perfusion and incubation with PLP overnight at 4°C, semi-thin 5mm sections were prepared. Sections were 1) immersed for 15min in three changes of 0.01% phosphate buffer saline (PBS; pH 7.4), 2) incubated with anti-MMP-1 diluted 1:100 or caveolin-1 diluted 1:200 or type 1 procollagen diluted 1:200 in 0.01M phosphate buffered saline containing 1% bovine serum albumin overnight at 4°C in a moisture chamber, 3) treated three times for 15min each time in PBS, 4) incubated in 1.4 nm colloidal gold-conjugated anti-mouse IgG antibody (Nanoprobes Inc., Yaphank, NY, USA) diluted 1:40 for 40min and treated three times at RT for 30min each time in 10 mM citrate buffer (pH 6.0), and 5) physically developed using a silver enhancement kit (Nanoprobes Inc.) for 5min, as described previously. Before transmission electron microscopy, the materials were treated three times with PBS for 15 min each time and fixed in 2.5% glutaraldehyde with 0.01% phosphate buffer (pH 7.4) for 1hr at 4°C, followed by a graded series of ethanol solutions and postfixation with 2% osmium tetroxide in 0.01% phosphate buffer (pH 7.4) for 90min at 4°C. After embedding in Epon, ultrathin sections were cut using a diamond knife with an ultramicrotome (LKB Bromma, Sweden). Then the sections were stained with uranyl acetate and observed by transmission electron microscope (JEM-1200 EX; JEOL, Tokyo, Japan).

Statistical analysis

Values are expressed as mean ± SD. The Mann–Whitney *U* test was used to evaluate differences between groups: a *p* value of less than 0.05 was regarded as statistically significant.

References

[1] Yamazaki H, Oda M, Takahashi Y, Iguchi H, Yoshimura K, et al. Relation between ultrastructural localization, changes of caveolin-1 and capillarization of liver sinusoidal endothelial cells in human hepatitis C-related cirrhotic liver. J Histochem Cytochem. 2013; 61(2):169–176.

[2] Yokomori H, Oda M, Yoshimura K, Kaneko K, Hibi T. Aquaporin-1 associated with hepatic arterial capillary proliferation on hepatic sinusoid in human cirrhotic liver. Liver Int. 2011; 31(10):1554–1564.

Appendix-II

Supplementary Table and Figures

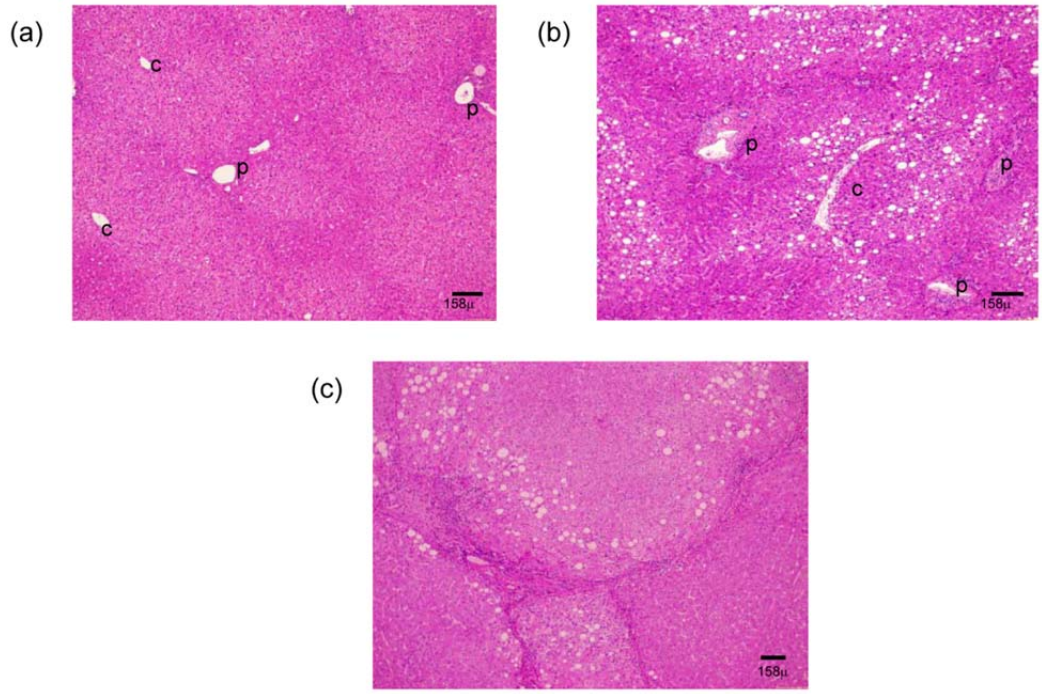
Supplementary Table 1 Background of normal controls, early and advanced NASH patients. Pathological findings were diagnosed independently by three pathologists.

	Case No.	*Age*	*Sex*	*BMI (kg/m^2)*	*Steatosis*	*Inflammation*	*Ballooning*	*Fibrosis*	*AST (IU/L)*	*ALT (IU/L)*	*γGT (IU/L)*	*TC (mg/dL)*	*LDL (mg/dL)*	*HDL (mg/dL)*	*TG (mg/dL)*	*BS (mg/dL)*	*HbA1c (%)*	
Early	1	36	F	28.6	2	2	1	1	54	105	73	225	156	46	200	115	5.5	†,*
	2	37	M	25.9	3	1	2	1	65	122	39	247	169	53	177	104	5.8	†,*
	3	40	M	31.4	3	1	1	1	96	176	69	249	124	50	375	107	5.8	†,*
	4	40	M	26.5	2	2	1	1	26	68	64	201	121	40	113	118	6.1	†,*
	5	44	M	31.7	2	3	1	2	57	133	54	201	141	42	207	127	6.6	†
	6	49	F	27.0	2	2	2	1	55	77	48	213	129	62	148	118	6.8	†,‡
	7	61	M	28.1	1	1	1	1	48	46	34	155	86	46	171	97	7.9	†
	8	62	F	25.8	2	1	1	1	83	87	71	184	117	48	94	93	6.1	†,‡
	9	67	F	35.2	2	1	1	1	53	46	37	134	59	54	75	166	6.8	†,‡
	10	68	M	25.6	1	1	1	1	24	37	32	150	98	39	64	111	5.7	†,‡
Advanced	11	46	M	24.0	2	3	1	4	29	58	144	141	79	47	113	78	5.5	†,*
	12	50	M	30.1	2	3	2	4	48	31	320	124	74	41	67	369	10.2	†,‡,*
	13	59	F	22.4	2	2	2	4	105	107	186	208	133	41	171	184	8.9	†,*
	14	67	F	24.1	1	2	1	3	65	85	85	154	86	50	92	109	6.6	†,*
	15	75	M	27.6	2	1	1	4	42	29	132	156	196	24	89	205	9.0	†
	16	62	F	24.4	2	3	1	4	100	70	72	152	65	67	77	167	10.9	†
	17	78	M	21.0	1	3	1	4	22	13	114	147	84	31	160	93	4.6	†,‡
	18	71	M	27.2	2	1	1	4	54	20	10	77	33	34	46	87	4.3	‡
	19	62	F	19.9	2	2	2	4	43	22	27	37	-	-	-	67	-	‡
Control	1	64	M	21.8					29	26	202	231	179	47	76	109	5.7	†,‡
	2	71	F	20.2					16	12	33	119	63	36	98	98	5.4	†,‡
	3	71	M	25.0					63	73	52	233	156	41	100	109	5.2	†,‡
	4	84	F	19.6					12	6	12	165	90	60	73	109	5.0	†

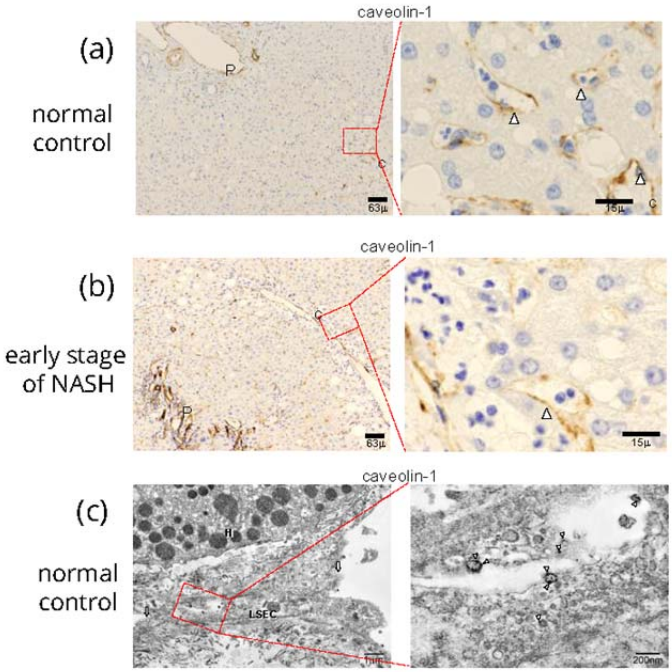
†, tissue: In this patient, liver tissues were analyzed for IHC and/or IEM of MMP-1.

‡, tissue: In this patient, liver tissues were analyzed for western blot.

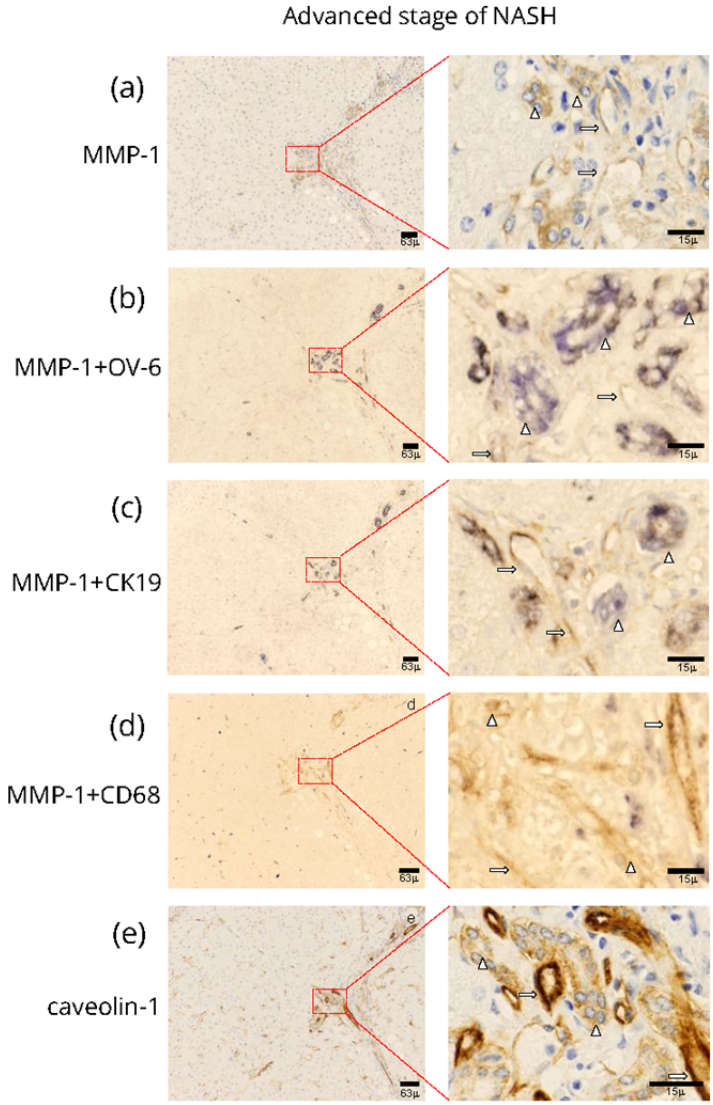
*, tissue: In this patient, liver tissues were analyzed for MMP-1 dual staining.



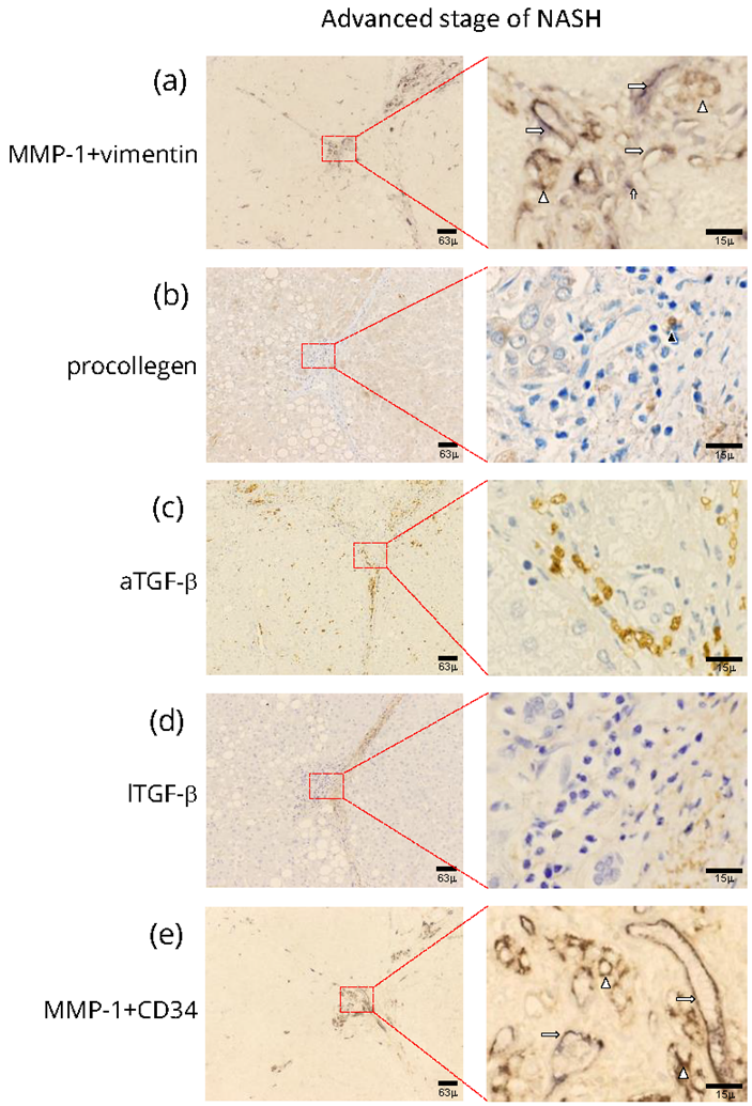
Supplementary Figure 1 Histologic features of NASH stage in this study according to Brunt's grading and staging. (a) Normal control (Case No. 1); (b) Stage 1 grade 1 NASH liver (Case No. 10); (c) Stage 2 grade 3 NASH liver Case No. 17); ×100 magnification, Hematoxylin-eosine stain.



Supplementary Figure 2 IHC and IEM: (a) IHC of caveolin-1 expression in normal control liver (Case No. 1). Caveolin-1 was observed on the walls of hepatic artery capillary venule and portal vein in the portal tracts of normal liver. The high magnification image in the inset shows caveolin-1 expression in hepatic sinusoidal lining cells around pericentral zone 3; (b) Caveolin-1 in early stage NASH (Case No. 10). In an early stage of NASH, caveolin-1 expression was similar to expression in control liver tissues. White arrows indicate sinusoidal lining cells with caveolin-1 expression in an early stage of NASH liver (brown). ×100 magnification. Column denotes high magnification (400). P, portal tract; C, central vein; (c) IEM expression of caveolin-1 in normal control liver (Case No. 1). Immunogold particles indicating caveolin-1 are found in non-coated vesicles in liver sinusoidal endothelial cells around zone 2. Arrowheads indicate caveolin-1 reaction products. Arrow denotes sinusoidal endothelial fenestrae. Scale bar shows magnification: H, hepatocyte; LSEC, liver sinusoidal endothelial cell. The scale bar shows magnification: H, hepatocyte; LSEC, liver sinusoidal endothelial cell.



Supplementary Figure 3 IHC expressions of advanced NASH liver (stage 2, grade 4) using serial sections (Case No. 17): (a) IHC expression of MMP-1 (brown); (b) Dual expression of MMP-1(brown) and OV-6 (blue); (c) IHC dual expression of MMP-1 (brown) and CK-19 (blue); (d) IHC dual expression of MMP-1 (brown) and CD68 (blue); (e) IHC expression of caveolin-1 (brown). The expression of MMP-1 is localized on sinusoidal lining cell in steatosis with features of significant cell injury or inflammation. Expression of MMP-1 increased proportionally with increase in hepatic progenitor cells and development of fibrosis. White arrowheads mark the hepatic progenitor cell. White arrows indicate proliferative capillary arteries or sinusoidal lining cells. Brown, immunoperoxidase staining was used for MMP-1. Blue, alkaline phosphatase staining was used for OV-6, cytokeratin 19, and CD68. Column denotes high magnification.



Supplementary Figure 4 IHC expressions of advanced NASH liver (stage 2, grade 4) using serial sections **(**Case No. 17): (a) IHC dual expression of MMP-1(brown) and vimentin (blue); (b) IHC expression of type 1 procollagen (brown); (c) IHC expression of aTGF-β (brown); (d) IHC expression of lTGF-β (brown); (e) IHC dual expression of MMP-1 (brown) and CD34 (blue). Expression of MMP-1 is localized in sinusoidal lining cells in steatosis with features of significant cell injury or inflammation. Black arrowheads mark vimentin or CD34-positive cells. White arrows indicate proliferative capillary arteries or sinusoidal lining cells. Brown, immunoperoxidase staining was used for MMP-1, latent TGF-β, active TGF-β, type 1 procollagen. Blue, alkaline phosphatase staining was used for CD34. Column denotes high magnification.