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ORIGINAL

Effects of laparoscopic sleeve gastrectomy on nonalcoholic fatty liver disease and TGF-β signaling pathway

Hideki Kumagai[1)], Akira Sasaki[1)], Akira Umemura[1)], Keisuke Kakisaka[2)], Takeshi Iwaya[3)] and Satoshi S Nishizuka[4)]

[1)] *Department of Surgery, School of Medicine, Iwate Medical University, Iwate 028-3695, Japan*
[2)] *Division of Gastroenterology and Hepatology, Department of Internal Medicine, School of Medicine, Iwate Medical University, Iwate 028-3695, Japan*
[3)] *Department of Clinical Oncology, School of Medicine, Iwate Medical University, Iwate 028-3695, Japan*
[4)] *Division of Biomedical Research and Development, Iwate Medical University Institute for Biomedical Sciences, Iwate 028-3694, Japan*

Abstract. Nonalcoholic fatty liver disease (NAFLD) develops as a result of unhealthy lifestyle but improves with laparoscopic sleeve gastrectomy (LSG). The transforming growth factor (TGF)-β signaling pathway reportedly contributes to liver fibrosis, mainly in animal experiments. The aim of the present study was to evaluate changes in serum proteins before and after LSG by proteomic analysis and to investigate their association with NAFLD. This study enrolled 36 severely obese patients who underwent LSG at our hospital from January 2020 to April 2022. As a pilot study, proteomic analysis was conducted on six patients using serum collected before and at 6 months after LSG, and significantly fluctuating proteins were extracted. Subsequently, verification by enzyme-linked immunosorbent assay (ELISA) using collected serum was performed on the remaining 30 patients. The mean weight of enrolled patients was 118.5 kg. Proteomic analysis identified 1,912 proteins, many of which were related to the TGF-β signaling pathway. Among these proteins, we focused on five TGF-β-related proteins: asporin, EMILIN-1, platelet factor-4, serglycin, and thrombospondin-1. Verification by ELISA revealed that asporin ($p = 0.006$) and thrombospondin-1 ($p = 0.043$) levels significantly fluctuated before and after LSG. Univariate analysis with a linear regression model showed that aspartate aminotransferase ($p = 0.045$), asporin ($p = 0.011$), and thrombospondin-1 ($p = 0.022$) levels were significantly associated with postoperative liver fibrosis. On multivariate analysis, asporin was an independent prognostic factor for postoperative liver fibrosis (95% confidence interval: 0.114–1.291, $p = 0.002$). TGF-β-related proteins dramatically fluctuated before and after LSG and were correlated with NAFLD pathogenesis. Asporin may be a useful prognostic marker of liver fibrosis in NAFLD after LSG.

Key words: Laparoscopic sleeve gastrectomy (LSG), Transforming growth factor-β (TGF-β), Asporin (ASPN), Nonalcoholic fatty liver disease (NAFLD), Liver fibrosis

NONALCOHOLIC FATTY LIVER DISEASE (NAFLD) is a lifestyle-related hepatic disease that develops as a result of an unhealthy diet or lack of exercise, and its prevalence has been increasing rapidly worldwide [1]. However, this rise in NAFLD diagnosis is becoming a global problem that cannot be ignored, as NAFLD may progress to nonalcoholic steatohepatitis (NASH), which in turn can causes liver cirrhosis and hepatocellular carcinoma (HCC) [2].

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Correspondence to: Akira Sasaki, Department of Surgery, Iwate Medical University, 2-1-1 Idaidori, Yahaba, Iwate 028-3695, Japan. E-mail: sakira@iwate-med.ac.jp

In response, metabolic surgery (MS), such as laparoscopic sleeve gastrectomy (LSG), which promotes weight loss, can effectually treat NAFLD [1, 3]. As we previously reported, LSG dramatically improves not only the clinical parameters related to NAFLD but also liver fibrosis, as assessed using the pericellular fibrosis score (PFS), a central vein fibrosis index [4]. In addition, though time-consuming, we also reported that fibrosis of NASH was improved by MS and resulted in a return to normal liver function [1]. Thus, expanding the indications for MS further is a desirable goal of future research.

One of the challenges in diagnosing NAFLD is that the histopathological evaluation of liver tissues obtained

via biopsy remains the gold standard [1]. A liver biopsy is required for a liver fibrosis evaluation, and while several non-invasive scoring systems have been introduced [5-7], they are somewhat complicated because they combine such indicators such as transaminases and type 4 collagen 7S. In addition, the accuracy of clinical scoring systems, such as the NAFIC score [5], Fibrosis 4 (FIB-4) index [6], and NAFLD fibrosis score [7], is reportedly to be low in patients with severe obesity [4]. Therefore, the development of simpler indicators is urgently needed.

The transforming growth factor (TGF)-β signaling pathway allegedly contributes to the pathogenesis of NAFLD, including liver fibrosis, despite only having been observed *via* animal experiments [2, 8, 9]. Yang *et al.* showed that liver fibrosis was significantly suppressed in TGF-β receptor type 2-deficient mice in comparison to control mice, even after consuming a high-fat diet [8]. Further, Pahk *et al.* revealed that SP-1154, a TGF-β signaling pathway inhibitor, improved liver steatosis in mice fed a high-fat diet [9]. From these findings, the TGF-β signaling pathway is predicted to be involved in the pathogenesis of NAFLD in humans; nonetheless, confirmatory reports are limited.

Proteomic analysis is a method for comprehensively analyzing proteins in biological samples such as blood, urine, and tissue. The purpose of this method is to search for new biomarkers, identify targets for new drug discoveries, and elucidate the pathogenesis of diseases. While some studies have evaluated protein fluctuation after MS in patients with severe obesity using proteomic analysis [10-12], few reports have investigated its relationship with obesity-related diseases such as NAFLD.

The aim of the present study was to evaluate changes in serum proteins before and after LSG by proteomic analysis and to investigate the association between proteins extracted by proteomic analysis and NAFLD using enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry (IHC) methods.

Materials and Methods

Study design

This single-institution prospective study was conducted at Iwate Medical University Hospital in accordance with the principles embodied in the Declaration of Helsinki. In addition, the study protocol was approved by the Institutional Ethics Committee of Iwate Medical University Hospital (approval number: MH2022-056), and written informed consent was obtained from all patients enrolled in this study.

Patients

In total, 45 severely obese patients underwent LSG at Iwate Medical University Hospital from January 2020 to April 2022. Of these 45 patients, nine were excluded due to a lack of samples, so a final sample of 36 patients was enrolled in the study. All patients met the insurance criteria for LSG in accordance with the 2021 consensus statement of the Japanese Society for the Treatment of Obesity, Japan Diabetes Society, and Japan Society for the Study of Obesity [13].

Data collection

Data on patients' age, sex, body weight, body mass index (BMI), and obesity-related diseases were analyzed, and NAFLD-related parameters such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltranspeptidase (γ-GTP), type 4 collagen 7S, hyaluronic acid, blood sugar (BS), hemoglobin A1c (HbA1c), immunoreactive insulin (IRI), homeostatic model assessment of insulin resistance (HOMA-IR), homeostasis model assessment for beta cell function (HOMA-β), NAFIC score [5], FIB-4 index [6], NAFLD fibrosis score [7], liver volume, the liver-to-spleen attenuation ratio, NAFLD activity score (NAS) [14], and PFS [4] were also examined. Liver volume and liver-to-spleen attenuation ratio were measured from computed tomography images using the SYNAPSE VINCENT imaging software (FUJIFILM, Tokyo, Japan). The NAS was used to determine the disease grade of NAFLD using histopathological specimens [14], whereas PFS was used to evaluate liver fibrosis, with a focus on fibrosis around the central vein [4]; both scoring systems were analyzed by more than two pathology specialists. Liver specimens were obtained from all patients, and a liver biopsy was performed during LSG. Patients diagnosed with NASH using the liver specimens obtained intraoperatively *via* a liver biopsy underwent an additional biopsy at 6 months after LSG. Patients with improved liver steatosis and inflammation, and undiagnosed NASH, were defined as NASH remission.

Proteomic analysis

We performed proteomic analysis on the first 6 patients who underwent LSG during the period. Their blood samples were obtained after 8 hours of fasting. Furthermore, blood samples were collected from these patients before and 6 months after LSG. Serum was separated by centrifugation (1,800 × g for 20 min) within 2 hours of blood collection and was immediately stored at –80°C. Prior to the proteomic analysis, serum samples additionally underwent heat treatment at a temperature of 56°C for 30 min and were immediately frozen. Unnecessary proteins present in the serum were removed using High Select Depletion Spin Columns (Thermo Fisher Scientific Inc., Massachusetts, USA), and 80 μL of the

collected samples was added to 120 μL of 100 mM tris-sodium dodecyl sulfate (pH 8.0, 4%) and 20 mM sodium chloride. Tris-(2-carboxyethyl) phosphine was added at a final concentration of 20 mM and incubated at 80°C for 10 min to cleave the S-S bond of the proteins. Iodoacetamide was added to achieve a final concentration of 30 mM, and the resultant concentration was incubated at room temperature (RT) in the dark for 30 min to alkylate the cysteine residues. Sera-Mag SpeedBead carboxylate-modified magnetic particles (hydrophilic) and Sera-Mag carboxylate-modified magnetic particles (hydrophobic) were mixed at a 1:1 ratio, washed three times with distilled water, and prepared in distilled water at 8 μg/μL (SP3 beads). Subsequently, 20 μL of SP3 beads and thrice the sample volume of ethanol were added to the alkylated samples and mixed at RT for 20 min. The beads were washed twice with 80% ethanol and mixed with 100 μL of 50 mM Tris-hydrogen chloride (pH 8.0); thereafter, the samples were incubated at 37°C overnight with the addition of 500 ng of Trypsin/Lys-C Mix (Promega K.K., Tokyo, Japan) to fragment the peptides. Samples containing 20 μL of 5% trifluoroacetic acid were sonicated, and after desalination with a reverse-phase spin column (GL-Tip SDB; GL Sciences K.K., Tokyo, Japan), they were dried using a centrifugal evaporator. Then, 2% acetonitrile and 0.1% trifluoroacetic acid were added to the samples, and the peptides were dissolved by sonication; meanwhile, the samples were adjusted to a peptide concentration of 200 ng/μL by 2% acetonitrile and 0.1% trifluoroacetic acid.

The adjusted samples were analyzed using the UltiMate 3000 RSLCnano LC System (Thermo Fisher Scientific Inc.) for nano-liquid chromatography and the Orbitrap Exploris 480 (Thermo Fisher Scientific Inc.) for mass spectrometry, and proteins and peptides were identified and quantified using the DIA-NN software [15].

Enzyme-linked immunosorbent assay

ELISA was performed to validate changes to the serum proteins extracted by proteomic analysis before and after LSG. In addition to the proteomic analysis, frozen serum obtained from 30 severely obese patients before and 6 months after LSG was used. Based on the proteomic analysis results, five proteins were examined using ELISA methods—namely, asporin (ASPN), EMILIN-1, platelet factor-4 (PF4), serglycin (SRGN), and thrombospondin-1 (THBS1). Human ASPN ELISA Kit (MBS760704; MyBioSource, Inc., San Diego, USA), The human EMILIN-1 ELISA Kit (MBS2883477; MyBioSource, Inc.), Human PF4 ELISA Kit (MBS722309; MyBioSource, Inc.), Human SRGN ELISA Kit (MBS2540306; MyBioSource, Inc.), and Human THBS1 ELISA Kit (RK00338; ABclonal, Inc., Massachusetts, USA) were used for ASPN, EMILIN-1, PF4, SRGN, and THBS1, respectively.

Immunohistochemistry

IHC was applied to confirm the expression of proteins extracted *via* the proteomic analysis in liver specimens. In this study, liver specimens obtained *via* biopsy during LSG were used, and similar to ELISA, five proteins (namely, ASPN, EMILIN-1, PF4, SRGN, and THBS1) were examined.

Liver specimens intraoperatively collected through liver biopsy were immediately stored at –80°C, and for preparation, nine 1-mm-diameter holes were created in frozen Tissue-Tek O.C.T. compound (compound 4,583; Sakura Finetek Japan Co., Ltd., Tokyo, Japan), and the collected liver tissues were buried in eight of these holes [16]. Only the small hole in the lower-right corner could not reliably recognize the specimen number. This method could simultaneously dye multiple tissues on a glass slide; thus, it was possible to match the staining conditions. After refreezing, they were sliced into 6–8-μm-thick slices using a Tissue-Tek Polar Cryostat/Microtome (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) and mounted on a slide glass.

Liver tissues mounted on a slide glass were fixated in acetone at 4°C for 10 min. To block endogenous peroxidase activity, the sections were dipped in 0.3% hydrogen peroxide in methanol for 20 min and rinsed twice with distilled water for 5 min each. IHC was performed using the ImmunoCruz ABC Staining System (sc-2018; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and sections were incubated in a 1.5% blocking serum solution with phosphate-buffered saline (PBS) at RT for 1 h. These were then incubated with primary antibodies at RT for 1 h and rinsed with PBS three times for 2 min each. Anti-ASPN (AB3) antibody (1:40, AV42487; Sigma-Aldrich Co. LLC, Missouri, USA), EMILIN-1 antibody (C-6) (1:40, sc-365737; Santa Cruz Biotechnology, Inc.), CXCL4/PF4 antibody (1:50, NBP2-46804; Novus Biologicals LLC, Colorado, USA), SRGN polyclonal antibody (1:50, PA5-51452; Thermo Fisher Scientific Inc.), and thrombospondin 1 antibody (C-8) (1:40, sc-393504; Santa Cruz Biotechnology, Inc.) were used as primary antibodies for ASPN, EMILIN-1, PF4, SRGN, and THBS1, respectively. Further, sections were incubated with biotinylated secondary antibodies at RT for 30 min and rinsed with PBS thrice for 2 min each. Furthermore, the sections were incubated with an AB enzyme reagent at RT for 30 min and rinsed with PBS thrice for 2 min each. The sections were also incubated with 1–3 drops of peroxidase substrate for 5 min and rinsed with distilled water for 5 min. The sections were counterstained with Mayer's hematoxylin solution (131-09665; FUJIFILM

Wako Chemical Corporation, Osaka, Japan) for 30 s and rinsed immediately with distilled water twice for 5 min each. Then, the sections were dehydrated twice in 95% ethanol for 10 s, twice in 100% ethanol for 10 s, and twice in xylene for 10 s. In total, 1–2 drops of PathoMount (164-28492; FUJIFILM Wako Chemical Corporation) were added to the sections, which were then covered with coverslips.

Statistical analysis

Data are presented as the median (interquartile range) for continuous variables and as the number (%) or median (interquartile range) for categorical variables. Continuous and categorical variables were analyzed using the paired *t*-test or Wilcoxon signed-rank test, and the relationship between serum proteins and NAFLD-related parameters was evaluated using Spearman's rank correlation coefficient. Three groups were compared according to the NAS or PFS using one-way analysis of variance with Tukey's *post hoc* test. Univariate and multivariate analyses with a linear regression model were conducted to analyze predictive factors associated with NAS and PFS; the variables were selected based on the results of univariate analysis and previous reports [1, 4]. All statistical analyses were performed using the EZR software version 1.61 (Jichi Medical University, Saitama Medical Center, Saitama, Japan) [17].

Results

Patient characteristics

Table 1 summarizes the patients' characteristics. The median patient age was 37 years, with a higher proportion of women than men (12/24). The median body weight and BMI in all patients were 113.5 kg and 44.2 kg/m^2, respectively, and overall, 17 (47.2%), 29 (80.6%), and 36 (100%) patients had type 2 diabetes, NASH, and obstructive sleep apnea, respectively. No significant differences in patients' characteristics were identified between the proteomic analysis group and the ELISA and IHC group.

Weight loss effect and NAFLD improvement after LSG

LSG led to good weight loss results, as shown in Table 2. Among all patients, the percentage of excess weight loss and percentage of total weight loss at 6 months after LSG were 48.8% and 24.5%, respectively.

Table 3 presents the changes in NAFLD-related parameters before and after LSG. While most variables significantly improved after LSG, those related to liver fibrosis, such as hyaluronic acid and PFS, did not significantly change, and the NASH remission rate at 6 months after LSG was 48.3% (14/29).

Proteome analysis

In total, 1,912 serum proteins were identified using proteomic analysis. Fig. 1A presents a volcano plot showing the changes in serum proteins before and after LSG. When focusing on proteins with $p < 0.1$, several proteins related to the TGF-β signaling pathway were observed to have fluctuated before and after LSG (Fig. 1B).

Validation by ELISA and the relationship between TGF-β-related proteins and NAFLD-related parameters

Based on the results of the proteomic analysis, we focused on five proteins reportedly be associated with the TGF-β signaling pathway namely, ASPN, EMILIN-1, PF4, SRGN, and THBS1. Among the five proteins, serum

Table 1 Baseline characteristics of analyzed patients in the proteomic analysis group and the ELISA and IHC group.

Variable	All patients (n = 36)	Proteome analysis (n = 6)	ELISA and IHC (n = 30)
Age (years)	36.5 (30.5, 44.8)	39.5 (30.5, 52.3)	36.5 (31.0, 44.0)
Gender, *n* (%)			
Male	12 (32.4)	2 (25.0)	10 (33.3)
Female	24 (67.6)	4 (75.0)	20 (66.7)
Body weight (kg)	113.5 (104.7, 131.8)	117.9 (107.5, 126.0)	113.5 (100.2, 134.6)
BMI (kg/m^2)	44.2 (39.4, 48.6)	43.8 (38.8, 47.5)	44.2 (39.9, 48.6)
Obesity-related diseases, *n* (%)			
T2D	17 (47.2)	4 (75.0)	13 (43.3)
NASH	29 (80.6)	5 (87.5)	24 (80.0)
OSA	36 (100)	6 (100)	30 (100)

Values are median (interquartile range).
ELISA, enzyme-linked immunosorbent assay; IHC, immunohistochemistry; BMI, body mass index; T2D, type 2 diabetes; NASH, nonalcoholic steatohepatitis; OSA, obstructive sleep apnea.

Table 2 Weight loss effects in the proteomic analysis group and the ELISA and IHC group before and after LSG.

Variable	Baseline	6 months after LSG	*p* value
All patients (*n* = 36)			
Body weight (kg)	113.5 (104.7, 131.8)	90.5 (74.6, 99.2)	<0.001
BMI (kg/m^2)	44.2 (39.4, 48.6)	32.6 (30.3, 35.7)	<0.001
EWL (%)	—	48.8 (38.1, 56.7)	—
TWL (%)	—	24.5 (18.7, 28.8)	—
SFA (cm^2)	472.2 (407.5, 560.7)	292.4 (231.1, 409.1)	<0.001
VFA (cm^2)	229.6 (192.2, 286.1)	140.1 (116.2, 169.0)	<0.001
Proteome analysis (*n* = 6)			
Body weight (kg)	117.9 (107.5, 126.0)	89.2 (81.3, 100.4)	—
BMI (kg/m^2)	43.8 (38.8, 47.5)	33.4 (31.5, 35.9)	—
EWL (%)	—	46.6 (37.5, 54.9)	—
TWL (%)	—	23.4 (19.4, 26.6)	—
SFA (cm^2)	460.8 (431.1, 492.5)	289.2 (276.5, 299.4)	—
VFA (cm^2)	265.8 (205.8, 385.3)	168.0 (115.5, 193.7)	—
ELISA and IHC (*n* = 30)			
Body weight (kg)	113.5 (100.2, 134.6)	90.5 (74.4, 98.0)	<0.001
BMI (kg/m^2)	44.2 (39.9, 48.6)	32.6 (30.2, 35.4)	<0.001
EWL (%)	—	48.8 (38.4, 59.3)	—
TWL (%)	—	24.5 (18.8, 29.1)	—
SFA (cm^2)	475.3 (394.6, 567.9)	295.0 (229.0, 415.6)	<0.001
VFA (cm^2)	229.6 (191.1, 251.0)	139.6 (120.5, 156.3)	<0.001

Values are median (interquartile range).
ELISA, enzyme-linked immunosorbent assay; IHC, immunohistochemistry; BMI, body mass index; EWL, excess weight loss; TWL, total weight loss; SFA, subcutaneous fat area; VFA, visceral fat area.

ASPN ($p = 0.006$) and THBS1 ($p = 0.043$) levels significantly decreased after LSG (Fig. 2). We investigated the association of these five proteins with weight loss, as well as with NAFLD-related parameters, by calculating Spearman's correlation coefficient and using a heat map (Fig. 3). These five proteins tended to correlate with the scoring systems, including the NAS and PFS, as assessed using the histopathological findings for liver tissues obtained *via* liver biopsy at 6 months after LSG.

The relationship of each TGF-β-related protein with the NAS and PFS is shown in Fig. 4A and B. Serum protein levels tended to increase as the NAS increased (Fig. 4A), whereas in contrast, ASPN was significantly correlated with PFS ($p = 0.017$, Fig. 4B). Univariate and multivariate analyses using linear regression model were conducted for NAS and PFS at 6 months after LSG. Univariate analysis of NAS revealed that AST, ALT, γ-GTP, HOMA-β, and THBS1 were associated with NAS (Table 4). In a multivariate analysis, AST was extracted as an independent predictive factor of NAS (regression coefficient: 0.130; 95% confidence interval [CI]: 0.061–0.198, $p < 0.001$; Table 4). On the other hand, simple linear regression analysis of PFS revealed a significant association of the PFS with AST, ASPN, and THBS1 (Table 5). In addition, a multiple linear regression analysis showed that ASPN was an independent predictive factor associated with liver fibrosis (regression coefficient: 0.703; 95% CI: 0.114–1.291, $p = 0.002$; Table 5). A receiver operating characteristic curve analysis indicated areas under the curve of 0.868 (95% CI: 0.714–1.000) for ASPN and 0.816 (95% CI: 0.582–1.000) for AST (Fig. 5). The most appropriate cutoff value for ASPN was 1.079 ng/mL (sensitivity: 78.9%, specificity: 100%).

Validation by IHC

IHC was performed to assess the expression of TGF-β-related proteins in liver tissues. The expression of all five proteins were confirmed in NASH cases (Fig. 6A), whereas ASPN tended to be expressed at an earlier stage than the other proteins in NAFLD cases (Fig. 6B).

Discussion

The present study revealed dynamic fluctuation in TGF-β-related proteins levels before and after LSG according to a proteomic analysis and ELISA, and their expression in the liver with NAFLD were confirmed.

Table 3 NAFLD-related parameters in the proteomic analysis group and the ELISA and IHC group before and after LSG.

Variable	Baseline	6 months after LSG	*p* value
All patients (*n* = 36)			
AST (IU/L)	36.5 (23.0, 56.5)	16.0 (12.5, 19.5)	<0.001
ALT (IU/L)	57.0 (31.3, 78.8)	14.0 (11.0, 22.5)	<0.001
γ-GTP (IU/L)	41.0 (26.8, 62.3)	17.0 (11.5, 23.0)	<0.001
Type 4 collagen 7S (ng/mL)	4.2 (3.7, 4.9)	3.7 (3.3, 4.5)	0.013
Hyaluronic acid (ng/mL)	11.8 (10.0, 30.7)	22.1 (10.0, 34.9)	0.157
BS (mg/dL)	102.0 (91.5, 112.0)	83.0 (79.8, 93.0)	<0.001
HbA1c (%)	6.2 (5.8, 7.2)	5.7 (5.4, 6.1)	<0.001
IRI (μU/mL)	18.4 (16.0, 23.8)	8.4 (6.8, 13.0)	<0.001
HOMA-IR	4.8 (3.5, 6.6)	1.7 (1.4, 2.6)	<0.001
HOMA-β	178.5 (127.1, 266.1)	148.8 (110.4, 193.2)	0.143
NAFIC score	1 (1, 2)	1 (0, 1)	<0.001
FIB-4 index	0.8 (0.5, 1.0)	0.7 (0.5, 0.9)	0.076
NAFLD fibrosis score	–1.3 (–2.4, 0.2)	–1.4 (–2.1, –0.4)	0.095
Liver volume (L)	2.2 (1.9, 2.4)	1.7 (1.5, 1.9)	<0.001
Liver/Spleen ratio	0.8 (0.7, 1.0)	1.3 (1.2, 1.3)	<0.001
NAFLD activity score	3 (2, 4)	1 (0, 2)	<0.001
Pericellular fibrosis score	2 (1, 3)	2 (1, 3)	0.223
Proteome analysis (*n* = 6)			
AST (IU/L)	43.5 (32.3, 63.8)	17.5 (15.3, 19.0)	—
ALT (IU/L)	79.0 (42.5, 96.8)	17.0 (15.0, 23.5)	—
γ-GTP (IU/L)	38.0 (29.8, 62.0)	21.0 (13.8, 27.5)	—
Type 4 collagen 7S (ng/mL)	5.0 (4.7, 5.6)	4.7 (4.4, 5.0)	—
Hyaluronic acid (ng/mL)	19.0 (11.0, 33.8)	28.0 (12.8, 35.4)	—
BS (mg/dL)	97.0 (90.0, 108.5)	88.0 (74.8, 93.0)	—
HbA1c (%)	7.0 (6.1, 7.5)	5.9 (5.5, 6.3)	—
IRI (μU/mL)	14.9 (11.9, 17.3)	11.3 (7.9, 13.4)	—
HOMA-IR	3.2 (2.7, 3.6)	2.3 (1.7, 2.7)	—
HOMA-β	129.1 (85.8, 190.7)	156.4 (122.9, 177.4)	—
NAFIC score	2 (1, 3)	1 (1, 2)	—
FIB-4 index	1.1 (0.7, 1.4)	0.8 (0.5, 1.0)	—
NAFLD fibrosis score	–0.7 (–1.6, 1.5)	–1.2 (–2.5, 0.5)	—
Liver volume (L)	2.3 (2.0, 2.7)	1.6 (1.6, 1.8)	—
Liver/Spleen ratio	0.8 (0.6, 0.9)	1.3 (1.3, 1.3)	—
NAFLD activity score	3 (3, 3)	2 (1, 2)	—
Pericellular fibrosis score	1 (1, 2)	2 (1, 2)	—
ELISA and IHC (*n* = 30)			
AST (IU/L)	36.5 (23.0, 54.0)	15.0 (12.0, 20.0)	<0.001
ALT (IU/L)	54.0 (30.0, 73.8)	14.0 (10.0, 22.0)	<0.001
γ-GTP (IU/L)	41.5 (26.3, 57.8)	16.0 (11.0, 22.0)	<0.001
Type 4 collagen 7S (ng/mL)	4.1 (3.4, 4.6)	3.6 (3.3, 4.4)	0.024
Hyaluronic acid (ng/mL)	10.2 (10.0, 28.3)	22.0 (10.0, 34.3)	0.251
BS (mg/dL)	102.0 (95.5, 112.0)	82.5 (80.0, 92.5)	<0.001
HbA1c (%)	6.1 (5.8, 6.8)	5.5 (5.2, 6.0)	<0.001
IRI (μU/mL)	19.5 (16.5, 25.0)	8.4 (6.5, 12.7)	<0.001
HOMA-IR	4.9 (4.1, 7.0)	1.7 (1.3, 2.6)	<0.001
HOMA-β	186.3 (135.5, 266.1)	146.3 (106.5, 200.1)	0.047
NAFIC score	1 (1, 2)	1 (0, 1)	<0.001
FIB-4 index	0.7 (0.5, 0.9)	0.6 (0.5, 0.9)	0.236
NAFLD fibrosis score	–1.3 (–2.4, 0)	–1.4 (–2.0, –0.5)	0.284
Liver volume (L)	2.1 (1.9, 2.4)	1.7 (1.5, 1.9)	<0.001
Liver/Spleen ratio	0.8 (0.7, 1.0)	1.3 (1.2, 1.3)	<0.001
NAFLD activity score	3 (2, 4)	1 (0, 2)	<0.001
Pericellular fibrosis score	2 (1, 3)	2 (1, 3)	0.149

Values are median (interquartile range).

ELISA, enzyme-linked immunosorbent assay; IHC, immunohistochemistry; NASH, nonalcoholic steatohepatitis; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, gamma-glutamyltranspeptidase; BS, blood sugar; HbA1c, hemoglobin A1c; IRI, immunoreactive insulin; HOMA-IR, homeostatic model assessment of insulin resistance; HOMA-β, homeostasis model assessment for beta cell function; NAFLD, nonalcoholic fatty liver disease.

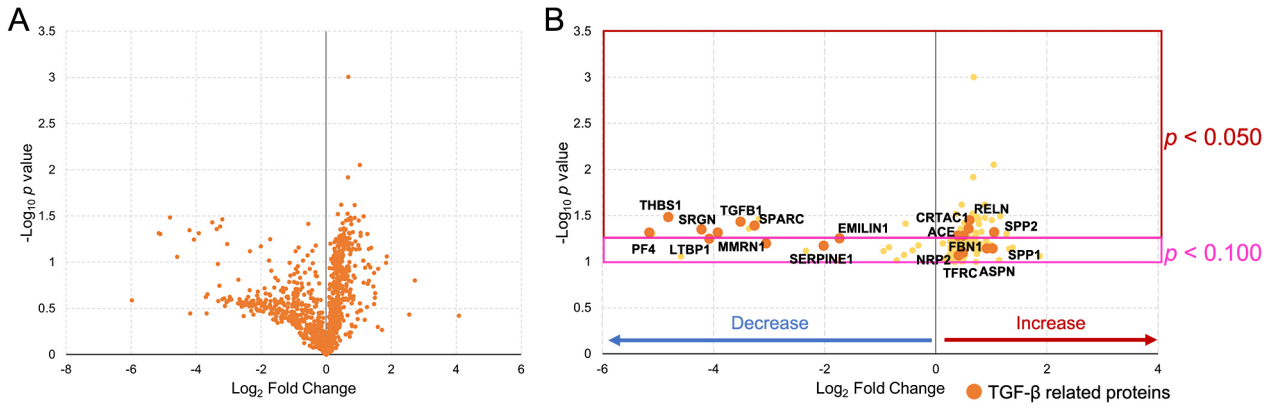

Fig. 1 Results of proteomic analysis using the patients' serum obtained before and at 6 months after LSG. A total of 1,912 serum proteins were identified using proteomic analysis. (A) A volcano plot showing the changes in serum proteins before and after LSG. (B) When focusing on proteins with $p < 0.1$, several proteins related to the TGF-β signaling pathway were observed to have fluctuated before and after LSG.

LSG, laparoscopic sleeve gastrectomy.

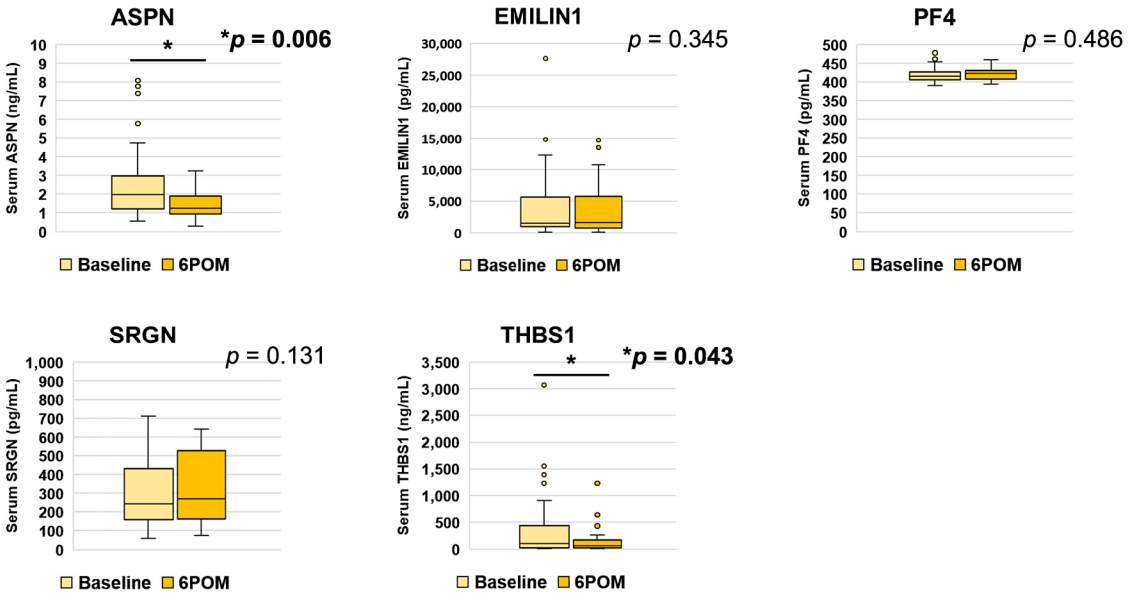

Fig. 2 Validation by ELISA for TGF-β-related proteins—namely, ASPN, EMILIN-1, PF4, SRGN, and THBS1. Among the five proteins, ASPN ($p = 0.006$) and THBS1 ($p = 0.043$) levels significantly decreased after LSG.

ELISA, enzyme-linked immuno sorbent assay; ASPN, Asporin; EMILIN1, EMILIN-1; PF4, Platelet factor 4; SRGN, Serglycin; THBS1, Thrombospondin-1.

In addition, our study results indicated that ASPN might be a useful predictor of liver fibrosis in NAFLD patients; that is, ASPN might be a helpful marker that can distinguish NASH from NAFLD.

ASPN was first identified in 2001 as an exocrine protein in human articular cartilages and periodontal tissues [18]. ASPN plays a role in the feedback mechanism with TGF-β1 in knee osteoarthritis, and it is involved in its pathogenesis [19]. However, in recent years, ASPN has been reported to be associated with various cancers, such as breast [20], gastric [21], and colorectal cancers [22]. There are few reports about a relationship between ASPN and NAFLD [23]. On the other hand, some reports have described a relationship between the TGF-β signaling pathway and NAFLD [2, 8, 9], where the former is reportedly associated with fibrosis and carcinogenesis in NAFLD [2]. In particular, the extracellular matrix is produced *via* the TGF-β signaling pathway in the livers of individuals with NAFLD, promoting the progression to cirrhosis. Furthermore, protein mutations related to the TGF-β signaling pathway and disruptions in the tumor-suppression function of the TGF-β signaling pathway

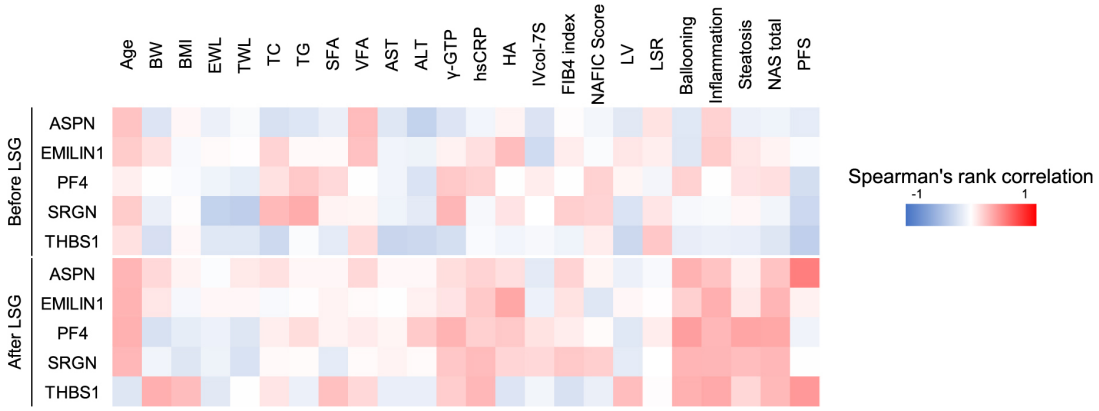

Fig. 3 Evaluation with a heat map for the association between the five proteins and weight loss- and NAFLD-related parameters. After LSG, the five proteins tended to correlate with histopathological scoring systems such as the NAS and PFS.

LSG, laparoscopic sleeve gastrectomy; ASPN, Asporin; EMILIN1, EMILIN-1; PF4, Platelet factor 4; SRGN, Serglycin; THBS1, Thrombospondin-1; BW, body weight; BMI, body mass index; EWL, excess weight loss; TWL, total weight loss; TC, total cholesterol; TG, triglyceride; SFA, subcutaneous fat area; VFA, visceral fat area; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, gamma-glutamyltranspeptidase; hsCRP, high-sensitivity C-reactive protein; HA, hyaluronic acid; IVcol-7S, type 4 collagen 7S; LV, liver volume; LSR, liver to spleen ratio; NAS, nonalcoholic fatty liver disease activity score; PFS, pericellular fibrosis score.

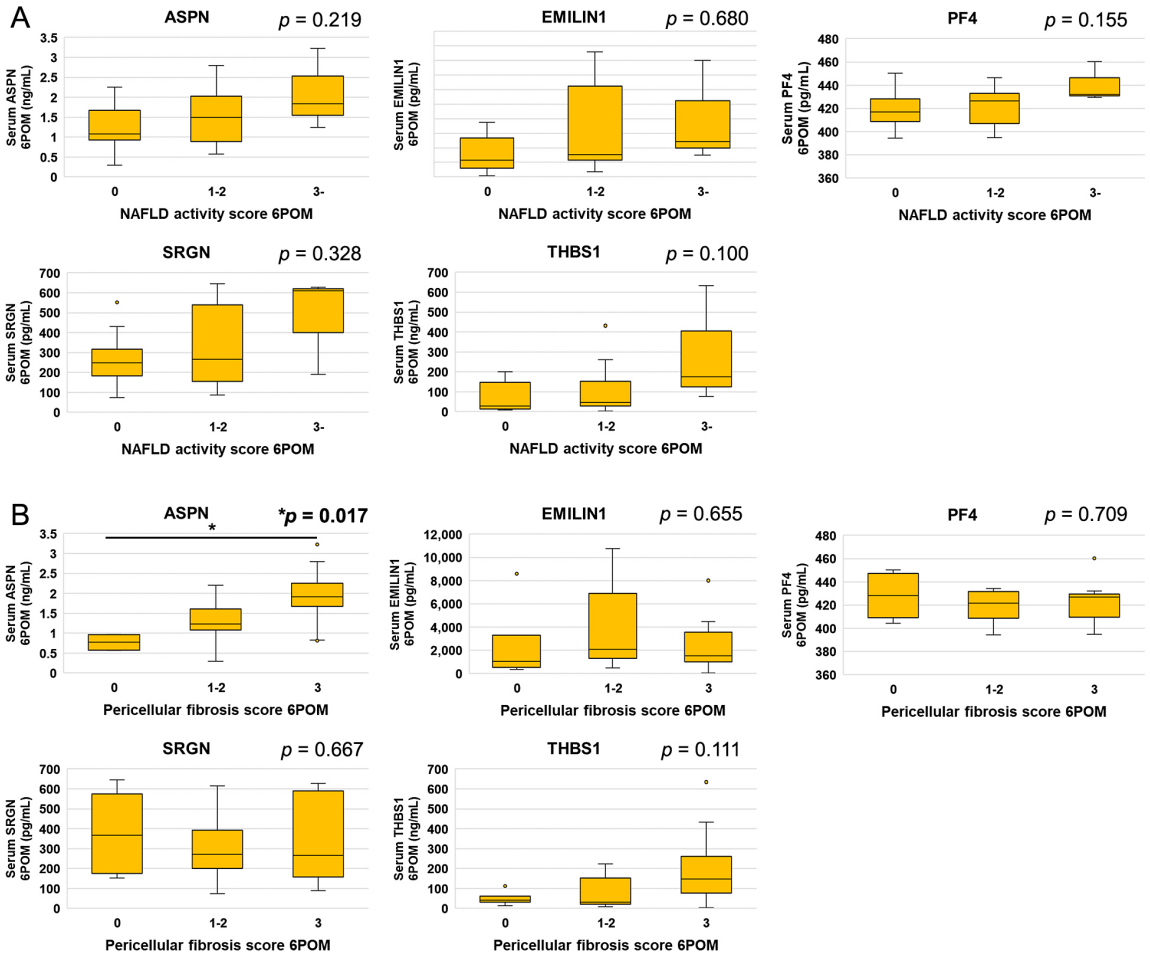

Fig. 4 Relationship between the TGF-β-related proteins and the NAS and PFS. (A) Serum protein levels tended to increase as the NAS increased, albeit with no significant difference. (B) In contrast, ASPN was significantly correlated with the PFS ($p = 0.017$).

ASPN, Asporin; EMILIN1, EMILIN-1; PF4, Platelet factor 4; SRGN, Serglycin; THBS1, Thrombospondin-1; NAS, nonalcoholic fatty liver disease activity score; PFS, pericellular fibrosis score.

Table 4 Univariate and multivariate linear regression analyses of the relationship between NAFLD-related parameters and the NAS after LSG.

Variables	Univariate analysis			Multivariate analysis		
	Regression Coefficient	95% CI	*p* value	Regression Coefficient	95% CI	*p* value
Age (years)	–0.007	–0.064, 0050	0.796			
BMI (kg/m^2)	0.079	–0.030, 0.188	0.147	0.029	–0.052, 0.110	0.464
AST (IU/L)	0.156	0.083, 0.230	<0.001	0.130	0.061, 0.198	<0.001
ALT (IU/L)	0.105	0.058, 0.152	<0.001			
γ-GTP (IU/L)	0.055	0.031, 0.079	<0.001			
Type 4 collagen 7S (ng/mL)	0.282	–0.330, 0.894	0.348			
Hyaluronic acid (ng/mL)	0.010	–0.011, 0.030	0.337			
BS (mg/dL)	0.006	–0.051, 0.062	0.837			
HbA1c (%)	1.060	–0.154, 2.273	0.084			
IRI (μU/mL)	0.078	–0.004, 0.160	0.060			
HOMA-IR	0.269	–0.079, 0.618	0.123			
HOMA-β	0.005	0.001, 0.009	0.017	0.003	>–0.001, 0.006	0.117
NAFIC score	0.155	–0.654, 0.964	0.694			
FIB-4 index	0.109	–1.581, 1.799	0.894			
NAFLD fibrosis score	–0.046	–0.469, 0.387	0.827			
Liver volume (L)	0.001	>–0.001, 0.003	0.247			
Liver/Spleen ratio	–2.614	–5.760, 0.532	0.098	–1.283	–4.376, 1.810	0.390
Asporin (ng/mL)	0.612	–0.110, 1.333	0.092			
EMILIN-1 (ng/mL)	0.001	>–0.001, <0.001	0.303			
Platelet factor 4 (ng/mL)	28.363	–1.193, 57.919	0.059			
Serglycin (μg/mL)	2.336	–0.398, 5.069	0.090			
Thrombospondin-1 (ng/mL)	0.004	<0.001, 0.007	0.032	0.002	>–0.001, 0.005	0.106

NAS, NAFLD activity score; LSG, laparoscopic sleeve gastrectomy; CI, confidence interval; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, gamma-glutamyltranspeptidase; BS, blood sugar; HbA1c, hemoglobin A1c; IRI, immunoreactive insulin; HOMA-IR, homeostatic model assessment of insulin resistance; HOMA-β, homeostasis model assessment for beta cell function; NAFLD, nonalcoholic fatty liver disease.

occur, resulting in HCC. As mentioned above, ASPN has a feedback mechanism for the TGF-β signaling pathway, specifically a negative feedback mechanism. Thus, the decrease in ASPN levels after LSG and the association of ASPN with fibrosis in the liver tissues at 6 months after surgery observed in the present study can be assumed to reflect the aforementioned feedback mechanism. Polyzos *et al.* reported a decrease in serum ASPN levels after vitamin E monotherapy or the combination of vitamin E and spironolactone therapy for NAFLD [23], which seems consistent with the results of this study. ASPN has also been suggested to be directly involved in the extracellular matrix [19], which may directly affect the fibrogenic response in liver tissues. We consider two hypotheses to explain why ASPN was expressed at an early stage of NAFLD in the liver in the IHC analysis. First, prior to progression to NASH, the suppressive effect of ASPN and other molecules on the TGF-β signaling pathway is dominant; nevertheless, upon progression to NASH, the promotive effect of other molecules on the TGF-β signaling pathway becomes dominant. Second, some mechanisms can directly suppress ASPN activity. This is consistent with a previous study that reported that serum ASPN levels decreased as NAFLD progressed [23]. Regardless, further studies on the dynamics of ASPN in NAFLD are required.

In this study, THBS1, another protein that fluctuated significantly before and after LSG, is associated with homeostasis of the extracellular matrix *via* the TGF-β signaling pathway [24], and several studies have confirmed

Table 5 Univariate and multivariate linear regression analyses of the relationship between NAFLD-related parameters and the PFS after LSG.

Variables	Univariate analysis			Multivariate analysis		
	Regression Coefficient	95% CI	*p* value	Regression Coefficient	95% CI	*p* value
Age (years)	–0.003	–0.056, 0.050	0.907			
BMI (kg/m^2)	0.024	–0.081, 0128	0.645			
AST (IU/L)	0.087	0.087, 0.171	0.045	0.066	–0.012, 0.144	0.091
ALT (IU/L)	0.036	–0.023, 0.095	0.216			
γ-GTP (IU/L)	0.023	–0.006, 0.053	0.118			
Type 4 collagen 7S (ng/mL)	0.067	–0.503, 0.637	0.810			
Hyaluronic acid (ng/mL)	–0.002	–0.021, 0.017	0.837	–0.008	–0.025, 0.009	0.325
BS (mg/dL)	–0.016	–0.067, 0.036	0.531			
HbA1c (%)	0.415	–0.762, 1.591	0.472			
IRI (μU/mL)	0.012	–0.069, 0.093	0.764			
HOMA-IR	0.008	–0.329, 0.345	0.962			
HOMA-β	0.003	–0.002, 0.007	0.210	<0.001	–0.003, 0.005	0.761
NAFIC score	0.202	–0.534, 0.937	0.575	0.076	–0.578, 0.730	0.809
FIB-4 index	0.138	–1.404, 1.680	0.854	–0.253	–2.808, 2.302	0.836
NAFLD fibrosis score	0.086	–0.308, 0.480	0.654	–0.026	–0.462, 0.410	0.900
Liver volume (L)	0.001	>–0.001, 0.003	0.195			
Liver/Spleen ratio	–0.850	–4.291, 2.591	0.611			
Asporin (ng/mL)	0.812	0.211, 1.414	0.011	0.703	0.114, 1.291	0.002
EMILIN-1 (ng/mL)	0.000	>–0.001, <0.001	0.970			
Platelet factor 4 (ng/mL)	–3.110	–32.497, 26.278	0.828			
Serglycin (μg/mL)	–0.072	–2.748, 2.603	0.956			
Thrombospondin–1 (ng/mL)	0.004	0.001, 0.007	0.022	0.002	–0.002, 0.005	0.351

PFS, pericellular fibrosis score; LSG, laparoscopic sleeve gastrectomy; CI, confidence interval; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, gamma-glutamyltranspeptidase; BS, blood sugar; HbA1c, hemoglobin A1c; IRI, immunoreactive insulin; HOMA-IR, homeostatic model assessment of insulin resistance; HOMA-β, homeostasis model assessment for beta cell function; NAFLD, nonalcoholic fatty liver disease.

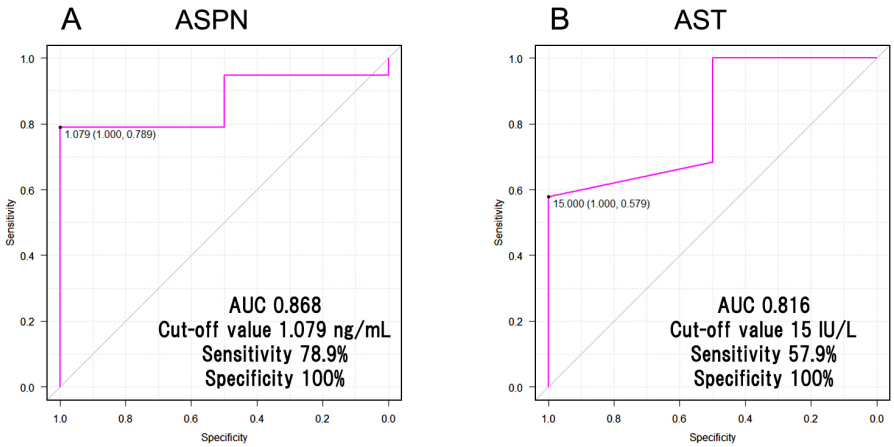

Fig. 5 ROC analysis revealed that ASPN was a good prognostic marker of NAFLD-related liver fibrosis.
ROC, receiver operating characteristic curve; AUC, area under the curve; ASPN, Asporin; AST, aspartate aminotransferase.

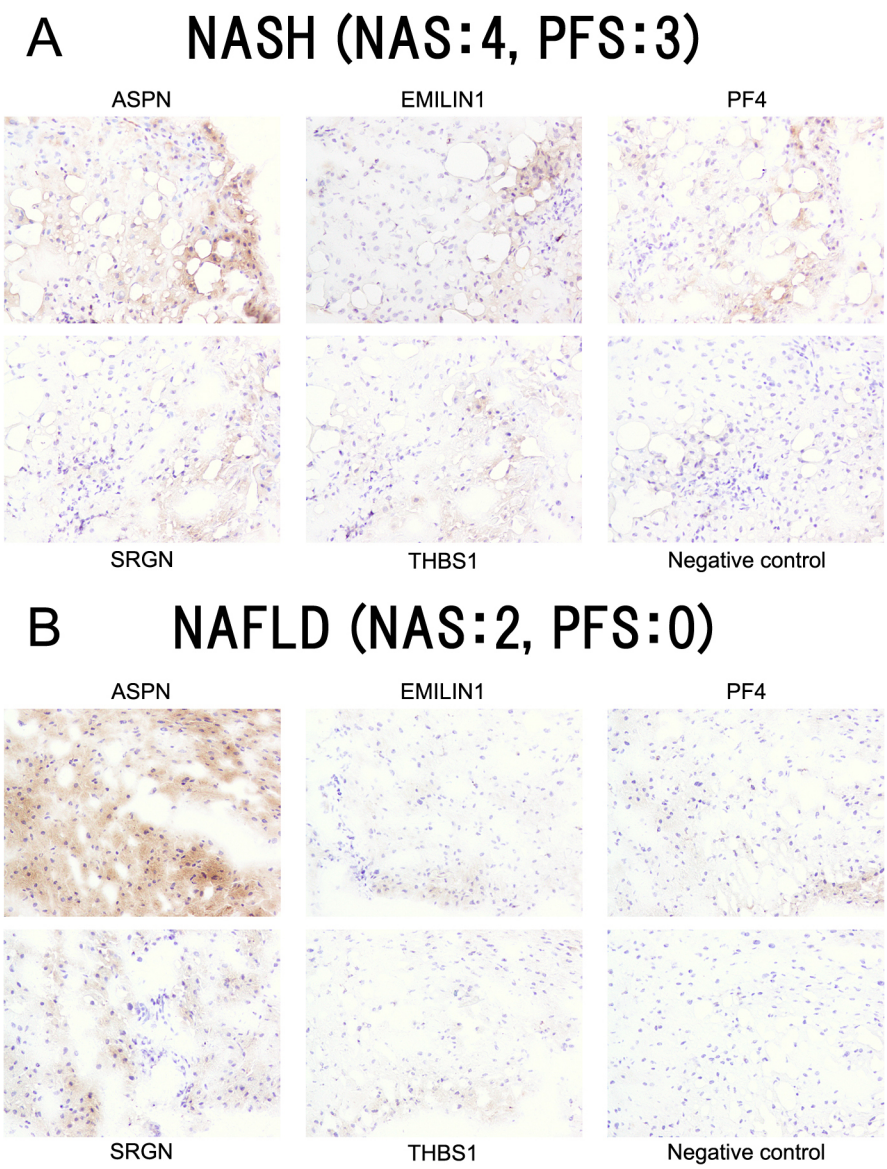

Fig. 6 IHC for assessing the expression of TGF-β-related proteins. (A) The expression of all five proteins was confirmed in NASH cases (44-year-old man, BW 125.8 kg, BMI 37.2 kg/m^2, with T2D). (B) ASPN tended to be expressed at an earlier stage than the other proteins in NAFLD cases (58-year-old woman, BW 92.2 kg, BMI 36.5 kg/m^2, without T2D).

IHC, immunohistochemistry; NASH, nonalcoholic steatohepatitis; NAS, nonalcoholic fatty liver disease activity score; PFS, pericellular fibrosis score; NAFLD, nonalcoholic fatty liver disease; BW, body weight; BMI, body mass index; T2D, type 2 diabetes; ASPN, Asporin; EMILIN1, EMILIN-1; PF4, Platelet factor 4; SRGN, Serglycin; THBS1, Thrombospondin-1.

its relationship with NAFLD [25-27]. An experiment using mice showed that liver fibrosis was suppressed by a high-fat diet in THBS1-deficient mice [25]. Another animal study revealed that the progression to obesity-related NASH *via* a high-fat diet was suppressed in macrophage-specific THBS1 knockout mice, whereas IHC verification indicated that the THBS1 expression in liver tissues increased as NAFLD progressed [26]. Furthermore, Bai *et al.* reported that serum THBS1 levels increased as liver steatosis worsened in patients with NAFLD [27], while in contrast, a study examining the association between NAFLD and thrombospondin-2 (THBS2), which belongs to the same family as THBS1, concluded that its presence in the liver was associated with the pathogenesis of NAFLD and that the serum THBS2 level was a valuable marker for predicting liver fibrosis [28]. These findings suggest that the thrombospondin family plays an important role in NAFLD progression. Further, THBS1 is involved not only in NAFLD but also in several other diseases, including type 2 diabetes and dyslipidemia [29]. Hence, it is necessary to recognize it as a protein that plays an important role in obesity-related diseases. In the present study, serum THBS1 levels tended to increase as the NAS and PFS

increased at 6 months after LSG, and the THBS1 expression in liver tissues increased as NAFLD progressed, which is consistent with the findings of previous reports.

EMILIN-1 is a protein belonging to the EMI domain-containing family in the extracellular matrix [30, 31], and it acts as a negative regulator of the TGF-β signaling pathway [30-32] and is associated with essential hypertension [30, 31]. A previous study observed that the peripheral arteries narrowed, and systemic blood pressure increased independently of cardiac output in EMILIN-1-deficient mice [31]. In the present study, we did not find any association between serum EMILIN-1 levels and NAFLD-related parameters; however, we did observe the EMILIN-1 expression in NASH livers. Considering its inhibitory effects on the TGF-β signaling pathway, EMILIN-1 may be involved in the suppression of disease progression in NAFLD, especially NASH. Thus, further investigations into this subject should be conducted.

SRGN is associated with the TGF-β signaling pathway and is conjugated with PF4 [33]. Although not presented in the Results section, serum SRGN and PF4 levels showed a high correlation with ELISA ($\rho = 0.813$, $p < 0.001$). SRGN has been reported to be involved in the regulation of epithelial-mesenchymal transitions (EMTs) *via* the TGF-β signaling pathway in breast cancer tissues [34]. In addition, HCC patients with a high SRGN expression exhibit a significantly shorter overall survival and higher recurrence rates than those with a low SRGN expression [35]. No direct association of SRGN and PF4 with NAFLD has been reported; however, our study revealed that the SRGN and PF4 expressions in liver tissues tended to increase as NAFLD progressed. These proteins may be involved not only in the EMTs regulation of malignant tumors but also in NAFLD progression.

Similar to several previous reports, in the present study, LSG led to significant weight loss, with a percentage total weight loss of 23.7% 6 months postoperatively, and most NAFLD-related clinical parameters improved dramatically. Moreover, our study revealed that proteins related to the TGF-β signaling pathway fluctuated dynamically before and after LSG in severely obese patients and that ASPN was associated with the pathogenesis of NAFLD, especially liver fibrosis. Many previous studies have focused on the relationship between fibrosis in NAFLD and the TGF-β signaling pathway [2, 8, 9], and the results of this study are consistent with their findings. PFS did not show significant changes before or 6 months after LSG; nevertheless, we previously reported that an improvement in liver fibrosis occurred at 1 year after LSG [4]. Thus, liver fibrosis in patients enrolled in this study would also improve at 6 months after LSG.

In our study, the association between PFS and the scoring systems previously reported to reflect fibrosis in NAFLD, such as the NAFIC and NAFLD fibrosis scores, could not be determined. Thus, rather, Brunt's criteria could explain this association, suggesting more advanced liver fibrosis than PFS [14, 36]. In other words, ASPN may reflect the earlier stage of liver fibrosis associated with NAFLD, as compared with the NAFIC and NAFLD fibrosis scores. In fact, ASPN tends to be expressed in liver tissues at the NAFLD stage, compared to other TGF-β-related proteins, which supports this hypothesis.

An unresolved problem in diagnosing NAFLD/NASH is that the gold standard is still invasive tests, including liver biopsy [1]. In this study, ASPN was demonstrated to be a better predictive factor in the pathogenesis of NAFLD, especially liver fibrosis, than the previously reported scoring systems [5-7]. In addition, as mentioned above, the TGF-β signaling pathway is confirmed involved in liver fibrosis, and we believe the use of TGF-β related proteins as predictive markers for liver fibrosis is reasonable. In particular, ASPN may serve as a novel biomarker for predicting liver fibrosis in the future.

Our study has some limitations. First, before LSG, the usefulness of proteins related to the TGF-β signaling pathway as markers of NAFLD could not be determined. There are two possible explanations for this finding. First, severely obese patients had several obesity-related diseases, including hypertension and diabetes, which might have generated a significant bias, and the TGF-β signaling pathway is also known to be associated with such pathologies as hypertension and diabetes [28-30]. Preoperative TGF-β-related proteins varied widely in our study compared to postoperative proteins, and obesity-related diseases, such as hypertension and diabetes dramatically improved at 6 months after surgery, simplifying comorbidities and reducing the bias. Thus, TGF-β-related proteins, such as ASPN, might accurately reflect the pathogenesis of NAFLD. As mentioned above, we should consider that the usefulness of ASPN as a fibrosis predictor is limited to after LSG at this stage. Second, this study was conducted at a single institution, and its limited sample size could not be ignored. In the future, studies with a larger number of samples and using comprehensive analyses, such as lipidomic and transcriptome analysis, should be planned and conducted to elucidate the mechanism underlying liver fibrosis. Second, the observation period of this study was 6 months, and evaluation over a longer period (*i.e.*, a year) is a future issue. Third, liver tissues at 6 months after LSG were not preserved because it was outside the protocol. The reason for this is that, unlike intraoperative liver biopsy, liver biopsy from the body surface is considered to be risky because it is difficult to deal with complications such as bleeding and biliary fistula at the

time of biopsy. Thus, the expression of TGF-β-related proteins in liver tissues at 6 months after LSG was not verified. Fourth, in the present study, we used frozen liver specimens for IHC analysis. As a result, cracks in the liver tissue and variations in the number of cells tended to occur during sectioning. Therefore, comparison of expression intensity of analyzed proteins and measurement of positive cells in the liver section was not possible. Solutions such as using paraffin-embedded tissues will be considered in future studies.

Conclusions

Proteins involved in the regulation of the TGF-β signaling pathway dramatically fluctuated before and after LSG in severely obese patients and were associated with the pathogenesis of NAFLD. An IHC analysis revealed the expressions of TGF-β-related proteins in liver tissues obtained from severely obese patients with NAFLD, and our study suggests that ASPN may be a more useful predictor of liver fibrosis in NASH than previously reported markers and scoring systems.

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Authorship Contribution Statement

Hideki Kumagai: Conceptualization, Writing – original draft, Data curation, Visualization.

Akira Sasaki: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Akira Umemura: Conceptualization, Funding acquisition, Methodology, Writing – review & editing.

Keisuke Kakisaka: Conceptualization, Methodology.

Takeshi Iwaya: Conceptualization, Methodology.

Satoshi S Nishizuka: Conceptualization, Methodology, Supervision.

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Ethical Approval

This study protocol was approved by the institutional ethics committee of Iwate Medical University Hospital (approval number: MH2022-056). The written informed consent was obtained from all patients enrolled in this study.

Competing Interest

None of the authors have any potential conflicts of interest associated with this research.

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