SUPPLEMENTARY MATERIALS AND METHODS

Materials

We obtained fine needle liver biopsies from four histologically diagnosed cases of early NASH (Brunt's classification stage 1, F1) (1) and compared the data from these biopsies with those of tiny laparoscopy resections of the liver edge from patients with metastatic liver cancer who were not undergoing treatment with chemotherapeutic agents.

This study was approved by the relevant ethics committees of the institutions involved. Informed consent was obtained from all participants.

All patients had evidence of NASH on abdominal ultrasonography and computed tomography. All were hyperglycemic and dyslipidemic. Furthermore, all patients had at least a 6 month history of high serum aminotransferase activities, with no other identifiable cause (e.g., viral infection, autoimmune disease, or other metabolic disorder). The men consumed <30 g/day alcohol. The women consumed <20 g/day alcohol. Blood tests and imaging studies revealed no evidence of other liver disease.

Methods

Immunohistochemistry for GLP-1R

Immunohistochemical analysis was conducted as reported earlier (2), with minor modifications. Serial 4 μm sections of 10% neutral formalin-fixed paraffin-embedded tissues were deparaffinized and rehydrated by immersion in xylene and graded concentrations of alcohol. Endogenous peroxidase activity was quenched by incubation in 0.3% (v/v) H_2O_2 in methanol for 30 min. Antigen retrieval was performed

by autoclaving in 10 mM citrate buffer, pH 6.0 for 15 min at 120°C. The sections were then blocked with 10% normal goat serum (Vector Laboratories Inc., Burlingame, CA, USA), incubated for 90–120 min with primary antibody in 2% serum, and then overnight at 4°C with 1:100 dilution of anti-GLP-1R and 1:100 caveolin-1, after which they were incubated with N-Histofine® Simple Stain MAX PO (Nichirei Corp., Tokyo, Japan) at RT for 30 min. After repeated washing with phosphate-buffered saline (PBS), the sections were reacted with diaminobenzidine in 0.01% H_2O_2 and were counterstained with hematoxylin before light microscopic examination.

Western blot analysis

Liver samples were homogenized in 10 volumes of homogenization buffer (20 µM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.1 mM phenylmethanesulfonyl fluoride, 20 µM pepstatin A, and 20 µM leupeptin). The obtained lysates, containing 30 µg/ml protein, 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 then incubated with anti-GLP-1R diluted 1:500 diluted 1:2,000 in 0.1% Tween-20 in PBS. After washing, the blots were incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin antibody (Jackson ImmunoResearch Inc., West Grove, PA, USA) for 1 h at RT. Protein bands were detected using an enhanced chemiluminescence detection system (ECL Plus; Amersham

Biosciences Corp., Uppsala, Sweden) and a CCD analyzer (General Electric Co., Fairfield, CT, USA). Anti-β-actin antibody (Merck Millipore Co., Billerica, MA, USA) was used as a loading control (2). Densitometric analysis of the Western blots was performed using Scion Image ver. Beta 4.0.2 (Scion Corp., Frederick, MD, USA). One-way ANOVA with Dunn's *post-hoc* analysis was used to assess differences between liver sample types (SuperANOVA; Abacus Concepts Inc., Berkeley, CA, USA). Differences for which $p < 0.05$ were considered to be significant.

Assessment of subcellular localization of GLP-1R using immunoelectron microscopy

To assess the subcellular localization of GLP-1R, we performed IEM, as described earlier (2). Briefly, wedge biopsies of approximately 3 cm × 1 cm × 1 cm or liver biopsies were obtained as quickly as possible from the margin of a liver lobe. The specimens were immersed immediately in PBS (pH 7.4) at 37°C and then perfusion-fixed by injection with periodate-lysine-paraformaldehyde (PLP) in a Petri dish filled with saline. Each specimen was held at a corner using forceps. PLP was then injected at multiple sites using a 26-G syringe until the tissue discolored and hardened. After perfusion and incubation with PLP overnight at 4°C, 5 mm sections were prepared. The sections were immersed for 15 min in three changes of 0.01% PBS, pH 7.4 and incubated with a 1:100 dilution of anti-GLP-1R in 0.01 M PBS containing 1% bovine serum albumin overnight at 4°C in a moisture chamber. After washing three times for 15 min in PBS, they were then incubated in 1.4 nm colloidal gold-conjugated anti-mouse IgG antibody (Nanoprobes Inc., Yaphank, NY, USA) diluted 1:40 for 40

min and washed three times at RT for 30 min in 10 mM citrate buffer (pH 6.0). Subsequently, the sections were physically developed using a silver enhancement kit (Nanoprobes Inc.) for 5 min, as described above, washed three times again with PBS for 15 min, fixed in 2.5% glutaraldehyde in 0.01% phosphate buffer (pH 7.4) for 1 h at 4°C, and then passed through a series of graded ethanol solutions. They were then post-fixed with 2% osmium tetroxide in 0.01% phosphate buffer (pH 7.4) for 90 min at 4°C and embedded in Epon. Ultra-thin sections were cut using a diamond knife and an ultramicrotome (LKB Bromma, Sweden), which were stained with uranyl acetate and examined using transmission electron microscopy (JEM-1200 EX; JEOL, Tokyo, Japan).

REFERENCES

1. Brunt EM, Janney CG, Di Bisceglie AM et al. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterol* 1999;94:2467–74.
2. Yokomori H, Inagaki Y, Ando W et al. Spatiotemporal expression of matrix metalloproteinase-1 in progression of nonalcoholic steatohepatitis. *J Mod Hum Pathol* 2016;1:11–20.