**Supplementary Material and Methods**

*Sandwich-type immunoassay for Inter-α-Inhibitor Heavy Chain 4 (ITIH4)*

Recombinant ITIH4 was expressed in HEK293 cells and purified to yield a protein preparation used for immunization of a rabbit for antiserum production (Supplemental Fig. 1A).8 Polyclonal IgG anti-human ITIH4 antibodies were purified from the rabbit serum. Wells of microtiter plates (Flouronunc, Merck) were coated with 0.25 µg rabbit anti-ITIH4 in 100 µL phosphate buffered saline (PBS) by overnight incubation at room temperature. Subsequently, residual binding sites in the wells were blocked by adding 200 µL of Tris buffered saline (TBS), 1 mg/mL HSA. After washing the wells thrice in TBS, 0.05% v/v Tween-20 (TBST), we added 100 µL of human serum or plasma diluted 10,000-fold in a buffer containing 25 mM Tris, 1 M NaCl, 1 mg/mL HSA, 100 µg/mL heat-aggregated human IgG and 100 µg/mL rabbit IgG (7406404; Lampire), pH 7.4. Upon overnight incubation at 4°C, the wells were washed in TBST, and 100 µL of 0.5 µg/mL biotinylated rabbit anti-ITIH4 diluted in TBST was added. After 2 h at room temperature, followed by washing with TBST, the wells received 0.1 µg/mL Eu3+-labeled streptavidin (Perkin Elmer) diluted in TBST, 25 µM EDTA for 1 h at room temperature. After washing, 200 µL of enhancement solution (Ampliqon A/S) was added to each well before measuring the fluorescence by time-resolved fluorometry using a Victor X5 plate reader (Perkin Elmer). A standard curve was made from a pool of citrate plasma in which the ITIH4 concentration was determined by comparing dilutions of the plasma with known concentrations of recombinant ITIH4. Three different citrate plasma samples were included as quality controls on every microtiter plate. The results were only accepted if the inter-assay coefficient of variation of these controls was below 15%. All samples, standards, and quality controls were added in duplicate, and measurement was only accepted if the coefficient of variation between the technical duplicates was below 15%.

As part of the validation of the specificity of the assay, 200 µL of EDTA plasma containing 2 mM of the serine protease inhibitor pefabloc (Merck) was fractionated using a Superose 6 Increase 10/300 GL column (GE Healthcare Life Sciences) equilibrated in TBS, 5 mM EDTA, 0.2 mM pefabloc. The fractions were diluted 400-fold and analyzed by the ITIH4 immunoassay or used for anti-ITIH4 western blotting, as described below.

*Western blotting*

To examine the status (i.e. size and fragmentation) of ITIH4 in the samples and to test if the ITIH4 levels determined by the immunoassays was also reflected in western blot analysis of subjects with high and low concentrations of ITIH4, we performed western blot analysis of EDTA plasma from a healthy donor with a low ITIH4 concentration (202 µg/mL), a PBC patient with a low ITIH4 concentration (256 µg/mL), and a PBC patient with a high ITIH4 concentration (524 µg/mL). Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 4-15% Criterion TGX Precast Midi Protein Gels (Bio-Rad), and plasma dilutions were loaded (corresponding to 125, 62.5, 31.3, 15.6, and 7.8 nL of plasma). Subsequently, proteins were transferred to nitrocellulose membranes using the semidry Trans-Blot Turbo Transfer System (Bio-Rad). The membranes were blocked by incubation in TBS, 0.1% v/v Tween-20 for 1 h. As the primary antibody, a mixture of 0.05 µg/mL monoclonal rabbit anti-ITIH4 (ab180139, Abcam) and 0.05 µg/mL polyclonal rabbit anti-ITIH4 (HPA001835, Sigma-Aldrich) diluted in 25 mM Tris, 0.05% v/v Tween-20, 1 mM EDTA, 1 mg/mL human serum albumin, 100 µg/mL human IgG, pH 7.4 was used to ensure that both N- and C-terminal fragments of ITIH4 were detected. The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit IgG (Dako, P0448), and SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) was used for detection. The signal was recorded by a CCD camera, and band intensities were estimated by densitometry using Fiji-ImageJ.30 Also, Western blot analysis of fractions from size-exclusion chromatography was performed as described above.

*Immunohistochemistry for ITIH4*

Formalin-fixed paraffin-embedded liver biopsy sections from controls and patients with PBC were examined for the presence of ITIH4. Four-micron thick sections of liver tissue were mounted on polylysine-coated slides (Thermo Scientific #10219280), deparaffinized, and hydrated (xylene for 10 m followed by an ethanol gradient), washed in distilled water for 5 m, and subjected to antigen retrieval by exposure for bacterial proteinase XXIV (Sigma-Aldrich #P8038) at 37°C for 30 m. After washing in PBS, sections were blocked with 30% normal goat serum (Sigma-Aldrich #G6767) in PBS for 30 m. The primary antibody (at 5 µg/mL) was the polyclonal rabbit anti-ITIH4 antibody described above for the immunoassay. After incubation O/N at 4°C, the sections were washed in PBS and blocked for 30 m at room temperature with peroxidase blocking solution (Dako REAL, #S2023) followed by washing and incubation with secondary antibody (1/500 (horseradish peroxidase-conjugated goat anti-rabbit IgG, Dako #p0448) for 60 m at room temperature in PBS with 1% goat serum. Subsequently, 3ʹ-diaminobenzidine substrate (Dako, #K3468) was applied for 4 m, and the sections were counterstained with filtered hematoxylin (Sigma-Aldrich #HHS32), washed, dehydrated, and cleared in xylene for 10 m before mounting using xylene-based media (Pertex, Histolab Products #PRC/R/750). As controls for staining on normal liver tissue, we used: (1) the full protocol, 2) PBS substituting the primary antibody, (3) rabbit Ig (Lampire #7406404) at 5 µg/mL substituting the primary antibody and (4) primary antibody pre-incubated with purified ITIH4 at 20 µg/mL to inhibit specific binding to ITIH4.

**Supplementary results**

*Development of a specific assay for detection and quantification of ITIH4*

To validate the specificity of the assay, EDTA plasma from a healthy donor was fractionated by size-exclusion chromatography, and fractions were tested in the assay. ITIH4 eluted as a well-defined peak (Supplemental Fig. 1B). Importantly, the fractions spanning the ITIH4 peak were confirmed to contain ITIH4 by western blot analysis using different anti-ITIH4 antibodies than those used in the immunoassay. The concentration of ITIH4 in a plasma pool was estimated by comparing dilutions of plasma with known concentrations of purified ITIH4 (Supplemental Fig. 1C). The limit of detection determined at two standard deviations above background corresponded to 2 ng/mL, which translates to a limit of detection of 20 µg/mL at the 10,000-fold dilution of samples used in the assay. The limit of linearity extracted from the assay standard curve was set at 400 µg/mL. Three internal controls (K1–K3) were used for inter-assay variability. The coefficient of variation was 7.4%, 5.5%, and 3.2% for K1, K2, and K3, respectively, based on calculations from ten consecutive assays. By western blotting analysis of plasma from a PBC patient with high ITIH4 and a PBC patient with low ITIH4 and a healthy donor with low ITIH4, we confirmed the difference seen in the immunoassay (Supplemental Fig. 2). ITIH4 was found as a 120 kDa protein in all samples, indicating that the difference in ITIH4 levels was not caused by, e.g. cleavage of ITIH4.

**Reference**

30. EASL Clinical Practice Guidelines: management of hepatitis C virus infection. J Hepatol 2014;60(2):392-420. doi: 10.1016/j.jhep.2013.11.003. PubMed PMID: 24331294