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Original Article

Highly Increased Levels of Inter-α-inhibitor Heavy Chain 4 (ITIH4) in Autoimmune Cholestatic Liver Diseases

Tea Lund Laursen[1*], Lars Bossen[1], Rasmus Pihl[2], Anne Troldborg[2,3,4], Thomas Damgaard Sandahl[1], Annette Gudmann Hansen[2], Trine Folserass[5], Mette Vesterhus[5,6,7], Henning Grønbæk[1] and Steffen Thiel[2]

[1]Department of Hepatology and Gastroenterology, Aarhus University Hospital, Aarhus, Denmark; [2]Department of Biomedicine, Aarhus University, Aarhus, Denmark; [3]Department of Rheumatology, Aarhus University Hospital, Aarhus, Denmark; [4]Department of Clinical Medicine, Aarhus University, Aarhus, Denmark; [5]Norwegian PSC Research Center, Department of Transplantation Medicine, Division of Surgery, Inflammatory Diseases and Transplantation, Oslo University Hospital Rikshospitalet, Oslo, Norway; [6]Haraldsplass Deaconess Hospital, Bergen, Norway; [7]Department of Clinical Science, University of Bergen, Bergen, Norway

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Abstract

Background and Aims: There is an unmet need for new biomarkers to improve diagnostics and prognostics in primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC). Inter-α-inhibitor heavy chain 4 (ITIH4) is an abundant, liver-produced protein, and its synthesis may be altered in liver diseases. We investigated whether ITIH4 plasma concentrations were affected in PBC and PSC patients. ***Methods:*** We developed an immunoassay specific for ITIH4 and determined ITIH4 plasma concentrations in 66 PBC, 126 PSC, 92 autoimmune hepatitis (AIH), 67 chronic hepatitis C (CHC), 33 alcoholic hepatitis (AH) patients and 138 healthy controls (HCs). Hepatic ITIH4 expression was investigated by immunohistochemistry in PBC. ***Results:*** The mean plasma concentration of ITIH4 was almost doubled in PBC [409 µg/mL (95% CI: 388–431)] and 35% higher in PSC [308 µg/mL, (95% CI: 296–319)] compared with HCs [226 µg/mL (95% CI: 221–231); $p<0.001$]. In PBC patients, ITIH4 correlated with IgM (rho=0.49, $p<0.001$). Responders to ursodeoxycholic acid treatment (UDCA) had lower levels of ITIH4 than incomplete responders [395 µg/mL (95% CI: 364–425)] vs. 460 µg/mL (95% CI: 421–498); $p=0.02$]. Four weeks of UDCA treatment had no effect ($p=0.19$). Increased ITIH4 immunohistochemical staining was seen in a liver biopsy from a PBC patient. ITIH4 levels in AIH [224 µg/mL (95% CI: 208–241)] and HCs were similar ($p=0.8$). ITIH4 levels were lower in AH [199 µg/mL (95% CI: 175–223)] and CHC [202 µg/mL (192–212)] patients than in HCs ($p<0.05$). ***Conclusions:*** The plasma concentration of ITIH4 was highly elevated in patients with PBC and PSC, suggesting that ITIH4 should be further investigated as a biomarker in cholestatic liver disease.

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Introduction

Primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) are cholestatic, chronic inflammatory liver diseases that progress to liver fibrosis and ultimately cirrhosis and liver failure.[1,2] Treatment with ursodeoxycholic acid (UDCA) slows PBC progression. There is no approved treatment available for PSC, and liver transplantation remains the only curative option for both diseases.[3,4] Therefore, new biomarkers are highly warranted to improve the accurate diagnosis and precise prognostication of these patients.[5,6]

Inter-α-inhibitor heavy chain 4 (ITIH4) is a liver-produced plasma protein that belongs to a family of proteins called the inter-α-inhibitor/ITIH family.[7] The biological function of ITIH4 has long remained unknown. However, we recently showed that ITIH4 is a broadly acting protease inhibitor that exploits a novel inhibitory mechanism.[8] Interestingly, ITIH4 is highly expressed during early liver development and is suggested to play an essential role in liver formation and regeneration.[9] Moreover, ITIH4 is an abundant plasma protein with reported concentrations ranging from 80–300 µg/mL.[10–12] Serum concentrations as low as the pg/mL range have also been reported.[13] ITIH4 has attracted attention as a promising biomarker in several diseases, and plasma levels of ITIH4 correlate with liver fibrosis in hepatitis C patients (HCV). However, the nature of this relationship is ambiguous since both increased and decreased ITIH4 concentrations have been observed to correlate with fibrosis.[13,14] Moreover, ITIH4 decreases during the progression of hepatocellular carcinoma (HCC).[15]

Despite ITIH4 being a liver-produced protein that circu-

Keywords: Primary biliary cholangitis; Primary sclerosing cholangitis; Autoimmune hepatitis; Ursodeoxycholic acid treatment; Alcoholic hepatitis; Chronic viral hepatitis.

Abbreviations: AH, alcoholic hepatitis; AIH, autoimmune hepatitis; ALP, alkaline phosphatase; ALT, alanine aminotransferase; BMI, body mass index; CHC, chronic hepatitis C; Hgb, hemoglobin; HC, healthy control; ITIH4, Inter-α-inhibitor heavy chain 4; INR, international normalized ratio; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; sCD163, soluble CD163; UDCA, ursodeoxycholic acid; WBC, white blood cell count.

[*]**Correspondence to:** Tea Lund Laursen, Department of Hepatology and Gastroenterology, Aarhus University Hospital, 99 Palle Juul-Jensens Boulevard, DK-8200 Aarhus N, Denmark. ORCID: https://orcid.org/0000-0003-2494-0526. Tel: +45-23715703, Fax: +45-78462860, E-mail: tealaurs@rm.dk, tealaurs@clin.au.dk

lates at high concentrations, the ITIH4 levels in liver diseases and the association with liver disease severity remains elusive. Here, we have established a novel specific immunoassay for quantitative measurement of ITIH4 and examined the plasma concentrations of ITIH4 in patients with cholestatic liver diseases, i.e. PBC and PSC. The levels were further compared to ITIH4 concentrations in other chronic inflammatory liver diseases, i.e. autoimmune hepatitis (AIH), chronic viral hepatitis C (CHC), and alcoholic hepatitis (AH). A group of healthy individuals was included for reference (HC). We also investigated the early effects of pharmacological treatment with UDCA in newly diagnosed PBC patients for changes in plasma concentration of ITIH4 before and after treatment.

Methods

Patients included in the project

We assessed five groups of patients with different chronic inflammatory liver diseases included in studies at the Department of Hepatology and Gastroenterology, Aarhus, Denmark, or the Norwegian PSC Research Center, Oslo University Hospital Rikshospitalet, Oslo, Norway.[16–18] For inclusion and exclusion criteria, we refer to the original studies and the supplementary material (Supplementary Table 1). We obtained baseline samples from all five patient groups and follow-up samples from PBC patients treated with UDCA. All patients and controls signed informed consent forms before inclusion in the studies. All studies complied with the Helsinki declaration. The Danish studies were approved by the local ethical review board and the Danish Data Protection Agency in the Central Denmark Region before study initiation. The Norwegian cohort was approved by the regional committee for research ethics in Southeastern Norway.

Samples from PBC patients

Patients with PBC were included from 2016–2017 and consisted of two groups of patients; one group with prevalent PBC patients (n=50, of whom 47 were treated with UDCA, one of whom also received obeticholic acid), and one group with incident PBC (n=16) with blood samples obtained before initiation of UDCA treatment and a repeat sample after 4 weeks of treatment. We considered patients as having cirrhosis if one of the following criteria were fulfilled: (1) they had cirrhosis on a previous liver biopsy, (2) they had a history of variceal bleeding or ascites, (3) they had liver stiffness >16.9 kPa.[19] This applied to eight patients, i.e. leaving 58 without cirrhosis. We considered patients with alkaline phosphatase (ALP) levels of >170 I/U after at least 1 year of UDCA treatment as incomplete responders summing up to 12 patients, leaving 32 as responders, while three patients had not been treated for an entire year, and therefore were excluded from analyses regarding treatment. Of the 66 PBC patients, 18 were antimitochondrial antibody (AMA)-negative at diagnosis, and five of them were newly diagnosed, i.e. 48 (73%) were AMA-positive in the entire group, and 11 (69%) were AMA-positive in the group of newly diagnosed PBC patients. None of the patients had AIH overlap disease.

Samples from PSC patients

Patients with large-duct PSC were included between 2008 and 2012, and comprised 126 patients who were sampled once.[16] Twenty-nine PSC patients had an enhanced liver fibrosis (ELF) score >11.3 and were considered as having cirrhosis.[20] None of the patients had AIH overlap disease.

Samples from control groups

Patients with AIH were included between January 2011 and January 2016 and comprised 92 patients with one sample each.[17] Thirty-four AIH patients had cirrhosis in liver biopsies. Twelve AIH patients were AMA-positive at inclusion. None of the patients had PBC or PSC overlap disease. Patients with CHC (n=67) were included between February 2015 and February 2017 in connection with a study examining the liver-related effects of direct-acting antiviral therapy on CHC infection.[18] Patients with AH were included between March 2013 and December 2017 and comprised 33 patients with a sample from the day of the AH diagnosis.[21] Samples were further obtained from 138 healthy blood donors with no signs of liver disease included at Aarhus University Hospital,[22] hereafter referred to as healthy controls (HCs).

Biochemical data

Standard blood parameters including alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, alkaline phosphatase (ALP), albumin, white blood cell count, hemoglobin, international normalized ratio (INR), prothrombin time, and IgM were analyzed using standard validated assays at the respective sites. Soluble (s)CD163 was analyzed as previously described.[23]

Liver disease severity

Liver stiffness was assessed using transient elastography in the PBC patients. When liver biopsies were available, they were used to assess liver inflammation and the stage of liver fibrosis. Liver disease severity was evaluated by the Child-Pugh score[24] and the model of end-stage liver disease (MELD).[25] The Mayo score was also calculated for the PSC patients.[26]

Immunoassay for ITIH4 in plasma

We developed a novel sandwich-type immunoassay for the determination of ITIH4 in plasma. The assay relies on specific polyclonal rabbit anti-ITIH4 antibodies for coating microtiter wells, followed by incubation with dilutions of samples and subsequent detection of bound ITIH4 with a labeled anti-ITIH4 antibody. For specific details, see Supplementary File 1.

Application of the immunoassay for ITIH4

In 138 HCs, we measured ITIH4 in paired serum and EDTA plasma samples from the donors. In a subgroup, we also measured the ITIH4 levels in paired citrate and heparin samples. All samples from the same donor were collected at the same time. Moreover, the diurnal variation of ITIH4 was tested by measuring the ITIH4 concentration in EDTA plasma drawn with 4 h intervals from six healthy blood donors. We also investigated ITIH4 levels in normal human serum and EDTA plasma after up to nine freeze (at −80°C)/thaw to room temperature cycles.

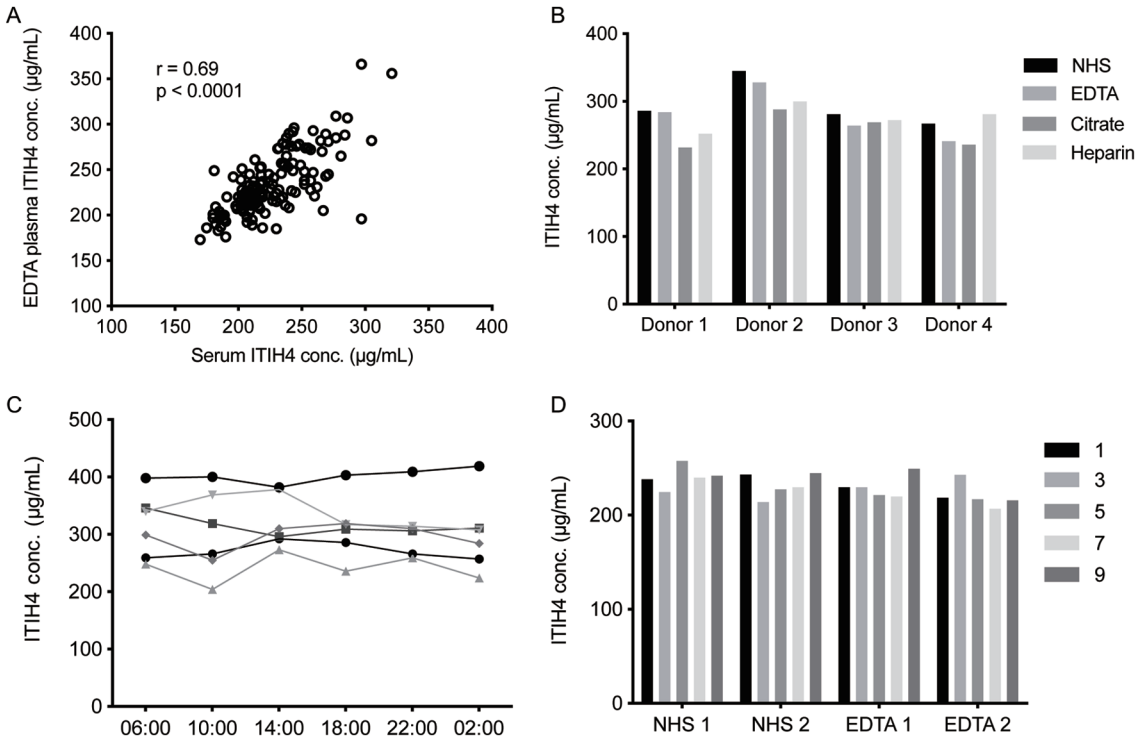

Fig. 1. Basic biomarker characteristics of Inter-α-inhibitor Heavy Chain 4 (ITIH4). (A) Correlation of ITIH4 concentrations in paired serum and EDTA plasma samples. Correlation was examined using a two-tailed Spearman test, yielding a rho value of 0.69 with a 95% CI of 0.59–0.77. (B) ITIH4 levels in different types of blood samples, i.e. serum and EDTA, citrate and heparin plasma, taken consecutively from four individuals. (C) Diurnal variation of ITIH4. EDTA plasma was drawn from six healthy donors with 4 h intervals, and the ITIH4 levels had no significant changes by repeated measures one-way analysis of variance (ANOVA) (p=0.49). (D) ITIH4 level was not affected by freeze-thaw cycles. Two serum samples and two EDTA plasma samples were subjected to repeated freeze-thaw cycles. The number of cycles is given in the figure to the right. ITIH4 levels were found to be stable by repeated measures one-way ANOVA (p=0.56).

Western blotting

We performed western blotting assays to examine the size and potential fragmentation of ITIH4 in the samples. Additionally, western blots were used to validate the ITIH4 levels determined by the immunoassays by analyzing plasma from a subject 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). For details, see Supplementary File 1.

Immunohistochemistry for ITIH4

Formalin-fixed paraffin-embedded liver biopsy sections from controls and a patient with PBC were examined for the presence of ITIH4 with the use of specific anti-ITIH4 antibody. For details, see Supplementary File 1.

Statistics

One-way analysis of variance was performed to assess differences among more than two groups, and student *t*-tests were used to compare two groups on log-transformed normally distributed data. For non-normally distributed data, the Wilcoxon rank-sum test was used. Paired data were analyzed using paired *t*-test or the Wilcoxon sign-rank test depending on the distribution. For changes over time, repeated measurement analyses of variance with mixed models were applied. For binary data, χ^2 or Fisher's exact tests were used depending on the sample size. We used Spearman's rank correlation analysis to investigate correlations between ITIH4 and relevant biochemical and clinical parameters as well as pertinent scores of the different liver diseases. The data were reported means with 95% confidence intervals (CIs) unless otherwise stated. Stata 14.2 (StataCorp, College Station, TX, USA) was used for the statistical analysis, and figures were drawn with GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA).

Results

Validation of the ITIH4 assay including reference interval, serum-plasma correlations, freeze-thaw cycles, and diurnal variation

To measure the concentration of ITIH4, we developed an immunoassay with the use of anti-ITIH4 antibodies generated following recombinant human ITIH4 for immunizations. The assay was found to be ITIH4-specific and highly sensitive (Supplementary Fig. 1). We measured the concentration of ITIH4 in paired EDTA plasma and serum samples from 138 healthy donors to establish a reference interval for ITIH4 in such samples. The mean levels of ITIH4 were 234 µg/mL (95% CI: 228–240) in serum (NHS) and 226 µg/mL (95% CI: 221–231) in EDTA plasma. To investigate basic characteristics of ITIH4 that are important for its potential use as a biomarker, we initially correlated the ITIH4 levels in NHS and EDTA plasma. We found a strong positive correlation (Spearman's rho=0.69, p <0.001; Fig. 1A).

Table 1. Baseline patient characteristics

	PBC		PSC	AIH	AH	CHC
	Prevalent	**Incident**				
Gender, F/M	47/3 (94/6)	12/4 (75/25)	31/95 (25/75)	65/27 (71/29)	14/19 (42/58)	25/42 (37/63)
Age, years	65 (60–68)	57 (52–66)	41 (38–45)	55 (47–59)	56 (51–58)	55 (53–58)
BMI, kg/m^2	24 (23–26)	28 (24–31)	–	–	27 (23–29)	25 (24–27)
ALT, IU/L	32 (27–39)	64 (36–109)	87 (74–112)	33 (29–37)	–	80 (60–110)
Bilirubin, µmol/L	8 (7–9)	10 (6–11)	20 (14–26)	8 (8–10)	–	8 (8–10)
ALP, IU/L	150 (130–166)	277 (222–399)	235 (208–273)	71 (63–85)	187 (148–264)	103 (87–112)
Albumin, g/L	37 (36–38)	38 (34–39)	41 (40–42)	–	22 (19–24)	36 (35–37)
Creatinine, µmol/L	65 (63–73)	66 (56–86)	65 (62–68)	69 (65–73)	70 (62–81)	71 (63–74)
Hgb, mmol/L	8.4 (8.2–8.6)	8.6 (8.0–9.5)	–	–	6.4 (5.6–7.0)	8.9 (8.8–9.2)
WBC, $\times10^9$/L	6.4 (6.0–6.8)	7.2 (5.3–8.1)	5.9 (5.6–6.5)	6.3 (5.5–6.9)	10.7 (8.7–13.9)	6.2 (5.5–6.6)
Platelets, $\times10^9$/L	254 (232–272)	271 (219–371)	290 (267–321)	213 (206–240)	140 (102–184)	137 (122–169)
PP - II, IV, X	1.1 (1.0–1.2)	1.1 (0.9–1.2)	–	–	0.9 (0.8–1.0)	0.8 (0.7–0.8)
INR	1 (1–1)	1 (1–1.1)	1 (1–1)	–	–	1.2 (1.1–1.2)
sCD163, mg/L	3.5 (3.0–4.2)	3.2 (2.6–5.5)	3.3 (3.0–3.9)	–	–	7.4 (5.9–7.9)
IgM, g/L	2.8 (2.1–3.1)	2.7 (1.3–4.0)	–	–	–	
IgG, g/L	11.9 (10.5–13.4)	13.4 (11.3–15.9)	–	12.2 (10.1–13.4)	–	–

Data are numbers (%) or medians (95% CI). AH, alcoholic hepatitis; AIH, autoimmune hepatitis; ALP, alkaline phosphatase; ALT, alanine aminotransferase; BMI, body mass index; CHC, chronic hepatitis C; Hgb, hemoglobin; INR, international normalized ratio; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; sCD163, soluble CD163.; WBC, white blood cell count.

In four donors, we measured ITIH4 in NHS, EDTA, citrate, and heparin plasma without observing any significant differences in such samples (Fig. 1B). We also measured the ITIH4 concentration in samples drawn during 24 h with 4-h intervals from six healthy donors, and ITIH4 displayed insignificant diurnal variation (p=0.49; Fig. 1C). To examine the stability of ITIH4 as a biomarker in samples submitted to cycles of freezing and thawing, we subjected two serum samples and two EDTA plasma samples from healthy donors to nine freeze-thaw cycles. The concentration of ITIH4 in these samples was stable and was not impacted by the freeze-thaw treatment (p=0.56; Fig. 1D). Thus, ITIH4 was an abundant plasma protein with promising characteristics as a biomarker, as the concentration was not affected by the sample type, timing of the blood draw, or freeze-thaw cycles. We found that the ITIH4 levels were similar in men and women, and it did not correlate with age in any patient group or in the healthy control group (data not shown).

Patient characteristics

The baseline patient characteristics are presented in Table 1. In general, patients had age and sex distributions, and biochemical characteristics as expected by their primary liver disease diagnosis.

ITIH4 levels in patients with PBC

The highest plasma ITIH4 concentrations were found in patients with PBC [409 µg/mL, 95% CI: (388–431)], which was nearly twice as high as that in HCs [226 µg/mL (95% CI: 221–231), p <0.001] and approximately 30% higher than that in PSC patients [308 (95% CI: (296–319), p <0.001; Fig. 2]. The increased concentrations measured by the immunoassay were validated by western blot analysis (Supplementary Fig. 2). In the PBC patients, the ITIH4 level tended to be lower in AMA-negative [381 µg/mL (95%

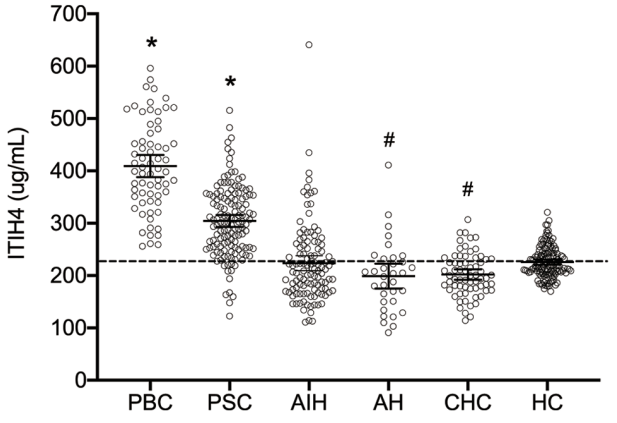

Fig. 2. Baseline Inter-α-Inhibitor Heavy Chain 4 (ITIH4) levels in the five groups of liver disease patients. Primary biliary cholangitis (PBC, n=66), primary sclerosing cholangitis (PSC, n=126), autoimmune hepatitis (AIH, n=92), alcoholic hepatitis (AH, n=33), and chronic hepatitis C, (CHC, n=67). The dashed line represents the mean (226 µg/mL) of the healthy controls (HCs, n=138). For each group, the line is at the mean, and whiskers represent the 95% CI. *Higher than HC, p<0.001. #Lower than HC, p <0.003.

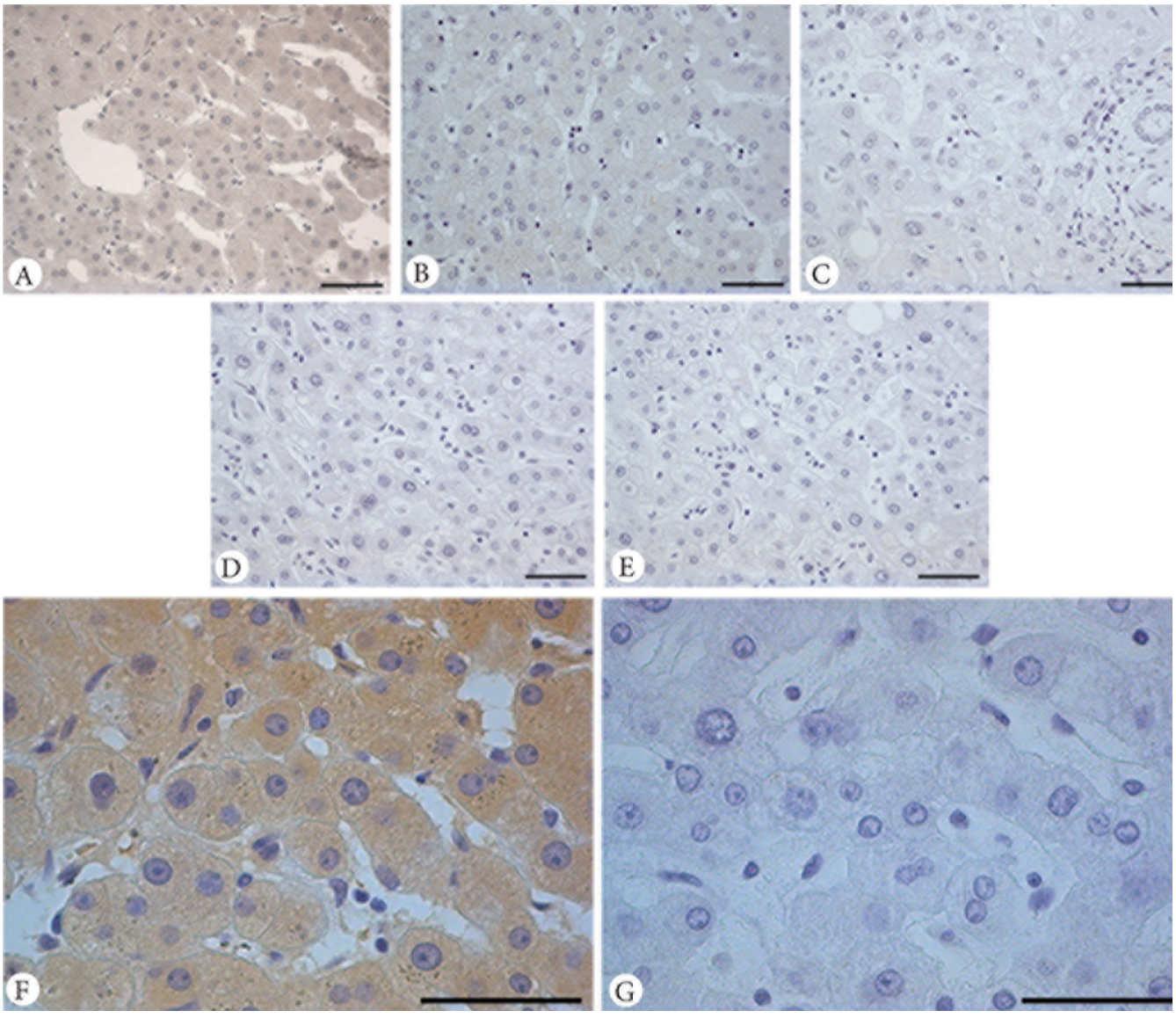

Fig. 3. Immunohistochemical staining of liver tissue. (A) Liver tissue from a patient with primary biliary cholangitis (PBC), magnification ×20. (B) Normal liver, magnification ×20. (C) Normal liver tissue stained after the protocol where the primary antibody is substituted by phosphate buffered saline, magnification ×20. (D) Normal liver tissue stained after the protocol with normal rabbit Ig substituting the primary antibody, magnification ×20. (E) Normal liver tissue stained after the protocol, but with the primary antibody preincubated with recombinant Inter-α-Inhibitor Heavy Chain 4 (ITIH4) to block the specific binding of the primary antibody, magnification ×20. (F) Liver tissue from a patient with PBC, magnification ×40. (G) Normal liver tissue stained after the protocol where the primary antibody is substituted by phosphate buffered saline, magnification ×40. Bars indicate 50 μm.

CI: 333–428)] than in AMA-positive (420 μg/mL (95% CI: 396–443)] patients (p=0.10); Supplementary Table 2], but the mean ITIH4 level in AMA-negative PBC patients was still higher than the mean ITIH4 level in AIH patients and in PSC patients (both p <0.001). There was an overlap of ITIH4 levels in PBC, PSC, and AIH patients, but uniquely to PBC, no patients had ITIH4 levels below 250 μg/mL (minimum=256 μg/mL; Fig. 2).

In the PBC patients, ITIH4 correlated with IgM (Spearman's rho=0.49, p <0.001), but not with ALP, liver stiffness, or MELD score (Supplementary Fig. 3). There was no difference in ITIH4 levels between PBC patients with and without cirrhosis (p=0.83). In the prevalent PBC patients, the responders to ursodeoxycholic acid treatment (UDCA) had lower levels of ITIH4 than incomplete responders [395 μg/mL (95% CI: 364–425) vs. 460 μg/mL (95% CI: 421–498), p=0.02; Supplementary Table 2]. Incident PBC patients had lower mean ITIH4 levels than prevalent PBC patients [367 μg/mL (95% CI: 323–412) vs. 423 μg/mL (95% CI: 399–446), p=0.02] and showed no significant change in ITIH4 levels after 4 weeks of UDCA treatment (367 μg/mL (95% CI: 323–412) vs. 402 μg/mL (95% CI: 354–450), p=0.19] despite a significant reduction in ALP (p<0.001) from 290 U/L (95% CI: 226–355) to 200 μg/mL (95% CI: 164–237). A liver biopsy from a patient with PBC was available for comparison with healthy liver tissue by immunohistochemistry for ITIH4. Reflecting the higher plasma levels of ITIH4 in PBC patients, the staining for ITIH4 was much more prominent in the biopsy from the PBC patient (Fig. 3).

ITIH4 levels in patients with PSC

The mean level of ITIH4 in PSC patients [308 µg/mL (95% CI: 296–319)] was 35% higher than in the HCs ($p<0.001$; Fig. 2). In PSC patients, ITIH4 correlated with ALP (Spearman's rho=0.29, $p<0.001$) and platelets (Spearman's rho=0.25, $p=0.01$) but not with Mayo score ($p=0.12$), disease duration ($p=0.19$) or cirrhosis status ($p=0.61$).

ITIH4 levels in patients with other liver diseases

AIH patients had a mean ITIH4 level of 224 µg/mL (95% CI: 208–241), which was similar to that in the HCs ($p=0.8$; Fig. 2). Disease duration did not influence ITIH4 levels in AIH patients ($p=0.06$), neither did corticosteroid therapy ($p=0.58$) or the presence of fibrosis or cirrhosis ($p>0.40$). In contrast to the cholestatic liver diseases, the mean ITIH4 levels in the CHC and AH patients were slightly lower than in the HCs [226 µg/mL (95% CI: 221–231)], CHC [202 µg/mL (95% CI: 192–212), $p<0.001$], and AH [199 µg/mL (95% CI: 175–223), $p=0.002$] patients.

Discussion

In this study, we systematically investigated ITIH4 levels in patients with liver diseases of different etiology by a new in-house assay specific for ITIH4. The main finding was highly elevated levels of ITIH4 in patients with autoimmune cholestatic liver diseases, particularly in PBC patients. ITIH4 levels were elevated in both AMA-positive and negative patients, and the levels correlated with IgM levels. Further, in UDCA-treated PBC patients, the ITIH4 levels were lower in responders compared with incomplete responders. There was no effect of short-term 4-week UDCA treatment in newly diagnosed PBC patients despite significant reductions in ALP.

There is an unmet need for new biomarkers of disease severity, progression, and prognosis in PBC and PSC. We developed a new, simple sandwich-type immunoassay specific for ITIH4. The assay had robust assay characteristics with similar ITIH4 concentrations measured in both serum and plasma (EDTA, citrate, and heparin) samples. We observed minimal diurnal fluctuations, and ITIH4 levels were stable during freeze/thaw cycles. Thus, the ITIH4 assay fulfills all requirements for an assay to be further investigated in the clinical setting of liver diseases.

In this study, we showed for the first time that ITIH4 was highly increased in the autoimmune cholestatic liver diseases PBC and PSC compared with HCs and other liver diseases. ITIH4 has previously been described as an acute phase reactant,[12] and elevated ITIH4 levels in PBC could be interpreted as an inflammation marker. However, ITIH4 was not associated with general chronic liver inflammation, as we observed similar or even lower levels in patients with AIH, AH, and CHC compared with the HC. Thus, the high ITIH4 levels observed in PBC and PSC are probably not due to inflammation of the liver *per se*, which is interesting, especially for PBC, as it is often characterized as a chronic inflammatory liver disease. These findings further increase the applicability of ITIH4 as a biomarker in PBC, as other potential markers may be influenced by inflammation.

If inflammation does not explain the elevated levels of ITIH4 observed in PBC and PSC, a different immunological mechanism may be at play. We observed a significant correlation between concentrations of IgM and ITIH4 in the PBC patients. Increased IgM is part of the autoimmune response in PBC, and elevated levels are suggested to be caused by long-term bacterial exposure,[27] which is supported by a study of PBC patients with decreased DNA methylation of the CD40L promoter, generally associated with hyper-IgM-syndrome.[28] IgM is a potent activator of the complement system through the classical pathway. Although activation of the classical complement pathway is not inhibited directly by ITIH4,[8] extensive complement activation can activate the kallikrein/bradykinin system,[29] of which ITIH4 is a significant inhibitor.[8] Such a mechanism may indicate a potential link between the elevated IgM and ITIH4 in the PBC patients. Future studies could advantageously include investigations of the ability of ITIH4 to influence the activities of enzymes that may be involved in cholestatic liver diseases, e.g. enzymes produced by the liver in such conditions.

ITIH4 was not strongly associated with the presence of fibrosis or cirrhosis, and there was only a nonsignificant inverse correlation between levels of ITIH4 and MELD score in PBC patients. This emphasizes that differences in disease severity do not explain the higher ITIH4 levels in prevalent PBC patients compared with incident PBC patients. In general, ITIH4 did not correlate well with disease severity as measured by clinical or biochemical parameters in any of the examined diseases, suggesting that ITIH4 is likely not an integral contributor to liver pathology. CHC patients were the exception to this, as ITIH4 correlated with platelet count and correlated inversely with liver stiffness and bilirubin. The lack of a clear, universal correlation between ITIH4 levels and disease severity observed here corroborates the conflicting observations regarding ITIH4 concentrations and fibrosis.[13,14] The results warrant studies with longer follow-up, and including other treatments, to better characterize the potential of ITIH4 as a prognostic or severity-specific marker.

UDCA is the primary drug of choice for treatment of PBC. We observed lower ITIH4 levels in the prevalent PBC patients who were responders to UDCA compared to incomplete responders. In incident PBC, ITIH4 levels were high but not as high as in the prevalent PBC patients where the majority were treated with UDCA (94%); and there was no significant effect of 4-week short-term UDCA treatment. To further investigate if UDCA treatment has significant effects on ITIH4 levels more extensive studies of both prevalent and incident PBC patients before and after UDCA treatment and with long-term follow-up in larger cohorts are needed. However, our data suggest that ITIH4 may be used as a biomarker to define a patient subset that does not benefit from UDCA treatment.

A major strength of this study is the high number of PBC and PSC patients included. Further, relevant disease controls were included across several different liver diseases representing potential differential diagnoses to our examined population. The study does not answer the question of a function for ITIH4 in liver diseases. However, ITIH4 staining was much more prominent in the biopsy from the PBC patient. This finding indicates that using our protocol makes it possible to detect ITIH4 in liver tissue, thereby paving the road for further studies aimed at quantifying ITIH4 in liver tissue and detect specific cells to secrete ITIH4. In addition, the highly increased levels in PBC and PSC indicate a role in chronic cholestatic liver disease involving the biliary tract. The higher level of a broad-acting enzyme inhibitor, i.e. ITIH4, could result in less activity of enzymes needed for the homeostasis of the biliary tract function. A weakness of our study is the retrospective design including prospective patient cohorts collected for different purposes. Only a few of the PBC patients had follow-up data with two measurements of ITIH4. Consequently, we could not make definite conclusions regarding the value of monitoring ITIH4 in patients with PBC and PSC, why implementation of ITIH4 as a biomarker in cholestatic liver disease remains unresolved. As shown in Figure 2, there was considerable overlap between ITIH4 levels in the different cohorts, which could be a limitation if it is intended as a diagnostic marker. How-

ever, ITIH4 has potential as a marker to rule out PBC, which could be highly relevant in a differential diagnostic setting, as none of the PBC patients had an ITIH4 value below 250 μg/mL.

In conclusion, we developed a robust immunoassay for ITIH4 detection and showed that ITIH4 levels were significantly elevated in patients with PBC and PSC, indicating a relation to chronic cholestatic liver disease. Further, in PBC patients, ITIH4 levels were lower in responders compared to incomplete responders suggesting that ITIH4 should be further investigated as a biomarker in cholestatic liver disease.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Designed the study (TLL, LB, RP, TDS, HG, ST), included the patients and collected the samples (TLL, LB, TDS, TF, MV), performed the experimental work (RP, AT, AGH), performed the data analysis (TLL and RP), interpreted the data (TLL, LB, RP, AT, TDS, HG, ST), and wrote the manuscript draft (TLL, LB, RP). All authors critically reviewed the manuscript and approved the final draft including the authorship list.

Ethical statement

All patients and controls signed informed consent forms before inclusion in the studies. All studies complied with the Helsinki declaration. The Danish studies were approved by the local ethical review board and the Danish Data Protection Agency in the Central Denmark Region before study initiation. The Norwegian cohort was approved by the regional committee for research ethics in Southeastern Norway.

Data sharing statement

The data used to support the findings of this study are available from the corresponding author at tealaurs@rm.dk upon request.

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