坂根貞嗣、榊原祐子、由雄敏之、中水流正一、外山隆、石田　永、三田英治	Telaprevir/Peg-IFN-α 2b/Ribavirin併用療法導入直後の腎機能低下機序に関する検討.	肝臓	53 (7)	434-5	2012
Takaguchi K, Moriwaki H, Doyama H, Iida M, Yagura M, Shimada N, Kang M, Yamada H, Kumada H.	Effects of branched-chain amino acid granules on serum albumin level and prognosis are dependent on treatment adherence in patients with liver cirrhosis.	Hepatol Res.			2012
正木尚彦.	肝炎をめぐる医療政策.	医学のあゆみ	240 (12)	997-9	2012
Miyagi Y, Nomura H, Yamashita N, Tanimoto H, Ito K, Masaki N, Mizokami M, Shibuya T.	Estimation of two real-time RT-PCR assays for quantitation of hepatitis C virus RNA during PEG-IFN plus ribavirin therapy by HCV genotypes and IL28B genotype.	J Infect Chemother.	19 (1)	63-9	2013
Saito H, Ito K, Sugiyama M, Matsui T, Aoki Y, Imamura M, Murata K, Masaki N, Nomura H, Adachi H, Hige S, Enomoto N, Sakamoto N, Kurosaki M, Mizokami M, Watanabe S.	Factors responsible for the discrepancy between IL28B polymorphism prediction and the viral response to peginterferon plus ribavirin therapy in Japanese chronic hepatitis C patients.	Hepatol Res.	42 (10)	958-65	2012
Ito K, Kuno A, Ikehara Y, Sugiyama M, Saito H, Aoki Y, Matsui T, Imamura M, Korenaga M, Murata K, Masaki N, Tanaka Y, Hige S, Izumi N, Kurosaki M, Nishiguchi S, Sakamoto M, Kage M, Narimatsu H, Mizokami M.	LecT-Hepa, a glyco-marker derived from multiple lectins, as a predictor of liver fibrosis in chronic hepatitis C patients.	Hepatology	56 (4)	1448-56	2012
Nomura H, Miyagi Y, Tanimoto H, Yamashita N, Ito K, Masaki N, Mizokami M.	Increase in platelet count based on inosine triphosphatase genotype during interferon beta plus ribavirin combination therapy.	J Gastroenterol Hepatol.	27 (9)	1461-6	2012
都築智之, 岩瀬弘明, 島田昌明, 平嶋　昇, 日比野祐介, 龍華庸光, 齋藤雅之, 玉置　大, 神谷麻子, 横井美咲, 横幕能行, 藤崎誠一郎, 杉浦　亙, 後藤秀実.	当院におけるHIV, HCV重複感染症例に対するペグインターフェロン, リバビリン併用療法の治療成績.	日本消化器病学会雑誌	109 (7)	1186-96	2012
杉　和洋.	クリティカルパスを活用した肝臓病チーム医療の実践.	Medical QOL		26-9	2013

Matsuzaki T, Ichikawa T, Kondo H, Taura N, Miyaaki H, Isomoto H, Takeshima F, Nakao K.	Prevalence of restless legs syndrome in Japanese patients with chronic liver disease.	Hepatol Res.	42 (12)	1221-6	2012
Miuma S, Ichikawa T, Arima K, Takeshita S, Muraoka T, Matsuzaki T, Ootani M, Shibata H, Akiyama M, Ozawa E, Miyaaki H, Taura N, Takeshima F, Nakao K.	Branched-chain amino acid deficiency stabilizes insulin-induced vascular endothelial growth factor mRNA in hepatocellular carcinoma cells.	J Cell Biochem.	113 (10)	3113-21	2012
Ichikawa T, Taura N, Miyaaki H, Matsuzaki T, Ohtani M, Eguchi S, Takatsuki M, Soyama A, Hidaka M, Okudaira S, Usui T, Mori S, Kamihira S, Kanematsu T, Nakao K.	Human T-cell leukemia virus type 1 infection worsens prognosis of hepatitis C virus-related living donor liver transplantation.	Transpl Int.	25 (4)	433-8	2012
Eguchi S, Hidaka M, Soyama A, Takatsuki M, Miyaaki H, Ichikawa T, Nakao K, Kanematsu T.	Is liver-targeted FOXp3 staining beneficial after living-donor liver transplantation?	Transpl Infect Dis.	14 (2)	156-62	2012
Yamaguchi T, Ichikawa T, Takeshita S, Taura N, Miyaaki H,Muraoka T, Shibata H, Honda T, Hamasaki K, Kato Y, Takeshima F, Nakao K.	Anti-hepatitis C virus activity of geranylgeranylacetone treatment in hepatitis C-infected patients.	Acta Medica Nagasakiensia.	57 (1)	1-4	2012
Takeshita S, Ichikawa T, Taura N, Miyaaki H, Matsuzaki T, Otani M, Muraoka T, Akiyama M, Miuma S, Ozawa E, Ikeda M, Kato N, Isomoto H, Takeshima F, Nakao K.	Geranylgeranylacetone has anti-hepatitis C virus activity via activation of mTOR in human hepatoma cells.	J Gastroenterol.	47 (2)	195-202	2012
Muraoka T, Ichikawa T, Taura N, Miyaaki H, Takeshita S, Akiyama M, Miuma S, Ozawa E, Isomoto H, Takeshima F, Nakao K.	Insulin-induced mTOR activity exhibits anti-hepatitis C virus activity.	Mol Med Report.	5 (2)	331-5	2012
Kamihira S, Usui T, Ichikawa T, Uno N, Morinaga Y, Mori S, Nagai K, Sasaki D, Hasegawa H, Yanagihara K, Honda T, Yamada Y, Iwanaga M, Kanematu T, Nakao K.	Paradoxical expression of IL-28B mRNA in peripheral blood in human T-cell leukemia virus type-1 mono-infection and co-infection with hepatitis C virus.	Virol J.	9		2012

Otani M, Honda N, Xia PC, Eguchi K, Ichikawa T, Watanabe T, Yamaguchi K, Nakao K, Yamamoto T.	Distribution of Two Subgroups of Human T-Lymphotropic Virus Type 1 (HTLV-1) in Endemic Japan.	Trop Med Health.	40 (2)	55-8	2012
Taura N, Fukuda S, Ichikawa T, Miyaaki H, Shibata H, Honda T, Yamaguchi T, Kubota Y, Uchida S, Kamo Y, Yoshimura E, Isomoto H, Matsumoto T, Takeshima F, Tsutsumi T, Tsuruta S, Nakao K.	Relationship of α-fetoprotein levels and development of hepatocellular carcinoma in hepatitis C patients with liver cirrhosis.	Exp Ther Med.	4 (6)	972-6	2012
Taura N, Ichikawa T, Miyaaki H, Kadokawa Y, Tsutsumi T, Tsuruta S, Kato Y, Inoue O, Kinoshita N, Ohba K, Kato H, Ohata K, Masuda J, Hamasaki K, Yatsuhashi H, Nakao K.	Baseline serum cholesterol is associated with a response to pegylated interferon alfa-2b and ribavirin therapy for chronic hepatitis C genotype 2.	Gastroenterol Res Pract.			2012
Kondo R, Yano H, Nakashima O, Tanikawa K, Nomura Y, Kage M.	Accumulation of platelets in the liver may be an important contributory factor to thrombocytopenia and liver fibrosis in chronic hepatitis C.	J Gastroenterol.			2012
Torimura T, Ueno T, Taniguchi E, Masuda H, Iwamoto H, Nakamura T, Inoue K, Hashimoto O, Abe M, Koga H, Barresi V, Nakashima E, Yano H, Sata M.	Interaction of endothelial progenitor cells expressing cytosine deaminase in tumor tissues and 5-fluorocytosine administration suppresses growth of 5-fluorouracil- sensitive liver cancer in mice.	Cancer Sci.	103 (3)	542-8	2012
Naito Y, Kusano H, Nakashima O, Sadashima E, Hattori S, Taira T, Kawahara A, Okabe Y, Shimamatsu K, Taguchi J, Momosaki S, Irie K, Yamaguchi R, Yokomizo H, Nagamine M, Fukuda S, Sugiyama S, Nishida N, Higaki K, Yoshitomi M, Yasunaga M, Okuda K, Kinoshita H, Nakayama M, Yasumoto M, Akiba J, Kage M, Yano H	Intraductal neoplasm of the intrahepatic bile duct: clinicopathological study of 24 cases.	World J Gastroenterol.	18 (28)	3673-80	2012
Komuta M, Govaere O, Vandecaveye V, Akiba J, Van Steenbergen W, Verslype C, Laleman W, Pirenne J, Aerts R, Yano H, Nevens F, Topal B, Roskams T.	Histological diversity in cholangiocellular carcinoma reflects the different cholangiocyte phenotypes.	Hepatology	55 (6)	1876-88	2012

Inoue K, Torimura T, Nakamura T, Iwamoto H, Masuda H, Abe M, Hashimoto O, Koga H, Ueno T, Yano H, Sata M.	Vandetanib, an inhibitor of VEGF receptor-2 and EGF receptor, suppresses tumor development and improves prognosis of liver cancer in mice.	Clin Cancer Res.	18 (14)	3924-33	2012
Abe M, Koga H, Yoshida T, Masuda H, Iwamoto H, Sakata M, Hanada S, Nakamura T, Taniguchi E, Kawaguchi T, Yano H, Torimura T, Ueno T, Sata M.	Hepatitis C virus core protein upregulates the expression of vascular endothelial growth factor via the nuclear factor-kappaB/hypoxia-inducible factor-1alpha axis under hypoxic conditions.	Hepatol Res.	42	591-600	2012
Tamada Y, Yatsuhashi H, Masaki N, Nakamuta M, Mita E, Komatsu T, Watanabe Y, Muro T, Shimada M, Hijioka T, Satoh T, Mano Y, Komeda T, Takahashi M, Kohno H, Ota H, Hayashi S, Miyakawa Y, Abiru S, Ishibashi H.	Hepatitis B virus strains of subgenotype A2 with an identical sequence spreading rapidly from the capital region to all over Japan in patients with acute hepatitis B.	Gut.			2012
Yatsuhashi H, Ohnishi Y, Nakayama S, Iwase H, Nakamura T, Imawari M.	Anti-hypoalbuminemic effect of branched-chain amino acid granules in patients with liver cirrhosis is independent of dietary energy and protein intake.	Hepatol Res.	41 (11)	1027-1035	2011
Kurokawa M, Hiramatsu N, Oze T, Yakushijin T, Miyazaki M, Hosui A, Miyagi T, Yoshida Y, Ishida H, Tatsumi T, Kiso S, Kanto T, Kasahara A, Iio S, Doi Y, Yamada A, Oshita M, Kaneko A, Mochizuki K, Hagiwara H, Mita E, Ito T, Inui Y, Katayama K, Yoshihara H, Imai Y, Hayashi E, Hayashi N, Takehara T.	Long-term effect of lamivudine treatment on the incidence of hepatocellular carcinoma in patients with hepatitis B virus infection.	J Gastroenterol.	47(5)	577-85	2012
Oze T, Hiramatsu N, Song C, Yakushijin T, Iio S, Doi Y, Oshita M, Hagiwara H, Mita E, Ito T, Inui Y, Hijioka T, Tamura S, Yoshihara H, Inoue A, Imai Y, Hayashi E, Kato M, Miyazaki M, Hosui A, Miyagi T, Yoshida Y, Tatsumi T, Kiso S, Kanto T, Kasahara A, Hayashi N, Takehara T.	Reducing Peg-IFN doses causes later virologic response or no response in HCV genotype 1 patients treated with Peg-IFN alfa-2b plus ribavirin.	J Gastroenterol.	47(3)	334-342	2012

Kanto T, Inoue M, Oze T, Miyazaki M, Sakakibara M, Kakita N, Matsubara T, Higashitani K, Hagiwara H, Iio S, Katayama K, Mita E, Kasahara A, Hiramatsu N, Takehara T, Hayashi N.	Dynamics of regulatory T cells and plasmacytoid dendritic cells as immune markers for virological response in pegylated interferon-α and ribavirin therapy for chronic hepatitis C patients.	J Gastroenterol.	47(2)	169-178	2012
Hiramatsu N, Inoue Y, Oze T, Kurashige N, Yakushijin T, Mochizuki K, Miyagi T, Tatsumi T, Kiso S, Kanto T, Kasahara A, Takehara T, Oshita M, Mita E, Hagiwara H, Inui Y, Katayama K, Tamura S, Yoshihara H, Imai Y, Hayashi N.	Efficacy of pegylated interferon plus ribavirin combination therapy for hepatitis C patients with normal ALT levels: a matched case-control study.	J Gastroenterol.	46	1335-1343	2011
Oze T, Hiramatsu N, Yakushijin T, Mochizuki K, Imanaka K, Yamada A, Oshita M, Kaneko A, Hagiwara H, Mita E, Ito T, Nagase T, Inui Y, Hijioka T, Tamura S, Yoshihara H, Hayashi E, Imai Y, Kato M, Hosui A, Miyagi T, Yoshida Y, Ishida H, Tatsumi T, Kiso S, Kanto T, Kasahara A, Takehara T, Hayashi N.	Efficacy of re-treatment with pegylated interferon plus ribavirin combination therapy for patients with chronic hepatitis C in Japan.	J Gastroenterol.	46	1031-1037	2011
Oze T, Hiramatsu N, Yakushijin T, Mochizuki K, Oshita M, Hagiwara H, Mita E, Ito T, Fukui H, Inui Y, Hijioka T, Inada M, Kaytayama K, Tamura S, Yoshihara H, Inoue A, Imai Y, Kato M, Miyagi T, Yoshida Y, Tatsumi T, Kiso S, Kanto T, Kasahara A, Takehara T, and Hayashi N.	Indications and limitations for aged patients with chronic hepatitis C in pegylated interferon alfa-2b plus ribavirin combination therapy.	J Hepatol.	54	604-611	2011
Inoue Y, Hiramatsu N, Oze T, Yakushijin T, Mochizuki K, Fukuda K, Mita E, Haruna Y, Inoue A, Imai Y, Hosui A, Miyagi T, Yoshida Y, Tatsumi T, Kiso S, Kanto T, Kasahara A, Takehara T, and Hayashi N.	Amino acid substitution in the core protein has no impact on relapse in hepatitis C genotype 1 patients treated with peginterferon and ribavirin.	J Med Virol.	83	419-427	2011

三田英治	C型肝炎 Hepatitis C among HIV-infected patients.	HIV感染症とAIDSの治療	2 (2)	37-42	2011
H. Kohno, H. Kouno, S. Aimitsu, Y. Aisaka, M. Kitamoto, H. Kawakami, K. Chayama	Impact of ribavirin dose reduction on the efficacy of pegylated interferon plus ribavirin combination therapy for elderly patients infected with genotype 1b and high viral loads.	Hepatol Res.	41	626-634	2011
平嶋 昇, 岩瀬弘明, 都築智之, 神谷麻子, 横井美咲, 斎藤雅之, 玉置 大, 龍華庸光, 日比野佑介, 島田昌明, 後藤秀実	前インターフェロン非治癒の1b高ウイルス量C型慢性肝炎に対する二重濾過血漿交換併用ペグインターフェロン・リバビリン療法(第2報).	肝臓	52 (9)	575-583	2011
平嶋 昇, 浦岡正尚, 神谷麻子, 横井美咲, 斎藤雅之, 玉置 大, 龍華庸光, 渡邉久倫, 日比野佑介, 島田昌明, 都築智之, 岩瀬弘明	発症2カ月で慢性肝炎の組織像を呈しインターフェロンで治癒したHBV Genotype A急性肝炎の1例.	医療	65 (8)	451-455	2011
Taura N, Ichikawa T, Miyaaki H, Yatsuhashi H, Ishibashi H, Nakao K	Prevalence of type 2 diabetes mellitus in Japanese patients with hepatocellular carcinoma.	Exp Ther Med.	2 (1)	81-84	2011
Taura N, Fukushima N, Yatsuhashi H, Takami Y, Seike M, Watanabe H, Mizuta T, Sasaki Y, Nagata K, Tabara A, Komorizono Y, Taketomi A, Matsumoto S, Tamai T, Muro T, Nakao K, Fukuizumi K, Maeshiro T, Inoue O, Sata M	The incidence of hepatocellular carcinoma associated with hepatitis C infection decreased in Kyushu area.	Med Sci Monit.	17 (2)	PH7-11	2011
Kawaguchi T, Kakuma T, Yatsuhashi H, Watanabe H, Saitsu H, Nakao K, Taketomi A, Ohta S, Tabaru A, Takenaka K, Mizuta T, Nagata K, Komorizono Y, Fukuizumi K, Seike M, Matsumoto S, Maeshiro T, Tsubouchi H, Muro T, Inoue O, Akahoshi M, Sata M	Data mining reveals complex interactions of risk factors and clinical feature profiling associated with the staging of non-hepatitis B virus/non-hepatitis C virus-related hepatocellular carcinoma.	Hepatol Res.	41 (6)	564-571	2011
Miyaaki H, Ichikawa T, Taura N, Miuma S, Shibata H, Isomoto H, Takeshima F, Nakao K	Predictive value of the fibrosis scores in patients with chronic hepatitis C associated with liver fibrosis and metabolic syndrome.	Intern Med.	50 (11)	1137-1141	2011

Hidaka M, Takatsuki M, Soyama A, Miyaaki H, Ichikawa T, Nakao K, Kanematsu T, Eguchi S	Living donor liver transplantation from a donor previously treated with interferon for hepatitis C virus: a case report.	J Med Case Reports.	5	276	2011
Ozawa E, Abiru S, Nagaoka S, Yano K, Komori A, Migita K, Yatsuhashi H, Taura N, Ichikawa T, Ishibashi H, Nakao K	Ferritin/alanine aminotransferase ratio as a possible marker for predicting the prognosis of acute liver injury.	J Gastroenterol Hepatol.	26 (8)	1326-1332	2011
Ichikawa T, Taura N, Miyaaki H, Matsuzaki M, Eguchi S, Takatsuki M, Kanematsu T, Nakao K	Successful pegylated interferon alpha2a monotherapy for hepatitis C virus infection in a transplanted patient who relapsed after the preceding course.	Transpl Infect Dis.	13 (4)	438-440	2011
Miyaaki H, Ichikawa T, Taura N, Miuma S, Kawaguchi M, Kohno R, Isomoto H, Takeshima F, Nakashima O, Nakao K	Imaging of focal nodular hyperplastic-like nodules in alcoholic liver cirrhosis patients using gadolinium-ethoxybenzyl-di ethylenetriamine pentaacetic acid magnetic resonance imaging.	Clin J Gastroenterol.	4 (4)	266-272	2011
Honda T, Miyaaki H, Ichikawa T, Taura N, Miuma S, Shibata H, Isomoto H, Takeshima F, Nakao K	Clinical characteristics of hepatocellular carcinoma in elderly patients.	Oncol Lett.	2 (5)	851-854	2011
Miyaaki H, Ichikawa T, Yatsuhashi H, Taura N, Miuma S, Usui T, Mori S, Kamihira S, Tanaka Y, Mizokami M, Nakao K	Suppressor of cytokine signal 3 and IL28 genetic variation predict the viral response to peginterferon and ribavirin.	Hepatol Res.	41 (12)	1216-1222	2011
Akiba J, Murakami Y, Noda M, Watari K, Ogasawara S, Yoshida T, Kawahara A, Sanada S, Yasumoto M, Yamaguchi R, Kage M, Kuwano M, Ono M, Yano H	N-myc downstream regulated gene1/Cap43 overexpression suppresses tumor growth by hepatic cancer cells through cell cycle arrest at the G0/G1 phase.	Cancer Lett.	310	25-34	2011
Iwamoto H, Torimura T, Nakamura T, Hashimoto O, Inoue K, Kurogi J, Niizeki T, Kuwahara R, Abe M, Koga H, Yano H, Kerbel RS, Ueno T, Sata M	Metronomic S-1 chemotherapy and vandetanib: an efficacious and nontoxic treatment for hepatocellular carcinoma.	Neoplasia	13 (3)	187-197	2011

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Association of *STAT4* Polymorphisms with Susceptibility to Type-1 Autoimmune Hepatitis in the Japanese Population

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Abstract

Background/Aims: Recent studies demonstrated an association of *STAT4* polymorphisms with autoimmune diseases including systemic lupus erythematosus and rheumatoid arthritis, indicating multiple autoimmune diseases share common susceptibility genes. We therefore investigated the influence of *STAT4* polymorphisms on the susceptibility and phenotype of type-1 autoimmune hepatitis in a Japanese National Hospital Organization (NHO) AIH multicenter cohort study.

Methodology/Principal Findings: Genomic DNA from 460 individuals of Japanese origin including 230 patients with type-1 autoimmune hepatitis and 230 healthy controls was analyzed for two single nucleotide polymorphisms in the *STAT4* gene (rs7574865, rs7582694). The *STAT4* rs7574865T allele conferred risk for type-1 autoimmune hepatitis (OR = 1.61, 95% CI = 1.23–2.11; $P = 0.001$), and patients without accompanying autoimmune diseases exhibited an association with the rs7574865T allele (OR = 1.50, 95%CI = 1.13–1.99; $P = 0.005$). Detailed genotype-phenotype analysis of type-1 autoimmune hepatitis patients with (n = 44) or without liver cirrhosis (n = 186) demonstrated that rs7574865 was not associated with the development of liver cirrhosis and phenotype (biochemical data and the presence of auto-antibodies).

Conclusions/Significance: This is the first study to show a positive association between a *STAT4* polymorphism and type-1 autoimmune hepatitis, suggesting that autoimmune hepatitis shares a gene commonly associated with risk for other autoimmune diseases.

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Introduction

Autoimmune hepatitis (AIH) is characterized by chronic inflammation of the liver, interface hepatitis, hypergammaglobulinemia and production of autoantibodies [1,2]. The etiology of AIH is unknown, but is thought to have both a genetic and an environmental basis [3]. Although the HLA DRB1 gene is a well-characterized susceptibility gene [4,5], non-HLA susceptibility genes may also contribute to genetic susceptibility to AIH and remain to be elucidated. Recently, with the emergence of genome-wide association studies (GWAS), there has been a dramatic increase in genetic discoveries for many complex genetic autoimmune diseases, such as type 1 diabetes and rheumatoid arthritis (RA) [6]. It is also interesting to note that evaluating the results from the study of one disease in other complex diseases can disclose common risk factors. Thus, there has been a marked overlap of loci between autoimmune diseases [7]. Of those, *STAT4* particularly has been confirmed in several studies and is clearly associated with autoimmune diseases such as RA or systemic lupus erythematosus (SLE) [8–10]. STAT4, a signal transducer and activator of transcription 4, is expressed in activated peripheral blood monocytes, dendritic cells and macrophages at the sites of inflammation in humans [11]. It is activated by interleukin (IL)-12, leading to T helper (Th) 1 and Th 17 differentiation, monocyte activation and interferon (IFN)-α production [12]. Since Th1 and Th17 cells have the capacity to cause autoimmunity [13], STAT4

may play a crucial role in the development of autoimmune diseases, including AIH.

The degree of risk for RA or SLE susceptibility observed with the *STAT4* haplotype was found to be similar in Caucasian and Japanese populations [14–16]. In addition, meta-analysis demonstrated that the *STAT4* rs7574865 T allele conferred susceptibility to various autoimmune diseases, suggesting an association between *STAT4* gene polymorphism and autoimmune diseases [17].

STAT4 is considered important in a mouse model of Th1-dependent liver injury [18]. Therefore, we hypothesized that *STAT4* polymorphisms may overlap in genetic susceptibility between AIH and other autoimmune diseases. To test this hypothesis, we investigated the association of *STAT4* with type-1 AIH susceptibility using a large series of Japan NHO-AIH registry [19]. We also tried to evaluate whether the gene was associated with type-1 AIH outcome measures in a Japanese AIH cohort.

Materials and Methods

Study population

Consecutive type-1 AIH patients were initially enrolled in the register of the Japanese National Hospital Organization (NHO) liver-network study, contributed to medical facilities in Japan, and prospectively followed since 2009 as a multicenter cohort population. All patients satisfied the 1999 revised criteria of International Autoimmune Hepatitis Group (IAIHG) diagnosis of type-1 AIH [20]. Patients were excluded from the study if there was histological evidence of cholangitis or non-alcoholic steatohepatitis. In addition, patients who were positive for hepatitis B virus (HBV)-surface antigen (HBsAg) or hepatitis C virus (HCV)-RNA were excluded. Patients with other causes of liver disease, such as excess alcohol or drug use, were excluded based on reviews of their appropriate history and investigations. The control group consisted of 230 gender-matched Japanese healthy subjects (34 men and 196 women). The mean ± SD age was 43.9±13.1 years. Among the cases (AIH) and controls, 156 patients and 163 controls were recruited from West Japan and 74 patients and 67 controls were recruited from East Japan. The study was approved by the Ethics committee of the Nagasaki Medical Center and participating NHO Liver-network hospitals ((NHO Sagamihara National Hospital, Tokyo National Hospital, Yokohama Medical Center, Nagoya Medical Center, Kure Medical Center, Osaka Minami Medical Center, Kyushu Medical Center, Minami Wakayama Medical Center, Shinshu Ueda Medical Center, Kanazawa Medical Center, Higashi Hiroshima Medical Center, Asahikawa Medical Center, Kokura Medical Center, Ureshino Medical Center, Higashi Nagoya National Hospital, Hokkaido Medical Center, Okayama Medical Center, Takasaki General Medical Center, Oita Medical Center, Beppu Medical Center, Osaka Medical Center, Kumamoto Medical Center, Nishigunma National Hospital). Written informed consent was obtained from each individual. This study was conducted with the approval of the ethical committees of Nagasaki Medical Center and participating NHO Liver-network hospitals. Written informed consent was obtained from each individual.

Variables at study entry

Demographic and other characteristics of the 230 retained patients were recorded in a database at the initial assessment. Data included sex, age at diagnosis, time of onset of symptoms or other evidence of liver disease, markers of infection with hepatitis viruses HBV and HCV, alcohol intake, coexisting autoimmune diseases, serum levels of ALT, AST, alkaline phosphatase and bilirubin, platelet count and prothrombin time. Anti-nuclear antibodies (ANA) and anti-smooth muscle antibodies (ASMA) were measured by indirect immunofluorescence on HEp-2 cells and cut-off titers for positivity were 1:40. Liver tissue from percutaneous biopsy performed at the referring facility was available for the majority of patients at the time of entry (192/230, 83.5%), but for only a few at the subsequent follow-up examination (7/230, 3.0%). The histological variables examined included degree of fibrosis (0; absent, 1; expansion of fibrosis to parenchyma, 2; portal-central or portal-portal bridging fibrosis, 3; presence of numerous fibrous septa, 4; multi-nodular cirrhosis). The histological diagnosis of cirrhosis required a loss of the normal lobular architecture, reconstruction of hepatic nodules and presence of regenerative nodules [21]. Liver biopsy was not performed for patients who had apparent biochemical, endoscopic and ultrasound features of liver cirrhosis. All phenotypic data were collected blind to the results of the genotypic data.

DNA extraction and genotyping

Blood samples were taken from all study participants, and genomic DNA was isolated from peripheral blood leukocytes using a DNA blood mini kit from Qiagen (Hilden, Germany) according to the manufacturer's guidelines. STAT4 SNPs (rs7574865, rs7582694) were determined by the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method [22,23]. The primers used for the PCR reaction were rs7574865, F:5′-AAAGAAGTGGGATAAAAAGAAGTTTG-3′, R:5′-CCACTGAAATAAGATAACCACTGT-3′, and rs7582694, F:5′-ATCCAACTCTTCTCAGCCCTT-3′, R:5′-TCATAATCAGGAGAGAGGAGT-3′.

Rs7574865 was a 147-bp PCR product and was digested with restriction enzyme *Hpa*I (New England Biolabs) and electrophoresed on a 2.5% polyacrylamide gel. Rs7574865 was a 338-bp PCR product was digested with restriction enzyme *Hpy*CH4III (New England Biolabs) and electrophoresed on a 3.0% polyacrylamide gel.

HLA-DRB1 genotyping was performed as described previously [24]. Briefly, the HLA-DRB1 genotype was determined by sequence-based typing (SBT) of group-specific PCR products.

Statistical analyses

Results are expressed as mean ± SD. The statistical significance of differences between groups was calculated by either the chi-square test or Fisher's exact test for categorical data and Mann-Whitney's *U*-test for quantitative data. Multivariate logistic regression analysis was performed with SPSS v.18 for windows (SPSS Statistics, Illinois). Deviation from Hardy-Weinberg equilibrium was assessed using the SNPAlyze software ver. 7.0 (Dynacom, Yokohama, Japan). Power calculations were performed by using an online power calculator [25]. A P value of <0.05 was considered significant.

Results

Baseline data at entry

Of the original 240 patients registered in the NHO-AIH study, 10 were excluded from analysis because of overlapping primary biliary cirrhosis (PBC). The remaining 230 patients were eligible for the study. Table 1 shows other demographic data for the cohort at entry. Among the enrolled type-1 AIH patients, 206 (89.6%) were positive for ANA (>1:40) and 96 (41.7%) for ASMA (>1:40). Some patients with lower serum aminotransferase or total bilirubin were managed with ursodeoxycholic acid (UDCA) therapy alone, which was demonstrated to be efficacious in Japanese patients with type I autoimmune hepatitis [26]. Among

Table 1. Baseline characteristics of type-1 AIH patients.

	n = 230
Gender (male/female)	23/207
Age at presentaion (years)	59.6±12.2
Other autoimune diseases	39(17.0%)
Baseline Laboratory Values	
AST (<40 IU/L)	432.5±444.1
ALT (<40 IU/L)	484.3±490.5
ALP (<112 IU/L)	463.5±210.3
Total Bilirubin (mg/ml)	3.83±6.14
Albumin (3.5–5.0 g/L)	3.85±0.67
IgG (870–1700 mg/dl)	2489.4±931.4
Platelets (15–40×10^4/μl)	18.6±7.1
ANA + (≥1:40)	206(89.6%)
ASMA + (≥1:40)	96(41.7%)
Cirrhosis at presentation	44(19.1%)
Received treatment	
Steroid alone	81(35.2%)
Steroid + UDCA	72(31.3%)
Steroid + Aza	15(6.5%)
UDCA alone	49(21.3%)

Abbreviations: AIH; autoimmune hepatitis, AST; aspartate aminotransferase, ALT; alanine aminotransferase, ALP; alkaline phosphate, IgG; immunoglobulin G, ANA; anti-nuclear antibody, ASMA; anti-smooth muscle antibody, UDCA; ursodeoxy cholic acid, Aza; azathioprine. Data are expressed as number (percentage) or mean ± standard deviations.
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230 eligible patients, 29 (12.6%) had liver cirrhosis at the time of diagnosis, and among the remaining 201 patients without liver cirrhosis, 15 developed liver cirrhosis during the follow-up. Two patients died because of complications (ruptured esophageal varices 1, hepatic failure 1) of liver cirrhosis during follow-up.

Association of *STAT4* polymorphisms with type-1 AIH

The genotype frequencies for *STAT4* rs7574865 and rs7582694 were in HWE (Hardy-Weinberg equilibrium) in both the patient and control populations (data not shown). Because of the strong linkage disequilibrium between rs7574865 and rs7582694 ($R^2 = 0.949$ and $D' = 0.981$), very similar results were observed between rs7574865 (Table 2) and rs7582694 (Table 3). We observed a significant difference in allele frequency and genotype distribution of *STAT4* polymorphisms (rs7574865) between type-1 AIH patients and controls. As shown in Table 2, the minor T allele and TT genotype frequencies at *STAT4* rs7574865 in the type-1 AIH group differed significantly from those in the control group.

To determine whether the observed association of the *STAT4* gene SNPs with disease susceptibility was caused by other autoimmune diseases associated with AIH, we stratified type-1 AIH patients without other overlapping autoimmune diseases. There was a significant association of *STAT4* rs7574865 with susceptibility to type-1 AIH even in the AIH patients without other overlapping autoimmune diseases (Table 4).

Associations between *STAT4* genotype status and type-1AIH phenotype

To examine the associations between HLA-DR and type-1 AIH, HLA-DR allele typing was performed in patients with type-1 AIH. In the analysis of HLA-DR alleles, the frequencies of DR *04 allele was significantly increased in type-1 AIH patients as compared with those in controls (Table 5). The STAT4 rs7574865 T allele and HLA-DR *04 allele for the progression to liver cirrhosis were subjected to multivariate logistic regression analysis. Neither HLA-DR *04 allele nor rs7574865 T allele did not contribute to the progression to liver cirrhosis (data not shown). Based on the significant association of the rs7574865 with susceptibility to type-1 AIH, we also performed a detailed genotype-phenotype analysis using the clinical data. However, we found no significant difference in the presence of autoantibodies (ANA or ASMA) and the peak levels of transaminases or total bilirubin (AST, ALT, TB) by laboratory tests among each genotype (data not shown).

Table 2. STAT4 rs7574865 polymorphism in patients with type-1 AIH and controls.

		Control (%)	AIH (%)	*p*-value[a]	OR (95%CI)
		n = 230	n = 230		
Genotype frequencies				0.001	
	G/G	103(44.8)	77(33.5)		
	G/T	108(47.0)	109(47.4)		
	T/T	19(8.3)	44(19.1)		
Allele				0.001	
	G	314(68.3)	263(57.2)		1
	T	146(31.7)	197(42.8)		1.611(1.230–2.109)

Abbreviation: AIH; autoimmune hepatitis, OR; odds ratio, CI; confidence interval, STAT4; signal transducer and activator or transcription.
[a]Genotype frequencies were determined by $\chi 2$ test using 2×3 contingency tables between patients with AIH and healthy controls. Allele frequencies were determined by $\chi 2$ test using 2×2 contingency tables between patients with AIH and healthy controls.
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Table 3. STAT4 rs7582694 polymorphism in patients with type-1 AIH and controls.

		Control (%)	AIH (%)	*p*-value[a]	OR (95%CI)
		n = 230	n = 230		
Genotype frequencies				0.001	
	G/G	101(43.9)	80(34.8)		
	G/C	109(47.4)	103(44.8)		
	C/C	20(8.7)	47(20.4)		
Allele				0.001	
	G	311(67.6)	263(57.2)		1
	C	149(32.4)	197(42.8)		1.563(1.195–2.046)

Abbreviation: AIH; autoimmune hepatitis, OR; odds ratio, CI; confidence interval, STAT4; signal transducer and activator or transcription.
[a]Genotype frequencies were determined by $\chi 2$ test using 2×3 contingency tables between patients with AIH and healthy controls. Allele frequencies were determined by $\chi 2$ test using 2×2 contingency tables between patients with AIH and healthy controls.
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Table 4. STAT4 rs7574865 polymorphism in patients with type-1 AIH without other autoimmune diseases.

		Control (%)	AIH without other autoimmune diseases (%)	*p*-value[a]	OR (95%CI)
		n = 230	n = 191		
Genotype frequencies				0.008	
	G/G	103(44.8)	68(35.6)		
	G/T	108(47.0)	89(46.6)		
	T/T	19(8.3)	34(17.8)		
Allele				0.005	
	G	314(68.3)	225(58.9)		1
	T	146(31.7)	157(41.1)		1.501(1.131–1.992)

Abbreviation: AIH; autoimmune hepatitis, OR; odds ratio, CI; confidence interval, STAT4; signal transducer and activator or transcription.
[a]Genotype frequencies were determined by $\chi 2$ test using 2×3 contingency tables between patients with AIH and healthy controls. Allele frequencies were determined by $\chi 2$ test using 2×2 contingency tables between patients with AIH and healthy controls.
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Discussion

AIH reflects a complex interaction between triggering factors, environmental factors, genetic predisposition and the immune regulatory network [3]. Most knowledge concerning the genetic factors of AIH comes from studies of the HLA genes [4,5]. Although multiple genes are probably involved, HLA genes appear to play a dominant role in the predisposition to AIH [27]. Genetic factors other than HLA genes that can affect the susceptibility of AIH are mainly polymorphisms in genes that encode proteins that affect cytokine pathways responsible for modulating immunity [27–29]. Although autoimmune diseases include a wide array of different organ involvement and symptoms, they all share a common component: the loss of immune tolerance toward "self antigen" [30]. Findings in recent genetic studies support the emerging concept that distinct clinical autoimmune diseases may share genetic susceptibility factors. STAT4 is a critical transcription factor involved in the regulation of Th1/Th2 cytokine balance [12]. *STAT4* polymorphisms have been found to be associated with various autoimmune diseases [8–10].

This study is the first to investigate a detailed correlation between *STAT4* gene polymorphisms and susceptibility to type-1 AIH in a Japanese nationwide AIH cohort study. In the current study, we confirmed an association of *STAT4* polymorphisms with susceptibility to type-1 AIH. Our data suggest that *STAT4* may be an "autoimmune disease susceptibility gene" and support the concept of deregulated pathways across multiple autoimmune diseases. In addition to their influence on autoimmune disease susceptibility, *STAT4* polymorphisms can also influence disease phenotypes. For example, rs7574865 in SLE patients was associated with severe disease manifestations, such as nephritis, high double stranded-DNA antibody production and younger age of disease onset. [31] For patients with systemic sclerosis, this polymorphism was associated with the presence of pulmonary

Table 5. Distribution of HLA-DR alleles distribution in patients with type-1 AIH.

HAL-DR alleles	AIH	Control	*P*	*Pc*	OR (95%CI)
	Alleles, No.(%)	Alleles, No.(%)			
	(n = 460 alleles)	(n = 460 alleles)			
*01	8(1.7)	24(5.2)	0.004	0.052	0.322(0.143–0.723)
*04	189(41.1)	118(25.7)	0.000001	0.000013	2.021(1.528–2.674)
*07	1(0.2)	4(0.9)	0.187	2.431	0.248(0.028–2.231)
*08	67(14.6)	42(9.1)	0.011	0.143	1.697(1.126–2.556)
*09	52(11.3)	70(15.2)	0.080	1.040	0.710(0.483–1.043)
*10	4(0.9)	2(0.4)	0.343	4.459	2.009(0.366–11.021)
*11	7(1.5)	7(1.5)	1.000	13.000	1.000(0.348–2.874)
*12	19(4.1)	26(5.7)	0.285	3.705	0.719(0.392–1.319)
*13	16(3.5)	47(10.2)	0.000052	0.000676	0.317(0.177–0.567)
*14	26(5.7)	28(6.1)	0.779	10.127	0.924(0.533–1.602)
*15	66(14.3)	88(19.1)	0.052	0.676	0.708(0.499–1.004)
*16	4(0.9)	2(0.4)	0.343	4.459	2.009(0.366–11.021)
*17	1(0.2)	2(0.4)	0.500	6.500	0.499(0.045–5.521)

HLA-DRB1 allele was assessed by cis-square test. The probability values were corrected (*Pc*) for multiple testing (Bonferroni correction).
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fibrosis [32]. Therefore, we examined possible associations between *STAT4* and the clinical phenotype of type-1 AIH. However, we did not find evidence of association between *STAT4* polymorphisms and disease progression or phenotype of type-1 AIH.

Regarding the disease-developing effect of genetic variants in the *STAT4* region on type-1 AIH observed in our study, it might be interesting to determine whether the *STAT4* risk alleles have different expression levels or functional effects in different effector cells [33]. The susceptibility SNP rs7574865 is located within intron 3 of *STAT4*, a non-coding region. It is suspected that it may influence the gene expression of STAT4 at the level of transcription or splicing variation [34]. A recent study reported that the expression level of STAT4 in peripheral blood mononuclear cells correlated with the risk allele of STAT4 rs7574865 [33]. This might indicate the effects of different *STAT4* gene variants on STAT4 expression levels. To date, the main alternative spliced isoforms of *STAT4* are STAT4α and STAT4β. STAT4β is a shorter form of the full-length STAT4α and is not as efficient as STAT4α for the direct induction of IFN-γ gene expression activated by IL-12 in Th1 cells [35]. However, expression of STAT4β, lacking the transactivation domain, was not affected by the STAT4 SNPs [33]. Additionally, a significant inverse correlation with T-risk alleles at rs7574865 and the methylation status of the *STAT4* promoter was demonstrated in inflammatory bowel disease [36]. The *STAT1* gene is located adjacent to *STAT4* suggesting it is also a candidate susceptibility gene for autoimmune disease [37]. To examine the role of the STAT1-STAT4 region, 52 tag SNPs encompassing this region in Japanese lupus patients [38]. The SNPs rs11889341 and rs10168266 were in linkage disequilibrium (LD) with rs7574865 and were significantly associated with SLE [38]. In contrast, significant association was not detected for SNPs in the *STAT1* region [38].

AIH pathogenesis are more complex than the traditional dichotomous Th1/Th2 paradigm, where STAT4 represents a transcription factor that induces IL-12, IL-23 and type 1 IFN-mediated signals to Th1 and Th17 differentiation, monocyte activation and interferon-γ production [39]. STAT4 is important for IL-22 production, which plays a pathological role in IL-17-dependent hepatitis [40].

A recent study showed that G allele at rs7574865 was associated with increased risk for HCC, suggesting dual roles of STAT4 in autoimmune diseases and HBV-related HCC [41]. Interestingly, subjects with GG genotype at rs7574865 had the lowest mRNA levels of *STAT4* in both HCC and non-tumor tissues compared with TG and TT genotypes [41]. Considering the role of STAT4 in Th1 immune responses, rs7574865 polymorphisms may affect the hepatic immune response against auto-antigen or viral antigen, contributing to the susceptibility of these related disorders. Further studies will be needed to examine the different possible mechanisms by which the variant haplotypes contribute to AIH.

The current study was limited because there were relatively small numbers of patients, and because some of the phenotypes examined were related to disease activity, and therefore may have fluctuated naturally or as a result of treatment. Additionally, it was difficult to perform a replication study due to the very low prevalence of type-1 autoimmune hepatitis and limited numbers of enrolled patients. In the current study, the power to detect a 1.6-fold increased risk, assuming an alpha value of 0.05, was 0.627 for rs7574865 T allele. Another limitation is the lack of complete information regarding the causal polymorphisms and their exact functional roles.

In summary, our results identified *STAT4* SNP rs7574865 as a disease-susceptible gene variant in type-1 AIH. Further studies on the expression and regulation of *STAT4* in the liver will be required to investigate the functional consequences of *STAT4* gene variants in more detail.

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Author Contributions

Conceived and designed the experiments: KM M. Nakamura H. Yatsuhashi HI. Performed the experiments: YJ MY. Analyzed the data: KM M. Nakamura MY. Contributed reagents/materials/analysis tools: SA SN AK SH SB K. Yamasaki TK MS HK TH M. Kohjima M. Nakamuta M. Kato K. Yoshizawa HO YN ET HN TS KA NH YO AN TM HS EM KS H. Yamashita FM. Wrote the paper: KM M. Nakamura MY HI.

References

1. Manns MP, Vogel A (2006) Autoimmune hepatitis, from mechanisms to therapy. Hepatology 43: S132–44.
2. Czaja AJ, Manns MP (2010) Advances in the diagnosis, pathogenesis, and management of autoimmune hepatitis. Gastroenterology 139: 58–72.
3. Longhi MS, Ma Y, Mieli-Vergani G, Vergani D (2010) Aetiopathogenesis of autoimmune hepatitis. J Autoimmun 34: 7–14.
4. Strettell MD, Donaldson PT, Thomson LJ, Santrach PJ, Moore SB, et al (1997) Allelic basis for HLA-encoded susceptibility to type 1 autoimmune hepatitis. Gastroenterology 112: 2028–35.
5. Yoshizawa K, Ota M, Katsuyama Y, Ichijo T, Matsumoto A, et al (2005) Genetic analysis of the HLA region of Japanese patients with type 1 autoimmune hepatitis. J Hepatol 42: 578–84.
6. Baranzini SE (2009) The genetics of autoimmune diseases: a networked perspective. Curr Opin Immunol 21: 596–605.
7. Lettre G, Rioux JD (2008) Autoimmune diseases: insights from genome-wide association studies. Hum Mol Genet 17: R116–21.
8. Remmers EF, Plenge RM, Lee AT, Graham RR, Hom G, et al (2007) STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus. N Engl J Med 357: 977–86.

9. Lee HS, Park H, Yang S, Kim D, Park Y (2008) STAT4 polymorphism is associated with early-onset type 1 diabetes, but not with late-onset type 1 diabetes. Ann N Y Acad Sci 1150: 93–8.
10. Sugiura T, Kawaguchi Y, Goto K, Hayashi Y, Tsuburaya R, et al (2012) Positive association between STAT4 polymorphisms and polymyositis/dermatomyositis in a Japanese population. Ann Rheum Dis 71: 1646–50.
11. Kaplan MH (2005) STAT4: a critical regulator of inflammation in vivo. Immunol Res 31: 231–42.
12. Watford WT, Hissong BD, Bream JH, Kanno Y, Muul L, et al (2004) Signaling by IL-12 and IL-23 and the immunoregulatory roles of STAT4. Immunol Rev 202: 139–56.
13. Bettelli E, Korn T, Kuchroo VK (2007) Th17: the third member of the effector T cell trilogy. Curr Opin Immunol 19: 652–7.
14. Taylor KE, Remmers EF, Lee AT, Ortmann WA, Plenge RM, et al (2008) Specificity of the STAT4 genetic association for severe disease manifestations of systemic lupus erythematosus. PLoS Genet 4: e1000084.
15. Kobayashi S, Ikari K, Kaneko H, Kochi Y, Yamamoto K, et al (2008) Association of STAT4 with susceptibility to rheumatoid arthritis and systemic lupus erythematosus in the Japanese population. Arthritis Rheum 58: 1940–6.
16. Namjou B, Sestak AL, Armstrong DL, Zidovetzki R, Kelly JA, et al (2009) High-density genotyping of STAT4 reveals multiple haplotypic associations with systemic lupus erythematosus in different racial groups. Arthritis Rheum 60: 1085–95.
17. Liang YL, Wu H, Shen X, Li PQ, Yang XQ, et al (2012) Association of STAT4 rs7574865 polymorphism with autoimmune diseases: a meta-analysis. Mol Biol Rep 39: 8873–82.
18. Gao B (2005) Cytokines, STATs and liver disease. Cell Mol Immunol 2: 92–100.
19. Migita K, Watanabe Y, Jiuchi Y, Nakamura Y, Saito A, et al (2012) Hepatocellular carcinoma and survival in patients with autoimmune hepatitis (Japanese National Hospital Organization-autoimmune hepatitis prospective study). Liver Int 32: 837–44.
20. Alvarez F, Berg PA, Bianchi FB, Bianchi L, Burroughs AK, et al (1999) International Autoimmune Hepatitis Group Report: review of criteria for diagnosis of autoimmune hepatitis. J Hepatol 31: 929–38.
21. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ (1994) Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology 19: 1513–20.
22. Zervou MI, Mamoulakis D, Panierakis C, Boumpas DT, Goulielmos GN (2008) STAT4: a risk factor for type 1 diabetes? Hum Immunol 69: 647–50.
23. Piotrowski P, Lianeri M, Wudarski M, Olesinska M, Jagodzin ski PP (2012) Contribution of STAT4 gene single-nucleotide polymorphism to systemic lupus erythematosus in the Polish population. Mol Biol Rep 39: 8861–6.
24. Yamazaki A, Yasunami M, Ofori M, Horie H, Kikuchi M, et al. (2011) Human leukocyte antigen class I polymorphisms influence the mild clinical manifestation of Plasmodium falciparum infection in Ghanaian children. Hum Immunol 72: 881–8.
25. Purcell S, Cherny SS, Sham PC (2003) Genetic Power Calculator: design of linkage and association genetic mapping studies of complex traits. Bioinformatics 19: 149–50.
26. Nakamura K, Yoneda M, Yokohama S, Tamori K, Sato Y, et al (1998) Efficacy of ursodeoxycholic acid in Japanese patients with type 1 autoimmune hepatitis. J Gastroenterol Hepatol 13: 490–5.
27. Czaja AJ (2008) Genetic factors affecting the occurrence, clinical phenotype, and outcome of autoimmune hepatitis. Clin Gastroenterol Hepatol 6: 379–88.
28. Agarwal K, Czaja AJ, Jones DE, Donaldson PT (2000) Cytotoxic T lymphocyte antigen-4 (CTLA-4) gene polymorphisms and susceptibility to type 1 autoimmune hepatitis. Hepatology 31: 49–53.
29. Czaja AJ, Cookson S, Constantini PK, Clare M, Underhill JA, et al (1999) Cytokine polymorphisms associated with clinical features and treatment outcome in type 1 autoimmune hepatitis. Gastroenterology 117: 645–52.
30. Oldstone MB (2005) Molecular mimicry, microbial infection, and autoimmune disease: evolution of the concept. Curr Top Microbiol Immunol 296: 1–17.
31. Sigurdsson S, Nordmark G, Garnier S, Grundberg E, Kwan T, et al (2008) A risk haplotype of STAT4 for systemic lupus erythematosus is over-expressed, correlates with anti-dsDNA and shows additive effects with two risk alleles of IRF5. Hum Mol Genet 17: 2868–76.
32. Dieudé P, Guedj M, Wipff J, Ruiz B, Hachulla E, et al (2009) STAT4 is a genetic risk factor for systemic sclerosis having additive effects with IRF5 on disease susceptibility and related pulmonary fibrosis. Arthritis Rheum 60: 2472–9.
33. Abelson AK, Delgado-Vega AM, Kozyrev SV, Sánchez E, Velázquez-Cruz R, et al (2009) STAT4 associates with systemic lupus erythematosus through two independent effects that correlate with gene expression and act additively with IRF5 to increase risk. Ann Rheum Dis 68: 1746–53.
34. Korman BD, Kastner DL, Gregersen PK, Remmers EF (2008) STAT4: genetics, mechanisms, and implications for autoimmunity. Curr Allergy Asthma Rep 8: 398–403.
35. Hoey T, Zhang S, Schmidt N, Yu Q, Ramchandani S, et al (2003) Distinct requirements for the naturally occurring splice forms Stat4alpha and Stat4beta in IL-12 responses. EMBO J 22: 4237–48.
36. Kim SW, Kim ES, Moon CM, Kim TI, Kim WH, et al (2012) Abnormal genetic and epigenetic changes in signal transducer and activator of transcription 4 in the pathogenesis of inflammatory bowel diseases. Dig Dis Sci 57: 2600–7.
37. Takeda K, Akira S (2000) STAT family of transcription factors in cytokine-mediated biological responses. Cytokine Growth Factor Rev 11: 199–207.
38. Kawasaki A, Ito I, Hikami K, Ohashi J, Hayashi T, et al (2008) Role of STAT4 polymorphisms in systemic lupus erythematosus in a Japanese population: a case-control association study of the STAT1-STAT4 region. Arthritis Res Ther 10: R113.
39. Murphy KM, Ouyang W, Szabo SJ, Jacobson NG, Guler ML, et al (1999) T helper differentiation proceeds through Stat1-dependent, Stat4-dependent and Stat4-independent phases. Curr Top Microbiol Immunol 238: 13–26.
40. Xu M, Morishima N, Mizoguchi I, Chiba Y, Fujita K, et al (2011) Regulation of the development of acute hepatitis by IL-23 through IL-22 and IL-17 production. Eur J Immunol 41: 2828–39.
41. Jiang DK, Sun J, Cao G, Liu Y, Lin D, et al (2013) Genetic variants in STAT4 and HLA-DQ genes confer risk of hepatitis B virus-related hepatocellular carcinoma. Nat Genet 45: 72–5.

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CIRRHOSIS AND LIVER FAILURE

Systemic and local expression levels of TNF-like ligand 1A and its decoy receptor 3 are increased in primary biliary cirrhosis

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Keywords
decoy receptor 3 – primary biliary cirrhosis – tumour necrosis factor-like ligand 1A – ursodeoxycholic acid

Abbreviations
AIH, autoimmune hepatitis; ALP, alkaline phosphatase; ALT, alanine aminotransferase; CHC, chronic hepatitis C; DcR3, decoy receptor 3; DR3, death domain receptor 3; FasL, Fas ligand; GWAS, genome-wide association study; IBD, inflammatory bowel diseases; PBC, primary biliary cirrhosis; PSL, prednisolone; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; TL1A, TNF-like ligand 1A; TLR, Toll-like receptor; UDCA, ursodeoxycholic acid.

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Abstract

Background & Aims: Through a genome-wide association study of a Japanese population, we recently identified *TNFSF15*, a gene encoding TNF-like ligand 1A (TL1A), as a susceptibility gene for primary biliary cirrhosis (PBC). We investigated the clinical significance of TL1A and one of its receptors, decoy receptor 3 (DcR3), in PBC. *Methods:* We analysed the systemic and local expression of TL1A and DcR3 in 110 PBC patients and 46 healthy controls using enzyme-linked immunosorbent assay, quantitative polymerase chain reaction and immunohistochemical staining. *Results:* Serum TL1A levels were significantly increased in PBC patients at both early and late stages as compared with healthy controls, and its levels were significantly decreased in early-stage PBC patients after ursodeoxycholic acid (UDCA) treatment. TL1A was immunohistochemically localized to biliary epithelial cells, Kupffer cells, blood vessels and infiltrating mononuclear cells in the PBC liver. In addition, TL1A messenger RNA expression was increased in the PBC liver as compared with the non-diseased liver. Serum DcR3 levels were also significantly increased in PBC patients, and were significantly decreased after UDCA treatment in early-stage PBC patients. *Conclusions:* These results indicate that TL1A and DcR3 may play an important role in the pathogenesis of PBC.

Primary biliary cirrhosis (PBC) is a chronic liver disease characterized by the destruction of intrahepatic bile ducts and progressive cholestasis that leads to cirrhosis and hepatic failure. Genome-wide association studies (GWAS) have recently revealed more than 20 PBC susceptibility genes, including *HLA*, *IL12A*, *IL12RB2*, *IRF5-TNPO3*, *STAT4*, *IL7R* and those at chromosome 17q12-21 in patients of European descent (1–4). Our

recent GWAS in a Japanese population identified *TNFSF15* and *POU2AF1* as new susceptibility genes for PBC (5). These results from individuals of different ethnicities indicate that there are two important disease pathways in the development of PBC, regardless of ethnicity: T-cell differentiation to Th1 cells and B-cell differentiation to plasma cells.

TNF-like ligand 1A (TL1A), which is encoded by *TNFSF15* on chromosome 9q32, is a tumour necrosis factor (TNF)-like cytokine that provides signalling to activated lymphocytes via binding to death domain receptor 3 (DR3) (6). TL1A protein is induced in endothelial cells by inflammatory cytokines such as TNF-α and IL-1 (6). TL1A is also induced in monocytes and dendritic cells by stimulation with microbial antigens (i.e., Toll-like receptor (TLR) 1, 2, 4, 6 and 9 ligands) and immune complexes (7, 8), and is involved in apoptosis (9), cell proliferation (10) and costimulation of T cells that lead to polarization to Th1 and Th17 effector cells(11). Increased levels of TL1A in both serum and involved tissues have been reported in human inflammatory bowel diseases (IBD) (12, 13), rheumatoid arthritis (RA) (14) and psoriasis (15). In addition, it has been shown that TL1A is involved in the development of various autoimmune diseases in mouse models, including experimental antigen-induced arthritis (16), experimental autoimmune encephalomyelitis (17, 18) and chronic ileitis and colitis (19, 20).

Decoy receptor 3 (DcR3), which binds to TL1A, LIGHT and Fas ligand (FasL), inhibits the apoptosis and lymphokine secretion mediated by these ligands (6, 21). DcR3 is overexpressed in various cancers (22) and is implicated in tumour progression via protection from either FasL-mediated apoptosis or the antitumour cytotoxic T-lymphocyte response (23, 24). DcR3 also modulates the function of various immune cells, contributing for instance to the differentiation and maturation of monocytes, macrophages (25) and dendritic cells; the polarization of naïve T cells into Th2 cells (26) and the negative regulation of B-cell activation induced by TLR ligands (27). Increased serum levels of DcR3 have been reported in conjunction with numerous conditions: various cancers; autoimmune diseases such as IBD (12, 28), systemic lupus erythematosus (SLE) (21) and RA (14); infections (29) and renal failure (30). In mouse models, administration of DcR3 protects against the development of autoimmune and cyclophosphamide-induced diabetes (31, 32), progressive immunoglobulin A nephropathy (33) and autoimmune crescentic glomerulonephritis (34), whereas DcR3-transgenic mice develop an SLE-like syndrome (35). Taken together, these findings indicate that DcR3 also plays an important role in the pathogenesis of various chronic inflammatory diseases that are associated with TL1A.

In this study, we examined the systemic and local expression of TL1A and DcR3 in PBC patients to elucidate their clinical significance in PBC.

Materials and methods

Subjects

The study subjects included 110 patients with PBC, 26 with chronic hepatitis C (CHC), 19 with autoimmune hepatitis (AIH) and 46 healthy controls, all of whom had been registered at the National Hospital Organization (NHO) Nagasaki Medical Center. Healthy controls were medical staff members working at the NHO in Japan who were free of apparent diseases, including chronic liver diseases and autoimmune diseases. PBC was diagnosed based on internationally accepted criteria (36): biochemical evidence of cholestasis based mainly on alkaline phosphatase (ALP) elevation, presence of serum antimitochondrial antibodies, histological evidence of chronic non-suppurative destructive cholangitis and destruction of interlobular bile ducts. PBC patients were classified into the following three clinical stages based on liver biopsy findings and/or clinical manifestations: clinical stage I – Scheuer's stage 1 or 2 on liver biopsy or unknown histological stage without any signs of portal hypertension or cirrhosis; clinical stage II – Scheuer's stage 3 or 4 on liver biopsy or any histological stage with signs of portal hypertension or cirrhosis, but without jaundice (total bilirubin <2 mg/dl) and clinical stage III – any Scheuer's stage with persistent jaundice (total bilirubin ≥2 mg/dl) (37). Clinical stage I was defined as the early stage, whereas clinical stages II and III were defined as late stages. Clinical stages I and II were also defined as non-jaundice stages, whereas clinical stage III was defined as the jaundice stage. PBC patients who had chronic hepatitis virus B or C infections, alcoholic or autoimmune liver diseases or hepatocellular carcinoma were excluded from this study. AIH patients were diagnosed based on established clinical criteria (38), and CHC patients were diagnosed by detection of serum hepatitis C virus ribonucleic acid (RNA) by polymerase chain reaction (PCR). CHC patients were classified into early and late stage based on the histological score of fibrosis in liver biopsy specimens assessed by Metavir scoring system as follows: early stage – fibrosis score 0 or 1; late stage – fibrosis score 2, 3 or 4.

Enzyme-linked immunosorbent assay (ELISA)

Soluble TL1A in serum was measured using the human TL1A ELISA kit (PeproTech, Rocky Hill, CT, USA) according to the manufacturer's protocol. In brief, the wells of flat-bottomed immunoplates (Thermo Fisher Scientific, Yokohama, Japan) coated with a rabbit anti-human TL1A antibody (1 μg/ml, PeproTech) were incubated for 2 h with serum samples that were diluted 1/10 in dilution buffer [0.05% Tween 20 in phosphate-buffered saline (PBS)]. After washing, the wells were incubated with a biotinylated rabbit anti-human TL1A antibody (1 μg/ml, PeproTech) for 2 h followed by incubation with horseradish peroxidase–conjugated

avidin (PeproTech) for 30 min. The wells were developed with ABTS Liquid Substrate Solution (Sigma-Aldrich, St. Louis, MO, USA) and the absorbance was measured at 405 nm. The detection limit of TL1A was 62 pg/ml. DcR3 was similarly measured using the human DcR3 DuoSet ELISA Development kit (R&D systems, Minneapolis, MN, USA) according to the manufacturer's protocol. In this ELISA, 1/10 diluted serum samples were used and absorbance was measured at 450 nm. The detection limit of DcR3 was 45 pg/ml. Antimitochondrial, anti-gp210 and anticentromere antibodies were measured as previously described (37). All samples were run in duplicate.

Histological examination of liver biopsy samples

The 4-μm-thick, formalin-fixed, paraffin-embedded sections were routinely stained with haematoxylin and eosin, Azan Mallory, reticulin silver impregnation and rhodamine. A new staging and grading system proposed by Nakanuma (39) was used to define histological scores (0–3) for bile duct loss and fibrosis and grading (0–3) for cholangitis activity (CA) and hepatitic activity (HA) in liver biopsy specimens from PBC patients. Immunohistochemical staining of TL1A in liver biopsy specimens was performed as previously described (40). Rabbit anti-human TL1A polyclonal antibody (Abcam, Cambridge, UK) was used at 1/200 dilution as a primary antibody, and a standardized two-step method with ENVISION+ (DAKO Japan, Tokyo, Japan) was used for TL1A antibody detection. The reaction products were visualized using 3,3′-diaminobenzidine as a chromogen (DAKO) and counterstained with Mayer's haematoxylin (DAKO). No positive staining was obtained when the primary antibody was replaced with an isotype-matched, non-immunized rabbit IgG used as a negative control for the staining procedures.

RNA extraction and quantification of mRNA

Total RNA was isolated from liver specimens of PBC and CHC patients and from non-diseased controls with metastatic liver cancers using the guanidinium thiocyanate–phenol–chloroform method as previously described (41). One microgram of total RNA was reverse transcribed with reverse transcriptase and an oligo-(dT) primer to synthesize complementary deoxyribonucleic acid (cDNA) (Qiagen, Valencia, CA, USA). Quantitative PCR was performed using an automated amplification and quantification system in real time (Light-Cycler 2.0 system; Roche, Basel, Switzerland). In brief, 5 μl of the diluted cDNA, 3 mM $MgCl_2$, FAST DNA SYBR Green I (Roche) and 500 mM of the specific primers for TL1A or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were reacted in a total volume of 20 μl as follows: initial denaturation at 95°C for 10 min followed by 40 amplification cycles of denaturation at 95°C for 10 s, annealing at 62°C for 10 s, extension at 72°C for 6 s for TL1A or 9 s for GAPDH and an additional step at 82°C for 1 s for TL1A. The quantification was performed by a standard melting curve analysis. The PCR primers used in this study were as follows: TL1A forward primer, 5′-GAAATGACAGTATCTGCG GAGTTTA-3′; TL1A reverse primer, 5′-CAACTAGCTA CTGTCTGGCACTGG-3′; GAPDH forward primer, 5′-TGAACGGGAAGCTCACTGG-3′; GAPDH reverse primer, 5′-TCCACCACCCTGTTGCTGTA-3′. Results were expressed as the ratio of TL1A cDNA to GAPDH cDNA copy numbers in each sample.

Ethics board

This study was approved by the Ethics Board at the Clinical Research Center in the National Hospital Organization Nagasaki Medical Center, and was conducted after obtaining informed consent from each subject for the use of their serum samples as well as liver biopsy and surgical samples.

Statistical analysis

Values are expressed as means ± standard deviations. Comparison of demographic and clinical characteristics between groups was performed using the Student's *t*-test or Fisher's exact test. Median values of serum TL1A, DcR3 and various serum and histological parameters were compared using Mann–Whitney's *U*-test. Spearman's rank correlation test was performed to assess correlations between serum TL1A and biochemical parameters in PBC patients. TL1A mRNA expression in liver tissues was compared using the Student's *t*-test. Serum levels of TL1A and DcR3 in the same patient before and after UDCA treatment were compared by Wilcoxon's single-rank test. A two-tailed *P*-value of <0.05 was considered significant. Statistical analyses were performed using StatFlex software version 5.0 (Artech, Osaka, Japan).

Results

Patient demographics and clinical characteristics

Demographics and clinical characteristics of PBC, CHC and AIH patients at the time of enrolment are shown in Table 1. Among PBC patients, 98.2%, 35.2% and 26.2% were positive for antimitochondrial, anti-gp210 and anticentromere antibodies respectively; 18% had already begun treatment with UDCA. Among AIH patients, 47%, 11% and 5% had been treated with prednisolone (PSL) alone, UDCA + PSL and UDCA alone respectively; 37% had undergone no treatment. Of CHC patients, 84% were not undergoing treatment; in the remaining 16%, treatment consisted of pegylated interferon-α alone (4%), UDCA alone (4%), UDCA + bezafibrate (4%) and stronger neo-minophagen C alone (4%).

Table 1. Demographics and clinical characteristics of PBC, CHC and AIH patients at study enrolment

	PBC n = 110	CHC n = 26	AIH n = 19
Age, mean ± SD (years)	59.8 ± 12.0	56.4 ± 10.6	58.6 ± 15.4
Women, n (%)	92 (83.6)	15 (57.7)	15 (78.9)
Early stage, n (%)	96 (87.3)	13 (50.0)	–
Late stage, n (%)	14 (12.7)	13 (50.0)	–
ALT, mean ± SD (IU/L)	55 ± 42	79 ± 50	196 ± 317
ALP, mean ± SD (IU/L)	684 ± 509	336 ± 224	461 ± 280
IgM, mean ± SD (mg/dl)	390 ± 307	119 ± 47	277 ± 375
No mediation, n (%)	90 (81.8)	22 (84.6)	7 (36.8)
UDCA alone treatment, n (%)	20 (18.2)	1 (3.8)	1 (5.3)
Pegylated interferon-α treatment, n (%)	0 (0)	1 (3.8)	0 (0)
Prednisolone alone treatment, n (%)	0 (0)	0 (0)	9 (47.4)

SD, standard deviation; ALT, alanine aminotransferase; ALP, alkaline phosphatase; UDCA, ursodeoxycholic acid

Serum TL1A levels are increased in both early- and late-stage PBC

Serum TL1A levels were significantly higher in PBC patients compared with healthy controls ($P = 8.0 \times 10^{-5}$, Fig. 1A). Serum TL1A levels were also significantly higher in CHC and AIH patients compared with healthy controls ($P = 0.04$ and $P = 0.003$ respectively). There were no significant differences in TL1A levels among PBC, CHC and AIH patients (Fig. 1A).

Figure 1B shows that serum TL1A levels in healthy controls were comparable with those in early-stage CHC patients ($P = 0.85$), but were significantly higher in patients with early-stage PBC ($P = 2.0 \times 10^{-4}$). Serum TL1A levels were significantly higher in both late-stage PBC and CHC patients as compared with healthy controls (PBC: $P = 0.002$; CHC: $P = 0.002$). Serum TL1A levels were significantly higher in late-stage than early-stage CHC patients ($P = 0.002$), a distinction not observed in PBC patients ($P = 0.15$).

Association of TL1A with biochemical parameters, prevalence of antinuclear antibodies and histological scores in PBC patients

There were no significant associations between serum TL1A levels and levels of either ALP ($P = 0.59$), alanine aminotransferase (ALT) ($P = 0.79$) or total immunoglobulin M (IgM) ($P = 0.32$) at the time of subject enrolment (Fig. S1). Serum TL1A levels were similar between PBC patients who were negative and positive for anti-gp210 antibodies and anticentromere antibodies (Fig. S2). Although serum TL1A levels showed a non-significant increase in chronic cholangitis of moderate grade (CA2) but not of minimal or mild grade (CA0–1) ($P = 0.06$), serum TL1A levels were not associated with histological scores reflecting bile duct loss, fibrosis or the grade of hepatitic change (HA) (Fig. 2).

Serum TL1A is decreased in response to UDCA treatment in patients with early-stage PBC

To evaluate the effect of UDCA on serum TL1A levels in PBC patients, we followed the levels of serum TL1A, ALP, ALT and total IgM in 76 PBC patients receiving various UDCA treatments. Specifically, these patients received UDCA alone (58; 76%), UDCA + bezafibrate (14; 18%), UDCA + bezafibrate + PSL (2; 3%) or

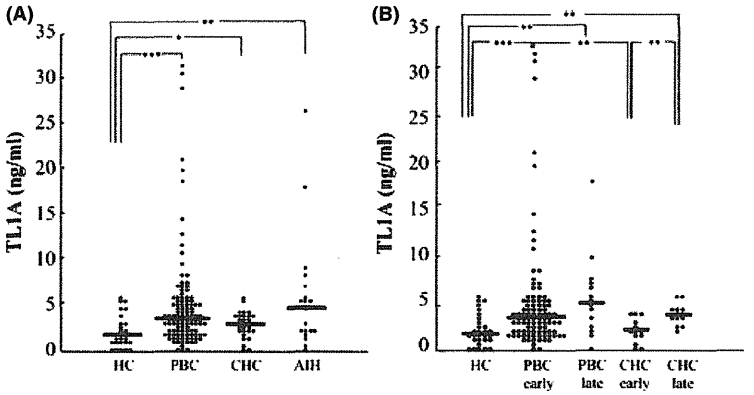

Fig. 1. Serum TL1A levels in PBC, CHC and AIH patients. (A) Serum TL1A levels were significantly higher in patients with PBC (n = 110, 4.9 ± 5.0 ng/ml), CHC (n = 26, 3.0 ± 1.5 ng/ml) and AIH (n = 19, 5.9 ± 6.4 ng/ml) as compared with healthy controls (n = 29, 2.2 ± 1.7 ng/ml). (B) Serum TL1A levels were significantly higher in both early-stage (n = 96, 4.7 ± 5.1 ng/ml) and late-stage PBC patients (n = 14, 5.7 ± 4.4 ng/ml) as compared with healthy controls, whereas serum TL1A levels were significantly higher in late-stage (n = 13, 3.8 ± 1.1 ng/ml) but not in early-stage CHC patients. Serum TL1A levels in early-stage PBC patients (n = 96, 4.7 ± 5.1 ng/ml) were significantly higher as compared with those of CHC patients (n = 13, 2.1 ± 1.3 ng/ml). Horizontal lines represent mean values for each group. Statistical differences were analysed using a two-tailed Mann–Whitney U-test; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. HC; healthy controls.

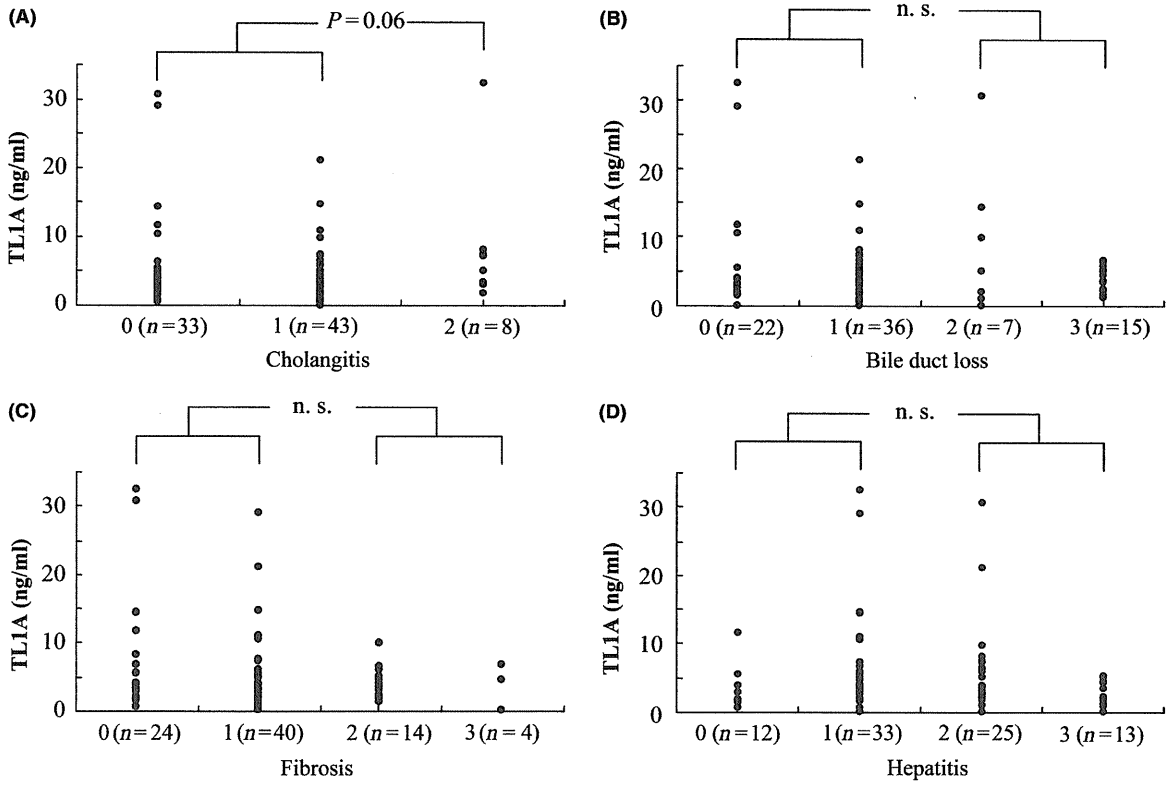

Fig. 2. Serum TL1A levels in PBC patients with different histological scores. (A) Serum TL1A levels tended to be higher in PBC patients with a cholangitis activity (CA) score of 2 as compared with those with a CA score of 0 or 1. There was no significant difference in serum TL1A levels among patients with different histological scores for bile duct loss (B), fibrosis (C) or hepatitis (D). Statistical differences were analysed using a two-tailed Mann–Whitney *U*-test. n.s.; not significant.

UDCA + PSL (2; 3%) during observation (median: 37.6 months; range: 8–148 months). The daily dose of UDCA was 600 mg (n = 67), 900 mg (n = 7) and 1200 mg (n = 2). Serum ALT, ALP and total IgM levels were significantly decreased in both early- and late-stage PBC patients receiving UDCA treatments (data not shown). On the other hand, serum TL1A levels were significantly decreased only in PBC patients classified as early stage (P = 0.04, Fig. 3B) at study entry, and not those categorized as late stage (P = 0.52, Fig. 3C). Thus, we noted a trend towards decreased serum TL1A levels (P = 0.13) under UDCA treatment in the total PBC patient population (Fig. 3A). By the end of the observation period, however, serum TL1A levels were sustained at significantly higher levels in early- and late-stage patients as compared with healthy controls (early stage: P = 0.06; late stage: P = 0.002).

Immunohistochemical analysis of TL1A in liver tissue

In the non-diseased liver, TL1A was localized in blood vessels, Kupffer cells, infiltrating mononuclear cells and intrahepatic bile ducts, but not in hepatocytes (Fig. 4A). In PBC and CHC patients, the localization of TL1A-positive cells was similar to that of non-diseased controls, however, the number of TL1A-positive cells was increased in both PBC (Fig. 4C, 4D) and CHC (Fig. 4B) patients as compared with non-diseased controls. There was no apparent difference between the PBC and CHC patients.

mRNA expression of TL1A in liver tissues and its association with histological activity

The expression of TL1A mRNA in the livers of PBC (P = 0.17) and CHC patients (P = 0.06) was higher as compared with those of controls (Fig. 5A), although this difference was not statistically significant. Compared with controls, the expression of TL1A mRNA was significantly increased only in late-stage CHC (P = 0.03, Fig. 5B). In addition, the expression of TL1A mRNA tended to be higher in late-stage than early-stage patients for both PBC (P = 0.07) and CHC (P = 0.16).

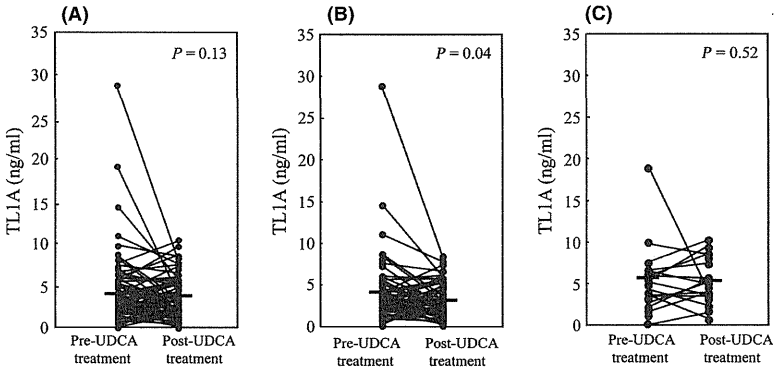

Fig. 3. Effect of UDCA treatment on serum TL1A levels in PBC patients. (A) In the PBC patient group as a whole (n = 76), serum TL1A levels tended to be decreased by UDCA treatment (pre: 4.5 ± 4.5 ng/ml, post: 3.6 ± 2.5 ng/ml). Serum TL1A levels were significantly decreased in early-stage PBC patients (n = 60) after UDCA treatment (pre: 4.0 ± 4.2 ng/ml, post: 3.0 ± 2.1 ng/ml) (B), but not in late-stage PBC patients (n = 16) (pre: 5.5 ± 4.3 ng/ml, post: 5.2 ± 2.3 ng/ml) (C). Horizontal lines represent mean values. Statistical analysis was performed using a two-tailed Wilcoxon's single-rank test.

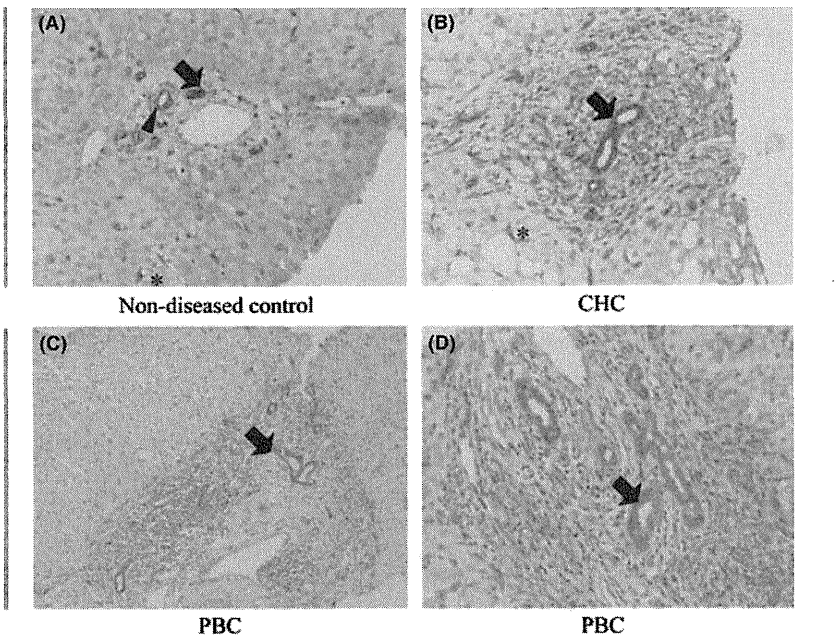

Fig. 4. Immunohistochemical staining of TL1A in the liver specimens of PBC and CHC patients. (A) TL1A was localized in intrahepatic bile ducts (arrow), blood vessels (arrowhead) and Kupffer cells (asterisk), but not in hepatocytes in non-diseased controls. In addition to these TL1A-positive cells, TL1A was primarily localized to mononuclear cells infiltrating the portal tract in both CHC (B) and PBC (C and D) liver sections. Original magnification: 400×.

The significance of DcR3 in PBC patients

At the time of enrolment, the number of patients with detectable serum DcR3 levels was significantly higher in patients with PBC (34%, $P = 1.0 \times 10^{-5}$), CHC (19%, $P = 0.02$) and AIH (47%, $P = 2.0 \times 10^{-5}$), as compared with healthy controls (2%). Serum DcR3 levels in PBC ($P = 0.004$), CHC ($P = 0.005$) and AIH ($P = 0.002$) patients were significantly higher than those in healthy controls (Fig. 6A). Serum DcR3 levels were also significantly higher in both early- and late-stage PBC (early stage: $P = 0.009$; late stage: $P = 0.003$) patients and CHC (early stage: $P = 0.008$; late stage: $P = 0.01$) patients as compared with healthy controls (Fig. 6B).

Serum DcR3 levels, when detectable, tended to be lower in PBC patients receiving UDCA treatment ($P = 0.15$, Fig. 7A). When these patients were stratified

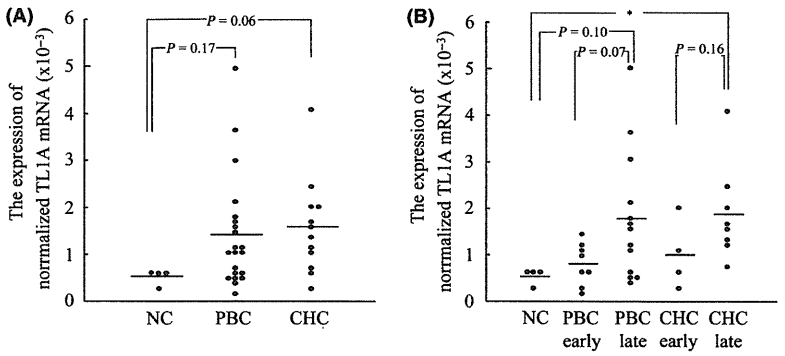

Fig. 5. Expression of TL1A mRNA in the liver specimens of PBC and CHC patients. The TL1 mRNA expression in the liver specimens of non-diseased controls (n = 4) as well as PBC (n = 21) and CHC (n = 12) patients was normalized to GAPDH mRNA expression. (A) The normalized TL1A mRNA expression in both PBC (0.0014 ± 0.0012) and CHC patients (0.0016 ± 0.0010) was higher than that in non-diseased controls (0.0005 ± 0.0002). (B) The normalized TL1A mRNA expression was higher in the late stage than in the early stage of both PBC (early: n = 8, 0.0008 ± 0.0004; late: n = 13, 0.0017 ± 0.0014) and CHC patients (early: n = 4, 0.0009 ± 0.0008; late: n = 8, 0.0019 ± 0.0010). A significant increase in TL1A mRNA was observed only in late-stage CHC patients. Horizontal lines represent mean values for each group. Statistical differences were analysed using a two-tailed Student's t-test; * $P < 0.05$. NC: non-diseased controls.

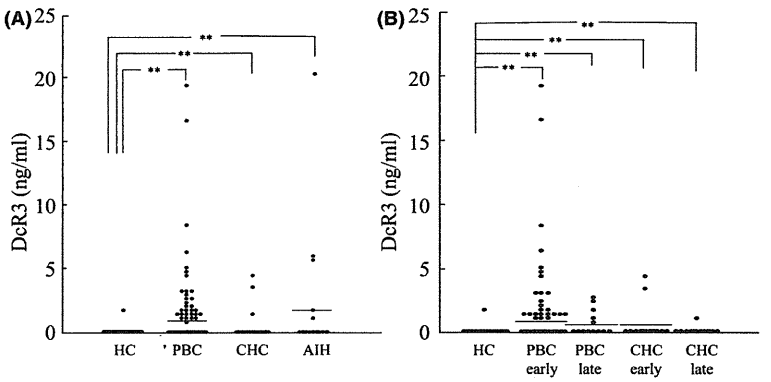

Fig. 6. Serum DcR3 levels in PBC, CHC and AIH patients. (A) Serum DcR3 levels were significantly higher in patients with PBC (n = 110, 0.92 ± 2.3 ng/ml), CHC (n = 26, 0.41 ± 1.1 ng/ml) and AIH (n = 19, 2.0 ± 4.8 ng/ml) as compared with healthy controls (n = 46, 0.04 ± 0.25 ng/ml). (B) Serum DcR3 levels were higher in both the early- and late-stage PBC (early: n = 96, 0.94 ± 2.4 ng/ml; late: n = 14, 0.71 ± 1.0 ng/ml) and CHC (early: n = 13, 0.62 ± 1.5 ng/ml; late n = 13, 0.20 ± 0.41 ng/ml) patients as compared with healthy controls. Horizontal lines represent mean values for each group. Statistical differences were analysed using a two-tailed Mann–Whitney U-test; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

by clinical stage at the end of the observation period, serum DcR3 levels were found to be significantly decreased only in patients with the non-jaundice stage (P = 0.004, Fig. 7B), whereas these levels were significantly increased in patients with the jaundice stage who progressed to hepatic failure (P = 0.04, Fig. 7C).

Discussion

In this study, we report for the first time that serum TL1A levels were significantly increased in both early- and late-stage PBC patients, and that these levels were significantly decreased after UDCA treatment in early-stage PBC patients. Interestingly, serum TL1A levels were also increased in other liver diseases such as CHC and AIH, although a significant increase was observed only in the late-stage CHC patients. These results may indicate that TL1A is involved not only in the early pathogenesis of PBC but also in CHC and AIH as a common denominator of chronic liver inflammation.

Previous reports have shown that serum TL1A levels are increased in various chronic inflammatory diseases such as IBD (12, 42) and RA (14). In these diseases, serum TL1A levels were higher in patients with active disease than those with inactive disease or in remission. In addition, serum TL1A levels were significantly higher in the late or terminal stages of these conditions as compared with their early stage. Previous studies have also