

**Title: Anti-programmed cell death-1 antibody as a new serological marker for type 1 autoimmune hepatitis.**

**Short running title: Anti-PD-1 antibody and type 1 AIH.**

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## **Abstract**

**Background and Aim:** Recently, the association of the dysfunction of programmed cell death (PD)-1 expressed on activated lymphocytes with the pathogenesis of autoimmune hepatitis (AIH) has been speculated. This study aimed to investigate the association of serum anti-PD-1 antibodies with clinical characteristics of type 1 AIH.

**Methods:** Serum samples before the initiation of prednisolone treatment were obtained from 52 type 1 AIH patients, 24 patients with drug-induced liver injury (DILI), 30 patients with acute viral hepatitis (AVH), 11 patients with primary sclerosing cholangitis (PSC), and 62 healthy volunteers. Titers of serum anti-PD-1 antibodies were measured by indirect enzyme-linked immunosorbent assay. The cut-off level was represented by a mean absorbance + 2 standard deviations in healthy volunteers.

**Results:** Prevalence of serum anti-PD-1 antibodies was 63% in type 1 AIH patients, 8% in DILI patients, 13% in AVH patients, 18% in PSC patients, and 3% in healthy volunteers. In type 1 AIH patients, titers of serum anti-PD-1 antibodies were correlated with serum levels of bilirubin ( $r = 0.31$ ,  $P = 0.030$ ) and alanine aminotransferase ( $r = 0.31$ ,  $P = 0.027$ ) but not serum immunoglobulin G levels. Positivity for serum anti-PD-1 antibodies was associated with the later normalization of serum alanine aminotransferase levels after the initiation of prednisolone and the disease relapse.

**Conclusions:** Serum anti-PD-1 antibodies would be useful for the discrimination of type 1 AIH from DILI, AVH and PSC as an auxiliary diagnostic marker. Furthermore,

anti-PD-1 antibodies may be associated with clinical characteristics of type 1 AIH.

**Keywords:** Acute viral hepatitis; Autoimmune hepatitis; Drug-induced liver injury; Primary sclerosing cholangitis; Programmed cell death-1.

## **Introduction**

Autoimmune hepatitis (AIH) is a progressive, autoimmune liver disease characterized by histological interface hepatitis, hypergammaglobulinemia, and circulating autoantibodies.<sup>1</sup> However, the pathogenesis of AIH has not been fully revealed yet, and the diagnosis is made based on the scoring systems for lack of specific diagnostic markers for AIH.<sup>2,3</sup>

Recently, co-stimulatory molecules with inhibitory properties expressed on activated T and B cells are revealed to be possibly associated with the pathogenesis of AIH. Programmed cell death (PD)-1 deficient mice thymectomized 3 days after birth develop massive hepatic necrosis with the appearance of serum anti-nuclear antibody (ANA).<sup>4</sup> Furthermore, a clinical trial using anti-PD-1 antibody as immunotherapeutic agent for advanced cancer shows the development of hepatitis, which required corticosteroid treatment, as adverse event.<sup>5</sup> Anti-PD-1 antibodies enhance allogeneic T cell proliferation.<sup>6</sup> Dysfunction of PD-1 may activate auto-reactive T cells and result in the development of autoimmune diseases.

Thus, we speculated that anti-PD-1 antibodies might exist in sera of type 1 AIH patients. This study aimed to confirm the presence of anti-PD-1 antibodies in sera of type 1 AIH patients and to investigate the usefulness of serum anti-PD-1 antibody for

the discrimination of type 1 AIH from drug-induced liver injury (DILI), acute viral hepatitis (AVH), and primary sclerosing cholangitis (PSC), and the association of serum anti-PD-1 antibodies with the clinical features of type 1 AIH.

## **Methods**

**Ethics.** This study complied with the Declaration of Helsinki and was approved by the Institutional Review Board at Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences. Serum samples and data were collected after each subject provided written informed consent.

**Patients and serum samples.** Serum samples before the initiation of corticosteroid treatment were obtained from 52 type 1 AIH patients, 24 DILI patients, 30 AVH patients, and 11 PSC patients. Thirty AVH patients consisted of 7 patients with acute hepatitis A, 21 patients with acute hepatitis B, and 2 patients with acute hepatitis C. Moreover, 62 serum samples from healthy volunteers were collected. Clinical characteristics of study population are shown in Table 1. Serum samples were stored -30°C until use.

All type 1 AIH patients underwent liver biopsy. All of type 1 AIH patients, DILI patients and PSC patients were seronegative for immunoglobulin M (IgM) antibody to hepatitis A virus, IgM antibody to hepatitis B core antigen, hepatitis B surface antigen,

and hepatitis C virus RNA identifiable by nested reverse transcription-polymerase chain reaction. Type 1 AIH was diagnosed based on the revised scoring system proposed by International Autoimmune Hepatitis Group.<sup>2</sup> None of type 1 AIH patients were positive for serum anti-liver kidney microsomal-1 autoantibodies. DILI was diagnosed based on the diagnostic criteria of the Digestive Disease Week-Japan 2004 workshop,<sup>7</sup> which usefulness in the diagnosis of DILI has been confirmed by the study with large sample size.<sup>8</sup> A diagnosis of hepatitis A, B and C was made based on the presence of IgM antibody to hepatitis A virus, IgM antibody to hepatitis B core antigen, and hepatitis C virus RNA, respectively. The diagnosis of PSC was made according to accepted criteria; typical cholangiographic findings or histological findings of cholangitis in combination with biochemical and clinical findings.<sup>9</sup>

Liver biopsy in type 1 AIH patients was performed before or just after commencing the initial treatment. Liver biopsy specimens were evaluated by two pathologists (YM, KY) and diagnosed as acute or chronic hepatitis.<sup>10</sup> Liver biopsy specimens diagnosed as chronic hepatitis underwent histological staging based on the classification of Desmet and colleagues.<sup>11</sup>

**Treatment for type 1 AIH.** As initial treatment, 43 patients (83%) received

prednisolone (PSL) treatment (20-40 mg/day), and 4 patients (8%) did monotherapy of ursodeoxycholic acid (UDCA). In the remaining 5 patients (9%), initial treatment was unknown because they were transferred to other hospitals without follow-up. Initial treatment was continued until the normalization of serum alanine aminotransferase (ALT) levels (30 IU/l or lower).

After the normalization of serum ALT levels, PSL was tapered by 2.5–5 mg every 1 or 2 weeks to a maintenance dose of 10 mg/day or less. PSL was halted when normal levels of serum ALT continued at the maintenance dose for more than 2 years.

Each patient underwent a comprehensive clinical review and physical examination at presentation and each follow-up visit. Conventional laboratory blood tests were performed every 1–2 months.

**Criteria for relapse in type 1 AIH.** Relapse was defined as an increase in serum ALT level to more than twofold of the upper normal limit (higher than 60 IU/l), following the normalization of serum ALT level with medical treatment.

**Peripheral blood mononuclear cell isolation and activation.** Approximately 30 ml of blood was obtained from a healthy volunteer in heparinized tubes. Purified peripheral

blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque density gradient centrifugation (GE Healthcare). PBMCs were resuspended in RPMI 1640 culture medium (Sigma-Aldrich) supplemented with 5% heat-inactivated human AB serum (MP Biomedicals), 2 mM L-glutamine (Sigma-Aldrich), and 1% penicillin/streptomycin solution (Sigma-Aldrich). CD3/CD28 Dynabeads (Life technologies) were added to obtain a bead-to-cell ratio of 1:1. The cultures were incubated for 7 days at 37 °C in 5% CO<sub>2</sub>.

**Activated PBMC lysate.** Activated PBMCs were washed twice in phosphate-buffered saline (PBS), resuspended in 500 µl of Pierce® IP lysis buffer (Pierce Biotechnology), and homogenized using FastPrep®-24 (MP Biomedicals). Lysates were cleared by a 10-min centrifugation at 10,000 × g at 4°C. Protein concentration was 2.8 µg/µl by using the Protein Assay Dye Reagent Concentrate (Bio-Rad).

**Co-immunoprecipitation.** Briefly, 200 µl of human sera diluted 1:10 in PBS with 0.02 % Tween 20 were incubated with Dynabeads Protein G (Invitrogen Dynal AS) for 1 hour at room temperature. The beads were washed with PBS and incubated with 200 µl PBS containing 20 µg activated PBMC lysate for 1 hour at room temperature. Beads

were then washed with PBS, and retained proteins were eluted with 2 x SDS sample buffer (20% glycerol, 4% SDS, 125mM tris-HCl pH6.8, 12% 2-mercaptoethanol, 0.004% bromophenol blue).

**Western blot.** Proteins were separated by SDS-polyacrylamide gel electrophoresis. The separated components were electroblotted onto an Immobilon-P polyvinylidene difluoride membrane (Millipore Corporation). Blot membranes were blocked in PVDF blocking reagent for Can Get Signal (TOYOBO CO., LTD.). The blots were washed with tris-buffered saline with Tween 20 and reacted with a 1:1,000 dilution of anti-human PD-1 antibody (R&D Systems) for 1 hour at room temperature, washed and then reacted with HRP-conjugated anti-mouse IgG for 1 hour. The blots were then developed by ECL (GE Healthcare) according to the manufacturer's instructions.

**Indirect enzyme-linked immunosorbent assay.** Titers of serum anti-PD-1 antibodies were measured by indirect enzyme-linked immunosorbent assay (ELISA) using Protein Detector ELISA Kit (Kirkegaard & Perry Laboratories). All serum samples were tested in duplicate.

Briefly, 96-well U-bottom microtiter plates (Greiner Bio-One GmbH) were coated

with 100  $\mu$ l of 1  $\mu$ g/ml recombinant PD-1 (Abnova) in PBS at room temperature for 1 hour. Unbound antigen was removed, nonspecific binding sites were blocked by incubation with 1% bovine-serum albumin (BSA) in PBS, and the wells were incubated with 100  $\mu$ l of human sera diluted 1:20 in PBS with 1% of BSA for 1 hour. Following incubation, the wells were incubated with anti-human IgG diluted 1:1,000 in PBS with 1% of BSA, covalently linked to alkaline phosphatase, and the reaction was visualized by adding 100  $\mu$ l of a substrate buffer (5-bromo-4-chloro-3-indolyl phosphate/ nitroblue tetrazolium). The optical densities at 630 nm were read with a Model 680 microplate reader (Bio-Rad Laboratories). In order to avoid inter-plate variability, we used a positive serum, assigned it 0.200 OD<sub>630nm</sub>, and read the optical densities of all samples against this positive serum. Intra-assay variability was found to be 8.4%.

**Statistical analysis.** Statistical analysis was performed using the SPSS statistical program. Continuous variables were expressed as median (range). Differences in continuous variables were evaluated by the Mann–Whitney U-test between two independent samples and the Kruskal–Wallis test among **five** independent samples. Dichotomous variables were compared by the  $\chi^2$ -test. The Spearman correlation coefficient was used to evaluate the consistency in the continuous variables between

two samples. Cumulative survival curves were analyzed using the Kaplan-Meier method, and the differences in the curves were tested using the log-rank test. The diagnostic accuracy of each factor was evaluated based on the area under the curve (AUC) using receiver operating characteristic (ROC) curve analysis. P-values <0.05 were considered significant.

## **Results**

**Western blot analysis of serum anti-PD-1 antibody.** We performed co-immunoprecipitation assay of activated PBMC lysate from a healthy volunteer and serum IgG from type 1 AIH patients, followed by western blot analysis (n = 3). Western blot analysis showed the protein band stained with anti-human PD-1 antibody (R&D Systems) (Fig. 1). This indicates that IgG-isotype antibodies binding to PD-1 molecules expressed on activated T cells exist in sera of some type 1 AIH patients.

**Titers and prevalence of serum anti-PD-1 antibody.** Titers of serum anti-PD-1 antibodies were significantly higher in type 1 AIH patients {0.101 (0.037-0.539) OD<sub>630nm</sub>} than in DILI patients {0.044 (0.005-0.104) OD<sub>630nm</sub>}, AVH patients {0.062 (0.015-0.186) OD<sub>630nm</sub>}, PSC patients {0.037 (0.020-0.357) OD<sub>630nm</sub>}, and healthy

volunteers {0.033 (0.002-0.144) OD<sub>630nm</sub>} (Fig. 2). When the cut-off level was represented by a mean absorbance + 2 SD in healthy volunteers (= 0.086 OD<sub>630nm</sub>), positivity for serum anti-PD-1 antibodies was shown in 63% of type 1 AIH patients, 8% of DILI patients, 13% of AVH patients, 18% of PSC patients, and 3% of healthy volunteers.

**Serum anti-PD-1 antibody and clinical characteristics.** In type 1 AIH patients, titers of serum anti-PD-1 antibodies were correlated with serum levels of bilirubin ( $r = 0.31$ ,  $P = 0.030$ ), aspartate aminotransferase (AST) ( $r = 0.29$ ,  $P = 0.042$ ), and ALT ( $r = 0.31$ ,  $P = 0.027$ ); however titers of serum anti-PD-1 antibodies were not correlated with serum IgG levels ( $r = 0.12$ ,  $P = 0.40$ ). In DILI patients, AVH patients, and PSC patients, titers of serum anti-PD-1 antibodies did not correlate with serum levels of bilirubin or AST, ALT.

The association of serum anti-PD-1 antibodies with ANA was analyzed. Type 1 AIH patients positive for ANA (1:40 or higher) had higher titers {0.113 (0.037-0.539) OD<sub>630nm</sub> versus 0.074 (0.038-0.439) OD<sub>630nm</sub>;  $P = 0.041$ } and higher prevalence (70% versus 33%;  $P = 0.039$ ) of serum anti-PD-1 antibodies than those negative for ANA.

Anti-smooth muscle cell antibodies (ASMA) was measured in 34 type 1 AIH

patients. Of them, 18 patients (53%) were positive for ASMA (1:40 or higher). ASMA positivity was not associated with either titers {0.119 (0.041-0.439) OD<sub>630nm</sub> versus 0.115 (0.038-0.411) OD<sub>630nm</sub>; P = 0.97} or prevalence (83% versus 69%; P = 0.32) of serum anti-PD-1 antibodies.

Histologically, of 52 type 1 AIH patients, seven were diagnosed with acute hepatitis, and the remaining 45 patients were diagnosed with chronic hepatitis. Patients with acute hepatitis showed higher titers of serum anti-PD-1 antibodies than those with chronic hepatitis {0.179 (0.076-0.439) OD<sub>630nm</sub> versus 0.097 (0.037-0.539) OD<sub>630nm</sub>; P = 0.016}. Of 7 patients diagnosed with acute hepatitis, 6 (86%) were positive for serum anti-PD-1 antibodies. Of 45 patients with chronic hepatitis, 27 showed early stage of liver fibrosis (F1 or F2), and the remaining 18 did the advanced stage (F3 or F4); however titer of serum anti-PD-1 antibodies {0.101 (0.041-0.539) OD<sub>630nm</sub> versus 0.093 (0.037-0.340) OD<sub>630nm</sub>; P = 0.58} were not differed between patients showing early stage of liver fibrosis and those showing the advanced stage.

**Serum anti-PD-1 antibody and the diagnosis of type 1 AIH.** The AUC of serum anti-PD-1 antibody for the discrimination of type 1 AIH from DILI, AVH, PSC, and healthy volunteers was 0.88 (95% confidence interval 0.80-0.96; P <0.001), 0.79 (95%

confidence interval 0.69-0.89;  $P < 0.001$ ), 0.80 (95% confidence interval 0.60-0.99;  $P = 0.002$ ), and 0.93 (95% confidence interval 0.89-0.97;  $P < 0.001$ ), respectively. **On the other hand, the AUC of ANA for the discrimination of type 1 AIH from DILI and PSC was 0.89 (95% confidence interval 0.81-0.96;  $P < 0.001$ ) and 0.91 (95% confidence interval 0.84-0.98;  $P < 0.001$ ), respectively.**

When patients positive for serum anti-PD-1 antibodies were diagnosed with type 1 AIH, the sensitivity, specificity, and positive and negative predictive values in the differential diagnosis between type 1 AIH and DILI were 63%, 92%, 94%, and 54%, respectively. Furthermore, those in the differential diagnosis between type 1 AIH histologically diagnosed with acute hepatitis and DILI were 86%, 92%, 75%, and 96%, respectively.

Similarly, the sensitivity, specificity, and positive and negative predictive values in the differential diagnosis between type 1 AIH and AVH were 63%, 87%, 89%, and 58%, respectively. In addition, those in the differential diagnosis between type 1 AIH histologically diagnosed with acute hepatitis and AVH were 86%, 87%, 60%, and 96%, respectively.

In the differential diagnosis between type 1 AIH and PSC, the sensitivity, specificity, and positive and negative predictive values were 64%, 82%, 94%, and 32%,

respectively.

**Serum anti-PD-1 antibody and treatment response.** Of 43 patients treated with PSL as the initial treatment, 7 were excluded from this analysis because their serum ALT level at the time of the initiation of PSL treatment was 30 IU/l or lower. So, in the remaining 36 patients, the association of positivity for serum anti-PD-1 antibodies with the normalization of serum ALT levels was investigated. There was no difference in serum ALT levels before the initiation of PSL treatment between 27 patients positive for serum anti-PD-1 antibodies and 9 negative for serum anti-PD-1 antibodies {335 (59-1783) IU/l versus 214 (59-2161) IU/l;  $P = 0.49$ }. Starting dose of PSL was similar between the two groups {40 (20-60) mg/day versus 40 (20-50) mg/day;  $P = 0.80$ }. The normalization of serum ALT levels after the initiation of PSL treatment was later in patients positive for serum anti-PD-1 antibodies (Fig. 3, log-rank test:  $p = 0.024$ ).

Of 47 patients achieving the normalization of serum ALT levels, 2 were transferred to other hospitals within 6 months from the normalization of serum ALT levels. So, in the other 45 patients, the association of positivity for serum anti-PD-1 antibodies with relapse of the disease was investigated. Of the 45 patients, 29 were positive for serum anti-PD-1 antibodies. There was no difference in the follow-up

duration after the normalization of serum ALT levels between 29 patients positive for serum anti-PD-1 antibodies and 16 patients negative for serum anti-PD-1 antibodies {89.1 (7.5-173.2) months versus 63.4 (11.4-209.6) months;  $P = 0.41$ }. In 19 of 29 patients (66%) positive for serum anti-PD-1 antibodies and 5 of 16 patients (31%) negative for serum anti-PD-1 antibodies, the disease relapsed ( $P = 0.027$ ).

## **Discussion**

In type 1 AIH patients, serum IgG levels are shown to be associated with disease activity,<sup>12,13</sup> relapse after drug withdrawal,<sup>14</sup> and recurrence of the disease after liver transplantation.<sup>15</sup> Serum IgG of type 1 AIH patients may contain some autoantibodies associated with the pathogenesis of the disease.

This study suggests that IgG-isotype PD-1 antibodies exist in sera of some type 1 AIH patients and that serum anti-PD-1 antibodies may be useful for the discrimination of type 1 AIH from DILI, AVH and PSC as an auxiliary diagnostic marker. Furthermore, serum anti-PD-1 antibodies were shown to be associated with the disease activity and the response to corticosteroid treatment. Patients positive for serum anti-PD-1 antibodies show severer disease and more frequently relapse. Patients negative for serum anti-PD-1 antibodies better respond to corticosteroid treatment. Recently,

repeated relapses have been reported to be associated with poor prognosis.<sup>16</sup>

Measurement of serum anti-PD-1 antibodies before the initiation of corticosteroid treatment may be also useful for the prediction of prognosis in type 1 AIH.

Serum IgG level and ANA are important markers for the diagnosis of type 1 AIH.

The diagnosis of type 1 AIH showing atypical features such as lower serum IgG levels and negativity for ANA is not easy.<sup>17,18</sup> A recent nationwide survey in Japan revealed

that approximately 10% of type 1 AIH patients showed histological features of acute hepatitis with lower serum IgG levels at presentation.<sup>19,20</sup> On the other hand,

approximately 20-30% of general population has been reported to be positive for ANA.<sup>21,22</sup> In this study, 4 of 7 type 1 AIH patients histologically diagnosed with acute

hepatitis showed serum ANA titers of 1:40 or less, and 3 of these 4 patients were positive for serum anti-PD-1 antibodies. And, of 6 patients showing serum IgG levels

below 2 g/dl and serum ANA titers of 1:40 or less, 3 were positive for serum anti-PD-1 antibodies. Furthermore, 27 of 40 patients whose serum titers of either ANA or ASMA

were 1:80 or higher showed positivity for serum anti-PD-1 antibodies, and 6 of 12 patients whose serum titers of both ANA and ASMA were 1:40 or less showed positivity

for serum anti-PD-1 antibodies. So, we speculate that serum anti-PD-1 antibodies may be useful for the diagnosis of type 1 AIH as an auxiliary diagnostic marker.

This study did not show functional effect of serum anti-PD-1 antibodies on lymphocytes although several studies have shown the following findings in type 1 AIH patients: 1) hyper-responsiveness of CD8<sup>+</sup> T cells to antigen,<sup>23</sup> 2) apoptosis-resistance in CD4<sup>+</sup> CD25<sup>-</sup> T cells and CD8<sup>+</sup> T cells,<sup>24</sup> 3) reduced expression of FOXP3 in CD4<sup>+</sup> CD25<sup>+</sup> T cells,<sup>24,25</sup> 4) decreased number of CD4<sup>+</sup> CD25<sup>+</sup> T cells,<sup>23,25</sup> 5) reduced ability of CD4<sup>+</sup> CD25<sup>+</sup> T cells to regulate CD8<sup>+</sup> T cells proliferation.<sup>23</sup> The similar phenomena are shown to be developed by using anti-PD-1 antibody. Anti-PD-1 antibody accelerates the proliferation of CD8<sup>+</sup> T cells and enhances the production of IFN- $\gamma$ , TNF- $\alpha$  and IL-2 from CD8<sup>+</sup> T cells.<sup>26</sup> Furthermore, anti-PD-1 antibodies decrease the number and protective effect of CD4<sup>+</sup> CD25<sup>+</sup> T cells.<sup>27-29</sup> In this study, titers of serum anti-PD-1 antibodies were correlated with serum levels of bilirubin and transaminase in type 1 AIH patients. Thus, we speculate that anti-PD-1 antibodies may be associated with the pathogenesis of type 1 AIH.

In summary, this study suggests that anti-PD-1 antibodies will exist in sera of some type 1 AIH patients, and that serum anti-PD-1 antibodies may be useful for the discrimination of type 1 AIH from DILI, AVH and PSC as an auxiliary diagnostic marker. Furthermore, anti-PD-1 antibodies may be associated with clinical features of type 1 AIH. In order to confirm these findings, further studies are required. The role of

anti-PD-1 antibodies in the pathogenesis of type 1 AIH may be worth investigating.

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**Table 1.** Clinical characteristics of study population.

|  | Type 1 AIH       | DILI             | AVH              | PSC              |
|--|------------------|------------------|------------------|------------------|
| Sample size, n                         | 52               | 24               | 30               | 11               |
| Age, yr                                | 52 (16-84)       | 41 (14-73)       | 42 (16-79)       | 35 (19-68)       |
| Gender, female (%)                     | 46 (88)          | 10 (42)          | 12 (40)          | 2 (18)           |
| Laboratory data                        |                  |                  |                  |                  |
| WBC ( $\times 10^3/\text{mm}^3$ )      | 5.1 (1.0-11.5)   | 5.9 (3.5-12.5)   | 6.3 (3.9-18.6)   | 5.7 (4.2-9.1)    |
| Hemoglobin (g/dl)                      | 12.6 (8.3-16.5)  | 13.3 (11.5-15.9) | 14.5 (10.8-18.4) | 12.3 (7.8-15.5)  |
| Platelet ( $\times 10^4/\text{mm}^3$ ) | 16.1 (5.6-36.7)  | 23.9 (10.7-44.0) | 18.2 (2.2-33.6)  | 26.1 (10.3-74.3) |
| Bilirubin (mg/dl)                      | 1.2 (0.5-22.6)   | 1.3 (0.5-26.0)   | 5.1 (0.8-25.4)   | 1.3 (0.4-14.8)   |
| AST (IU/l)                             | 216 (45-2330)    | 118 (30-3590)    | 1445 (112-5540)  | 72 (35-138)      |
| ALT (IU/l)                             | 229 (33-2161)    | 205 (38-6170)    | 2501 (266-7990)  | 60 (49-293)      |
| IgG(g/dl)                              | 2.55 (1.34-6.56) | 1.33 (0.77-3.15) | 1.48 (0.60-2.37) | 1.83 (1.30-3.45) |
| ANA (%)                                |                  |                  |                  |                  |
| <1:40                                  | 9 (17)           | 21 (87)          | —                | 11 (100)         |
| $\geq 1:40$ and $\leq 1:80$            | 11 (21)          | 3 (13)           | —                | 0 (0)            |
| $\geq 1:160$                           | 32 (62)          | 0 (0)            | —                | 0 (0)            |

**Table 1.** Continued.

| Healthy    | P value |
|------------|---------|
| 62         | —       |
| 44 (24-78) | 0.026   |
| 40 (65)    | <0.0001 |
| —          | 0.018   |
| —          | 0.0004  |
| —          | 0.0073  |
| —          | 0.0013  |
| —          | <0.0001 |
| —          | <0.0001 |
| —          | <0.0001 |
| —          | <0.0001 |
| —          |         |
| —          |         |
| —          |         |

AIH, autoimmune hepatitis; ALT, alanine aminotransferase; ANA, anti-nuclear

antibody; AST, aspartate aminotransferase; AVH, acute viral hepatitis; DILI, drug-induced liver injury; IgG, immunoglobulin G; WBC, white blood cell; PSC, primary sclerosing cholangitis.

P value is based on the statistical analysis by the Kruskal–Wallis test assessing overall group differences.

**Figure legends**

**Figure 1.** Identification of serum anti-programmed cell death-1 antibodies by western blot analysis. Serum anti-programmed cell death-1 antibodies in type 1 autoimmune hepatitis patients were identified by co-immunoprecipitation of activated peripheral blood mononuclear cell lysate and serum immunoglobulin G followed by western blot analysis (n = 3). Activated peripheral blood mononuclear cell lysate was used as positive control.

**Figure 2.** Titers of serum anti-programmed cell death-1 antibodies in study populations. Closed circles show positivity for serum anti-programmed cell death-1 antibodies. Open circles show negativity for serum anti-programmed cell death-1 antibodies.

**Figure 3.** Cumulative incident rates of the normalization of serum alanine aminotransferase levels after the initiation of prednisolone treatment.