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ORIGINAL ARTICLE

# Association of a single nucleotide polymorphism upstream of ICOS with Japanese autoimmune hepatitis type 1

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**Autoimmune hepatitis (AIH) is an uncommon chronic autoimmune liver disease. Several studies reported the association of polymorphisms between CD28, CTLA4 and ICOS gene cluster in 2q33.2 with autoimmune or inflammatory diseases. The previous genome-wide association study on type 1 AIH in a European population has reported a risk G allele of a single nucleotide polymorphism (SNP), rs4325730, in this region. Here, we conducted an association study of this SNP with type 1 AIH in a Japanese population, as a replication study.An association study of rs4325730 was conducted in 343 Japanese AIH patients and 315 controls.We found that rs4325730 is associated with AIH ($P=0.0173$, odds ratio (OR) 1.30, 95% confidence interval (CI) 1.05–1.62, under the allele model for G allele, $P=0.0070$, OR 1.62, 95% CI 1.14–2.31, under the dominant model for G allele). This SNP was strongly associated with definite AIH ($P=0.0134$, OR 1.36, 95% CI 1.07–1.74; under allele model for G, $P=0.0035$, OR 1.85, 95% CI 1.22–2.81, under dominant model for G).This is the first replication association study of rs4325730 upstream of ICOS with AIH in the Japanese population and rs4325730G is a risk allele.**
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## INTRODUCTION

Autoimmune hepatitis (AIH) is an uncommon chronic autoimmune liver disease that can cause cirrhosis.[1–3] AIH is characterized by the elevated transaminases and immunoglobulin levels and the presence of auto-antibodies and interface hepatitis. Type 1 AIH is distinguished by the presence of serum anti-nuclear antibodies or anti-smooth muscle antibodies and type 2 AIH by type 1 liver-kidney microsomal antibodies. AIH in Japanese populations is consisted mainly of type 1. The genetic risk factors are thought to be associated with AIH. AIH is associated with human leukocyte antigen (*HLA*)-*DRB1*03:01*, and *DRB1*04:01* in European populations[4] and *DRB1*04:05* in Japanese populations.[5–7] Polymorphisms in non-*HLA* genes also confer the genetic risk of AIH. A recent genome-wide association study of type 1 AIH in a European population has identified genetic risk factors in *HLA* and non-HLA regions.[8] Genetic associations of SNPs in non-*HLA* genes with AIH in Japanese populations were also investigated; associations were detected in *STAT4*[9] and *PTPN22*,[10] but not in *CARD10*,[11] *CTLA4*[12] or *FCRL3*.[13]

*CD28*, *CTLA4* and *ICOS* genes are clustered in human chromosome 2q33.2. The products of these genes were CD28-family member molecules and play important roles on the T cell stimulation, transducing co-stimulatory or inhibitory signals. It was predicted that these genes play key roles in autoimmune disease or tumor bearing patients. The previous genome-wide association study on type 1 AIH in a European population has reported a risk G allele of a single nucleotide polymorphism (SNP), rs4325730, in this region.[8] This SNP is located upstream of *ICOS* and is in strong linkage disequilibrium in European with rs4675374 ($r^2=1$)[8] that was reported to be associated with celiac disease.[14,15] Here, we conducted an association study of this SNP with type 1 AIH in a Japanese population, as a replication study.

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**Table 1 Genotype and allele frequencies of rs4325730 in the AIH patients and the healthy controls**

| | | *Genotype* | | | *Allele* | *Allele model* | | | *Dominant model* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *n* | *[G/G]* | *[G/A]* | *[A/A]* | *[G]* | *P-value* | *OR* | *95% CI* | *P*-value | *OR* | *95% CI* |
| Overall AIH, *n* (%) | 343 | 81 (23.6) | 189 (55.1) | 73 (21.3) | 351 (51.2) | 0.0173 | 1.30 | (1.05–1.62) | 0.0070 | 1.62 | (1.14–2.31) |
| Definite AIH, *n* (%) | 214 | 51 (23.8) | 122 (57.0) | 41 (19.2) | 224 (52.3) | 0.0134 | 1.36 | (1.07–1.74) | 0.0035 | 1.85 | (1.22–2.81) |
| Control | 315 | 62 (19.7) | 157 (49.8) | 96 (30.5) | 281 (44.6) | | | | | | |

Abbreviations: AIH, autoimmune hepatitis; CI, confidence interval; OR, odds ratio.
Genotype and allele frequency are shown in parenthesis (%). Association was tested by chi-square analysis using 2×2 contingency tables under the indicated models for G allele.

## MATERIALS AND METHODS

### Patients and controls

Type 1 AIH patients ($n=343$; median age (interquartile range), 64 (55–73), 41 male (12.0%)) who satisfied the criteria of International Autoimmune Hepatitis Group (IAIHG score>9)[16] without any other types of liver diseases were enrolled from the register of Japanese National Hospital Organization (NHO) Liver Registry. Among 343 AIH patients, 214 were definite AIH patients (IAIHG score>15, 62.4%). The healthy controls ($n=315$; median age (interquartile range), 36 (31–46), 2 male (0.6%)) were recruited at Sagamihara Hospital or by the Pharma SNP Consortium (Tokyo, Japan).[17] All the patients and healthy individuals were native Japanese living in Japan. This study was reviewed and approved by the NHO central Institutional Review Board and University of Tsukuba Research Ethics Committee. Written informed consent was obtained from each individual. This study was conducted in accordance with the principles expressed in the Declaration of Helsinki.

### Genotyping

Genotyping of rs4325730 (G/A) upstream of *ICOS* was performed using Custom TaqMan SNP Genotyping Assay (Thermo Fisher Scientific Inc., Waltham, MA, USA) on 7500 Fast Real-Time PCR System (Thermo Fisher Scientific Inc.), according to the manufacturer's instructions. Thermal cycling conditions consisted of initial denaturation at 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s followed by 60 °C for 30 s. A representative allelic discrimination plot of rs4325730 is shown in Supplementary Figure 1.

### Statistical analysis

The distribution of allele and genotype frequencies was compared between AIH patients and healthy controls by chi-square analysis using 2×2 contingency tables. Overall AIH or definite AIH subset were compared with healthy control. Association of clinical features of the AIH patients with or without G allele of rs4325730 was tested by chi-square analysis using 2×2 contingency tables or Mann–Whitney's U Test. This study had 80% statistical power to detect associations when the genotype relative risk was higher than 1.36.[18]

## RESULTS

### Association analysis of rs4325730 upstream of ICOS with AIH

The genotyping of rs4325730 was performed in Japanese AIH patients and healthy controls. No deviation from Hardy-Weinberg equilibrium was detected in the controls ($P=0.8790$) or overall AIH patients ($P=0.0573$). The number of heterozygout AIH patients tended to be higher than expected. A significant association was found for rs4325730 ($P=0.0173$, odds ratio (OR) 1.30, 95% confidence interval (CI) 1.05–1.62, Table 1) under the allele model for G allele. This SNP was also significantly associated with the overall AIH ($P=0.0070$, OR 1.62, 95% CI 1.14–2.31) under the dominant model for G allele. We further explored associations between rs4325730 and definite AIH and rs4325730 was more significantly associated with definite AIH ($P=0.0134$, OR 1.36, 95% CI 1.07–1.74; under allele model for G, $P=0.0035$, OR 1.85, 95% CI 1.22–2.81, under dominant model for G). Thus, the association of rs4325730 with AIH was detected in the Japanese population and more important roles of the SNP in definite AIH were suggested.

**Table 2 Comparison of the demographics between AIH patients with or without rs4325730G allele**

| | *rs4325730G (+)* | *rs4325730G (−)* | *P-value* |
|---|---|---|---|
| Number | 270 | 73 | |
| Male, *n* (%) | 34 (12.6) | 7 (9.6) | 0.4828[a] |
| Age at onset, years (IQR) | 60 (50–69) | 61 (52–67) | 0.8920 |
| Albumin (g $dl^{-1}$) (IQR) | 3.9 (3.5–4.2) | 3.9 (3.4–4.2) | 0.6562 |
| Total bilirubin (mg $dl^{-1}$) (IQR) | 1.3 (0.8–4.5) | 1.1 (0.7–3.4) | 0.1703 |
| AST(IU $l^{-1}$) (IQR) | 298 (104–725) | 184 (75–722) | 0.2148 |
| ALT(IU $l^{-1}$) (IQR) | 314 (109–819) | 238 (73–693) | 0.2517 |
| ALP(IU $l^{-1}$) (IQR) | 424 (313–566) | 447 (320–587) | 0.9026 |
| IgG (mg $dl^{-1}$) (IQR) | 2234 (1777–2865) | 2117 (1833–2955) | 0.7969 |
| Platelets ($10^4$ $\mu l^{-1}$) (IQR) | 19 (13–24) | 17 (13–21.2) | 0.0729 |
| Anti-nuclear antibodies ⩾1:40, *n* (%) | 238 (88.1) | 65 (89.0) | 0.8330[a] |
| Anti-smooth muscle antibodies ⩾1:40, *n* (%) | 93 (39.7) | 23 (33.8) | 0.3770[a] |
| Cirrhosis, *n* (%) | 35 (13.0) | 14 (19.2) | 0.1782[a] |

Abbreviations: AIH, autoimmune hepatitis; IQR, interquartile ranges.
[a]Chi-square analysis was employed. Numbers or median values of each group are shown. Percentages or IQRs are shown in parenthesis.
Association was tested between AIH patients with or without rs4325730G allele by chi-square analysis using 2×2 contingency tables or Mann–Whitney's U Test.

Finally, we analyzed the clinical phenotypes of AIH patients with or without G allele of rs4325730. No significant difference of demographic features of the AIH patients with or without G allele of rs4325730 was detected (Table 2).

## DISCUSSION

The previous genome-wide association study reported an association of SNPs in non-*HLA* regions, rs3184504 in *SH2B3* and rs6000782 in *CARD10,* with type I AIH.[8] Polymorphism of rs3184504 was not detected in Japanese and rs6000782 was not associated with AIH in Japanese.[11] The present study showed that rs4325730G is a risk allele for type I AIH in the Japanese population. To the best of our knowledge, we were the first to replicate the association of SNPs in non-*HLA* region reported in the genome-wide association study with Japanese AIH.

Several studies reported the association of polymorphisms between *CD28*, *CTLA4* and *ICOS* gene cluster in 2q33.2 with autoimmune or inflammatory diseases. However, most of the disease-associated variants reported were located between *CD28* and *CTLA4*.[19–22] A few studies identified disease-associated SNPs in *ICOS* gene.[14,15] These studies reported a celiac disease associated SNP, rs4675374, located in the first intron of *ICOS*. The location of rs4325730 is 3 kbps upstream of *ICOS* gene. Two SNPs, rs4675374 and rs4325730 are in strong linkage disequilibrium in European ($r^2=1$)[8] and Japanese ($r^2=0.981$, http://www.ensembl.org/), indicating the shared susceptible SNPs between celiac disease and type I AIH. These data suggested

the common signaling pathways in the pathogenesis of these diseases. Future fine mapping studies of this region should be performed.

AIH shares some clinical features with systemic lupus erythematosus (SLE)[23,24] and also shares some susceptible genes, *HLA*[4–7,25] and *STAT4*.[9,26] In the present study, rs4325730 upstream of *ICOS* was associated with AIH, but the association of SNPs in *CTLA4* was not detected in Japanese AIH.[12] On the other hand, it was reported that SNPs upstream of *ICOS* was not associated with SLE in Japanese.[27] An association was reported between SLE and SNPs located in *CTLA4*.[28] These results suggested that SLE and type I AIH do not share susceptible SNPs in this region.

We first reported the association of polymorphisms upstream of *ICOS* with Japanese AIH. However, functional influences and pathological roles of the SNP in AIH are still unknown. The SNP, rs4325730, might be in histone marks sites (http://www.broadinstitute.org/mammals/haploreg/haploreg.php)[29] and influence the expression pattern of *CD28* and *ICOS* genes (http://www.genenetwork.nl/bloodeqtlbrowser/).[30] ICOS molecules are expressed on activated T cells and play important roles in the maintenance of the T cell activation. Since ICOS-deficient mice have reduced germinal center formation,[31] elevated expression levels of ICOS molecules may change the development of T follicular helper cells and the formation of germinal centers, leading to the altered development of autoreactive B cells.

We also detected a stronger association of rs4325730 with definite AIH patients than overall AIH. It was suggested that the predisposing effects of rs4325730 was enhanced in AIH patients diagnosed more strictly, though other each clinical manifestation of AIH is not correlated with the presence of rs4325730 risk allele (Table 2). Since rs4325730G allele frequencies in other ethnic populations are different, the association of the SNP with AIH in other populations should be replicated. Although it is difficult to increase the sample size, the associations of rs4325730 should be confirmed in future large scale studies.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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