Table 1. *TNFSF15* SNP rs4979462 and *POU2AF1* SNP rs4938534 Associated with Susceptibility to PBC

dbSNP rsID	Nearest Gene	Risk Allele	Allele (1/2)	Stage	PBC Cases				Healthy Controls				OR[a]	
					11	12	22	RAF	11	12	22	RAF	95% CI	p Value[b]
rs4979462	*TNFSF15*	T	T/C	GWAS	154 (31.8)	244 (50.4)	86 (17.8)	0.57	98 (20.7)	230 (48.5)	146 (30.8)	0.45	1.63 (1.36–1.95)	1.21×10^{-7}
				Replication	253 (32.3)	390 (49.7)	141 (18.0)	0.57	131 (21.6)	305 (50.3)	170 (28.1)	0.47	1.52 (1.30–1.76)	5.79×10^{-8}
				Combined	407 (32.1)	634 (50.0)	227 (17.9)	0.57	229 (21.2)	535 (49.5)	316 (29.3)	0.46	1.56 (1.39–1.76)	2.84×10^{-14}
rs4938534	*POU2AF1*	A	G/A	GWAS	114 (23.6)	229 (47.3)	141 (29.1)	0.53	151 (31.8)	247 (52.0)	77 (16.2)	0.42	1.53 (1.28–1.83)	3.51×10^{-6}
				Replication	179 (22.8)	391 (49.8)	215 (27.4)	0.52	179 (29.4)	299 (49.2)	130 (21.4)	0.46	1.29 (1.11–1.50)	9.32×10^{-4}
				Combined	293 (23.1)	620 (48.9)	356 (28.1)	0.52	330 (30.5)	546 (50.4)	207 (19.1)	0.44	1.39 (1.24–1.56)	2.38×10^{-8}

Parenthetical numbers indicate the percentage of allele 11, 12, or 22 among total alleles in PBC cases or healthy controls. The following abbreviations are used: PBC, primary biliary cirrhosis; RAF, risk allele frequency; and GWAS, genome-wide association study.
[a]Odds ratio (OR) of minor allele from the two-by-two allele frequency table.
[b]p value of Pearson's chi-square test for the allelic model.

of 1,402 samples (787 Japanese PBC cases and 615 Japanese healthy controls) and the original set of 963 samples (487 PBC cases and 476 healthy controls) using the DigiTag2[13] and custom TaqMan SNP genotyping assays. Two SNPs, rs6890853 and rs9303277 located in loci *IL7R* and *IKZF3*, respectively, showed significant associations and the *STAT4* locus (rs7574865) showed suggestive association with PBC in 2,365 Japanese samples (1,274 PBC cases and 1,091 healthy controls) (rs6890853, combined p value = 3.66×10^{-8}, OR = 1.47 for *IL7R*; rs9303277, combined p value = 3.66×10^{-9}, OR = 1.44 for *IKZF3*; rs7574865, combined p value = 1.11×10^{-6}, OR = 1.35 for *STAT4*) (Tables S5 and S8).

Moreover, we genotyped 16 additional associated SNPs, all of which were the same SNPs as identified in previous studies,[4–7] and revealed that six out of 16 SNPs (located on *CXCR5*, *NFKB1*, *CD80*, *DENND1B*, *MAP3K7IP1*, and *TNFAIP2*) were replicated ($p < 0.05$) in 2,365 Japanese samples (Table S8). The SNP rs2293370, located in the *CD80* locus, showed a significant association and the *NFKB1* locus (rs7665090) showed a suggestive association with PBC in the Japanese population (rs2293370, combined p value = 3.04×10^{-9}, OR = 1.48 for *CD80*; rs7665090, combined p value = 1.42×10^{-7}, OR = 1.35 for *NFKB1*). Although further study for determining the primary SNP at each locus is necessary, the remaining ten loci (*RAD51L1*, *PLCL2*, *IL12RB2*, *IRF5*, *SPIB*, *RPS6KA4*, *CLEC16A*, *TNFRSF1A*, *IL12A*, and *MMEL1*) did not show significant association ($p < 0.05$) with PBC in the Japanese population (Table S8).

In the current GWAS in the Japanese population, we identified two significant susceptibility loci for PBC, *TNFSF15* (rs4979462) and *POU2AF1* (rs4938534), which had not been identified in the previous GWAS in populations of European descent. In addition, of the 21 PBC susceptibility loci that have been identified in populations of European descent, three loci (*IL7R*, *IKZF3*, and *CD80*) showed significant associations and two loci (*STAT4* and *NFKB1*) showed suggestive associations with PBC in the Japanese population. Eight other loci (*RAD51L1*, *CXCR5*, *PLCL2*, *IL12RB2*, *DENND1B*, *MAP3K7IP1*, *TNFAIP2*, and 7p14) also showed marginal associations with PBC in the Japanese population. These results indicate the presence of additional important disease pathways (via TNFSF15 and POU2AF1)—differentiation to T helper 1 (Th1) cells (via IL7R and STAT4), B cell differentiation (via IL7R and IKZF3), T cell activation (via CD80), and NF-κB signaling—in addition to the previously reported disease pathways in the development of PBC in Japanese populations.

TNFSF15 is a newly described member of the TNF superfamily that interacts with death receptor 3 (*DR3* [MIM 603366], also known as *TNFRSF25*) not only to promote effector T cell expansion (i.e., Th1 and Th17 cells) and cytokine production (i.e., interferon-γ [IFN-γ, MIM 147570]) at the site of inflammation, but also to induce apoptosis in cells that overexpress DR3.[15] Interestingly, genetic polymorphisms in *TNFSF15* are associated with susceptibility to CD, UC, ankylosing spondylitis (AS, MIM 106300), and leprosy (MIM 609888)[16–20] (Table S8). Strong association of five SNPs (rs3810936, rs6478108, rs6478109, rs7848647, and rs7869487) in the *TNFSF15* region with CD was first reported for a Japanese population,[16] and the finding was replicated in an independent Japanese population and in European-descent and Korean populations.[21–25] Another SNP within *TNFSF15* (rs4263839) is also associated with susceptibility to CD in populations of European descent.[17,20,26] In addition, the risk alleles of the SNPs were significantly associated with *TNFSF15* mRNA expression in peripheral blood.[27,28] Given that there exists strong LD among SNPs in *TNFSF15*, including those in the promoter region (rs6478109 and

rs7848647) and introns (rs4263839 and rs4979462), it is very probable that the PBC susceptibility haplotype containing rs4979462 also influences *TNFSF15* mRNA expression. Additionally, TNFSF15 signaling via DR3 synergizes with interleukin-12 (IL-12) and IL-18 to promote IFN-γ production.[15] The IL-12 signaling pathway includes *IL12A* and *IL12RB* (MIM 601604), variants of which have been identified as PBC susceptibility loci in previous GWASs of peoples of European ancestry, and has been implicated as a key player in the pathogenesis of PBC.[4–7] STAT4 is essential for IL-12 signal transduction via the IL-12 receptor (IL12R) for IFN-γ production and Th1 polarization.[29] Thus, the evidence that *TNFSF15* and *STAT4* were identified and confirmed as PBC susceptibility loci in the present study might indicate that the IL-12 signaling pathway via IL12R is also operative in PBC pathogenesis in Japanese populations, as it is in populations of European descent.

POU2AF1 is a B cell-specific transcriptional factor that coactivates octamer-binding transcriptional factors *POU2F1* (MIM 164175) and *POU2F2* (MIM 164176) on B cell-specific promoters; thus, *POU2AF1* is essential for B cell maturation and germinal center formation.[30] The E-twenty six transcription factor *Spi-B* was recently identified as a direct target of the coactivator *POU2AF1*.[31] *Spi-B* is an important mediator of both B cell receptor signaling and early T cell lineage decisions.[32,33] *Spi-B* also induces IL7R-induced CD40 (MIM 109535, MIM 300386) expression.[34] Given that *Spi-B* has been identified as a PBC susceptibility gene in previous GWASs of peoples of European ancestry,[6,7,35] variation of *POU2AF1* might function along with *Spi-B* in this pathway of B cell signaling and differentiation. The lack of *POU2AF1* reportedly prevents the development of autoimmunity in *Aiolos* (also known as *IKZF3*) mutant mice, which have a systemic lupus erythematosus (MIM 152700)-like phenotype, and in MRL-lpr mice.[36,37] *IKZF3* and *IL7R* were both replicated and confirmed as PBC susceptibility loci in this study; *IKZF3* functions as a transcription factor that participates in the generation of high-affinity bone marrow plasma cells responsible for long-term immunity, and *IL7R* participates in pre-B cell expansion.[38,39] Collectively, these results strengthen the notion that the B cell signaling pathway is involved in the development of PBC.

In conclusion, *TNFSF15* and *POU2AF1* were identified as significant susceptibility loci for PBC in a Japanese population. Our results provide further evidence for the presence of (1) ethnic differences in genetic susceptibility loci (i.e., *TNFSF15*, *IL12A*, and *IL12RB2*), (2) a new autoimmune pathway (i.e., TNFSF15 signaling) shared with other autoimmune diseases (CD, UC, and AS), and (3) common pathogenic pathways such as B cell differentiation (i.e., *POU2AF1*, *IKZF3*, and *SPIB*), IL-12 signaling (i.e., *IL12A*, *IL12RB2*, and *STAT4*), and T cell activation (i.e., *CD80*) for the development of PBC in individuals of European descent and Japanese individuals (Table S8). Functional analysis of these genetic loci, as well as the identification of additional susceptibility loci associated with PBC in eastern Asian populations, should facilitate the analysis of the pathogenesis of PBC worldwide and aid the development of rationale for therapies in the future.

Supplemental Data

Supplemental Data include two figures, eight tables, and Supplemental Acknowledgments and can be found with this article online at http://www.cell.com/AJHG/.

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Web Resources

The URLs for data presented herein are as follows:

MEXT Integrated GWAS Database, https://gwas.biosciencedbc.jp/cgi-bin/gwasdb/gwas_top.cgi
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org

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Long-term Outcome of Japanese Patients With Type 1 Autoimmune Hepatitis

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The long-term outcome of patients with autoimmune hepatitis (AIH) in Japan has not been well-defined. The aim of this study was to clarify the outcome of this disease over a long follow-up period compared with that of the general Japanese population as well as that among patients. A total of 203 AIH patients were enrolled for a mean follow-up period of 131 months. All patients were treated with corticosteroids with or without azathioprine. The overall survival of AIH patients was similar to that of the general population in Japan. The prognosis of AIH subgroups divided according to disease severity, sex, incidence of relapse, liver histology, presence of cirrhosis, probable or definite AIH score, antibody to hepatitis B core antigen antibody positivity, or human leukocyte antigen DR4–positivity did not differ greatly among patients. However, the prognosis of patients experiencing two or more relapses was significantly poorer than that of patients with remission or a single relapse both in univariate ($P < 0.001$) and multivariate ($P = 0.020$) analyses. The development of liver malignancy was also a possibility among AIH patients with multiple relapses. Severe adverse effects of corticosteroids were rare, even in patients who underwent long-term treatment. *Conclusion:* Repeated relapses of AIH are significantly associated with a poorer long-term prognosis in Japan. AIH patients can expect a similar prognosis to that of the general population, provided they are adequately managed with continuous low doses of immunosuppressive therapy, especially after the first relapse. (HEPATOLOGY 2012;56:668-676)

Autoimmune hepatitis (AIH) is an organ-specific autoimmune disease characterized by chronic inflammation of the liver, hypergammaglobulinemia, and autoantibodies.[1-3] Autoimmunity is believed to play a crucial role in AIH, although the mechanisms of disease development and progression remain unclear. Immunosuppressive therapies using corticosteroids and/or azathioprine have been established for AIH treatment[1-4] that lead to remission in most patients. However, more than half of patients relapse after treatment withdrawal,[5-8] so the emerging issue facing clinicians is if and when treatment should be withdrawn. In Japan, the withdrawal criteria for immunosuppressive therapies of AIH are not clearly established, and most practitioners continue treatment for long periods.

The long-term outcome of patients with AIH treated with corticosteroids and/or azathioprine has not been fully evaluated in Japan, but some reports have analyzed prognostic factors. Miyake et al.[9] showed that the prognosis of Japanese AIH patients was good if aminotransferases were sustained at normal levels, and Hino et al.[10] revealed the importance of achieving a good response to immunosuppressive therapies at treatment onset. No definitive reports exist as well on the survival of AIH patients compared with the general population in Japan. Among Caucasian patients with AIH, Kanzler et al.[11] reported that long-

Abbreviations: AIH, autoimmune hepatitis; ALT, alanine aminotransferase; AMA, antimitochondrial antibody; ANA, antinuclear antibody; anti-HBc, antibody to hepatitis B core antigen; CCC, cholangiocellular carcinoma; CI, confidence interval; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HLA, human leukocyte antigen.

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term survival in well-managed patients was excellent compared with that of the general population in Germany, although long-term or permanent immunosuppressive therapy was required. Montano-Loza et al.[12] revealed that multiple relapses were associated with poorer prognosis than sustained remission or single relapse in the United States.

In this study, we evaluate the long-term outcome of patients with AIH compared with the general population in Japan for the first time. We also clarify the factors affecting the outcome of this disease and the development of hepatic malignancies over a long follow-up period, especially with regard to age, sex, presenting symptoms, histology at presentation, human leukocyte antigen (HLA) phenotype, probable or definite AIH score, antibody to hepatitis B core antigen (anti-HBc) positivity, and incidence of relapse.

Patients and Methods

Study Design. This was a prospective cohort study performed at Shinshu University Hospital and its affiliated hospitals in Nagano prefecture and Ehime University Hospital dating from 1990. The medical records of patients diagnosed from 1974 to 1989 were retrospectively re-estimated using the international criteria for AIH[13] and diagnosed as having probable or definite AIH. This study was approved by the Ethics Committees of the Shinshu University School of Medicine and Ehime University School of Medicine. Written informed consent was obtained from each subject.

Study Population. Between 1974 and 2010, a total of 246 patients of Japanese ethnicity were diagnosed as having probable or definite AIH. Twenty patients were excluded because of incomplete medical records or hepatitis C virus (HCV) infection. Patients referred from other hospitals for liver transplantation were also excluded. Eleven patients died from fulminant hepatic failure due to poor treatment outcome within several weeks of diagnosis. Three patients died from hepatic failure after they stopped taking prednisolone without prior consultation with a doctor. Nine patients were treated with ursodeoxycholic acid only and thus excluded, leaving a total of 203 patients for evaluation of the long-term outcome of AIH for a mean period of 131 months (range, 13-432 months) (see Table 1 and Fig. 1), 95 of whom were followed for more than 10 years. Follow-up began from the time of diagnosis and was terminated with the most recent outpatient appointment at the hospital or at the time of death. No patients had a history of excess alcohol consumption. All patients were seronegative for hepatitis B surface antigen, anti-HCV antibody (second and third generation), and HCV RNA. Hepatitis C viral markers of patients diagnosed before 1992 were tested using sera at diagnosis that had been stored at −80°C. Anti-HBc was positive at a low titer in 26 patients (13.5%), in whom serum hepatitis B surface antigen or hepatitis B virus DNA never appeared during corticosteroid therapy, even at a relapse. In Japan, anti-HBc positivity is approximately 10%-15% in the general population above 30 years old.[14] Antinuclear antibodies (ANAs) were detected by indirect immunofluorescence on HEp-2 cells. ANAs were found in 95.6% of patients at a titer of ×40 or more and in 91.1% at a titer of x80 or more. Only 9 patients did not have detectable ANAs, although 5 of them were positive for anti-smooth muscle antibodies (×80 or more). One hundred twenty-three patients were tested for liver-kidney microsomal antibody type 1, and all but one patient, who was also ANA-positive and diagnosed with type 1 AIH, were negative. Type 1 AIH patients

Table 1. Characteristics of the Long-term Follow-up AIH Cohort (n = 203)

Characteristics	Values
Age at diagnosis, years	55.5 (12-86)
Sex, women/men	177/26
Mean observation period, months	131 (13-432)
IAIHG score before treatment	17.4 (10-22)
Definite AIH	172 (84.7)
Acute onset	142 (70.0)
Chronic onset	61 (30.0)
Severe symptom at diagnosis*	72 (35.5)
Cirrhosis at diagnosis	26 (12.8)
Stage F3 or F4 at diagnosis (n = 198)	88 (44.4)
HLA-DR4 (n = 169)	121 (71.6)
Overlap with primary biliary cirrhosis	8 (3.9)
Achieved remission	203 (100)
Treatment withdrawal	13 (6.4)
Relapse	48 (23.6)
Relapse two or more times	27 (13.3)
Death	22 (10.8)
Liver-related death	7 (3.4)
ANA ×40 or more	194 (95.6)
AMA or M2-positive	11 (5.4)
Platelet count, $\times 10^4/mm^3$	17.3 (3.9-33.6)
Bilirubin level, mg/dL†	5.7 (0.3-30.4)
ALT level, U/L‡	622.1 (47-5,586)
ALP level, U/L§	498.1 (101-1,984)
IgG level, mg/dL§§	2,981.2 (1,110-7,600)
Anti-HBc (n = 193)	26 (13.5)

Abbreviations: ALP, alkaline phosphatase; IAIHG, International Autoimmune Hepatitis Group; IgG, immunogloblin G.

Data are presented as median (range) or no. (%) unless noted otherwise.

*Severe symptom: total bilirubin >5.0 mg/dL and/or prothrombin time >40%.

†Normal range: 0.3-1.2 mg/dL.

‡Normal range: 7-45 U/L.

§Normal range: 124-367 U/L.

§§Normal range: 800-2,000 mg/dL.

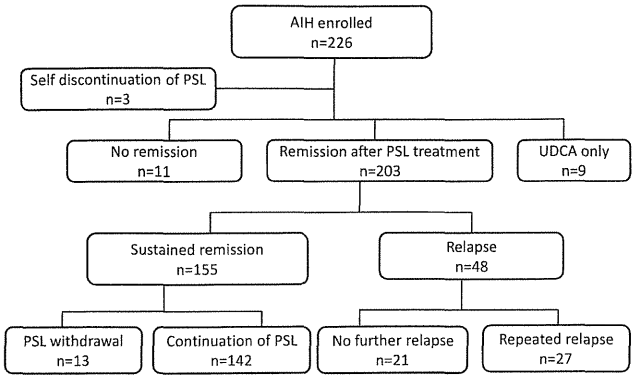

Fig. 1. Schematic of patient selection. PSL, prednisolone; UDCA, ursodeoxycholic acid.

were classified based on antibody profiles or liver histology. Histological material at diagnosis was available in 198 of 203 patients. Five patients received a biopsy after treatment, revealing abnormal activated partial thromboplastin time in one and prolonged prothrombin time or ascites in the others, all of which were improved by treatment. Histological examination was performed by a pathologist and classified according to the Metavir scoring system.[15] A follow-up liver biopsy was done in only a few nonrelapse patients who were judged to be in remission using laboratory data. Six of 19 patients underwent biopsy following a single relapse, which revealed histological relapse with no progression of staging in most cases. A repeated biopsy was performed in 13 of 27 patients with two or more relapses and showed histological stage progression in the majority of cases after the second or third relapse. All patients were assessed clinically and subjected to biochemical tests every 1 or 2 months. Twenty-one patients died during follow up (Table 2). One hundred sixty-nine patients were tested for HLA class I and II genotypes.

Treatment. Most patients with AIH were initially treated with prednisolone at an oral dosage of 20-50 mg/day that was gradually tapered after remission. Eighteen patients with fulminant hepatic failure or severe hepatitis were treated with a 500 to 1,000 mg methyl-prednisolone pulse for 3 days, followed by oral doses of 50-60 mg/day. Azathioprine at a dose of 50 mg/day was added for nine patients whose serum alanine aminotransferase (ALT) level did not normalize within 3 months or severe side effects were seen from prednisolone therapy. If patients exhibited an increase in serum ALT levels to more than twice the upper limit of normal (>90/L) during follow-up, prednisolone therapy was reinstituted. Most patients also took bisphosphonate, vitamin D, and/or vitamin K supplements.

Outcomes. We evaluated the outcomes of this disease for total patients, patients with definite AIH score, and patients with histological stage F3-F4 or grade A3 over a long follow-up period compared with survival rates of the general Japanese population. For evaluation of long-term disease outcome among AIH patients, we compared age, sex, disease severity, histological stage and activity, HLA-DR positivity, anti-HBc positivity, and incidence of relapse as indicators of liver-related death. Remission was defined as a return of ALT level ($\leq$45 U/L), serum immunoglobulin G level ($\leq$1,700 mg/dL), and bilirubin values to normal levels that was sustained for at least 6 months. A relapse of AIH was defined as an increase in serum ALT level to more than twice the upper limit of normal (>90 U/L). The maintenance of serum ALT levels of less than twice the upper limit of normal during follow-up constituted a sustained remission. Withdrawal of therapy was considered if remission was achieved and maintained for at least 2 years and the patient agreed to cessation.

Statistical Analysis. A cumulative survival curve was constructed for AIH patients followed for more than 1 year. Because the general population sample size was very large, the widths of its 95% confidence intervals (CIs) were nearly zero. Comparisons between patients and the age- and sex-matched general population in Japan were calculated using Kaplan-Meier statistics, and differences between groups were analyzed using a log-rank test. Information about the general population in Japan was obtained from the life tables published from 1974 to 2010 by the Japanese Ministry of Health, Labor, and Welfare.

Cutoff values were decided using receiver operating characteristic curve analysis, and results were evaluated by measuring the area under the curve. Fisher's exact and Pearson's chi-square tests were adopted to test for differences between subgroups of patients. To compare continuous data, the Mann-Whitney U test was employed. The Kaplan-Meier method was used to

Table 2. Causes of Death Among Long-Term Follow-up AIH Patients (n = 203)

Cause	Values
Hepatic malignancy (HCC or CCC)	6 (3.0)
Malignancy of other organs	7 (3.4)
Liver failure	1 (0.5)
Rheumatoid arthritis	1 (0.5)
Cerebral bleeding	1 (0.5)
Old age	3 (1.5)
Other	3 (1.5)

Data are presented as no. (%).

estimate rates of liver-related death or development of hepatocellular carcinoma (HCC). The log-rank test was used to test hypotheses concerning differences in liver-related death or development of HCC between selected groups. Multivariate analyses were performed using the Cox regression model. Variables associated with a $P < 0.5$ in univariate analyses were included in a stepwise Cox regression analysis to identify independent factors associated with liver-related death or development of HCC. All tests were performed using the IBM SPSS Statistics Desktop for Japan (version 19.0; IBM Japan Inc., Tokyo, Japan). P values of less than 0.05 were considered statistically significant.

Results

Table 1 shows the baseline characteristics of the 203 patients treated with immunosuppressive therapy who were followed for more than 12 months. Of the 214 patients treated with corticosteroids, 203 (94.9%) achieved remission and 11 died without remission in several weeks. One hundred forty-two patients had acute onset of symptoms prior to initial presentation. Seventy-two patients had severe disease symptoms (total bilirubin >5.0 mg/dL and/or prothrombin time <40%). Sixty-one patients had chronic onset AIH, with 35 of them having symptoms (fatigue, anorexia, or arthralgia) for more than 3 months before presentation. The remaining patients were asymptomatic at presentation, but abnormal liver findings were detected during health screening or examination for coexisting autoimmune diseases or other diseases that were not thought to affect AIH development, such as hypertension or diabetes mellitus. Twenty-three patients had a coexisting autoimmune feature, including rheumatoid arthritis in nine (4.4%), primary biliary cirrhosis in eight (3.9%), Sjögren's syndrome in eight (3.9%), autoimmune thyroiditis in 13 (6.4%), systemic lupus erythematosus in two (1.0%), and hyperthyroidism and CREST syndrome in one each (0.5%). Antimitochondrial antibody (AMA) or AMA M2 was positive in 11 patients who met the international AIH group criteria,[13] and eight of them were thought to have overlap syndrome based on their liver histology. Histology or laboratory data at diagnosis revealed cirrhosis in 26 patients, and histological findings showed F3 or F4 status in 88. Two hundred patients achieved remission within 3 months of prednisolone treatment. The remaining three patients entered remission within 6 months after the addition of azathioprine, which was administered to a total of nine patients because of poor response or adverse reactions to prednisolone. Immunosuppressive therapy was successfully withdrawn without relapse for more than 1 year in only 13 patients. In the remaining patients, prednisolone treatment was continued at 2.5-10.0 mg/day until the study endpoint. Forty-eight patients experienced at least one relapse during tapering or after cessation of immunosuppressive therapy. Twenty-seven patients experienced relapses two or more times (Fig. 1). The occurrence of relapse did not differ between acute and chronic onset patients.

As shown in Table 2, 22 patients died during the follow-up period. Five died from HCC and 1 from cholangiocellular carcinoma. One patient who experienced repeated relapses died from hepatic failure. We defined these seven patients as having succumbed to a liver-related death. Two patients developed HCC but were alive at the study endpoint. Seven patients died from a malignancy of other organs. Three elderly patients who presented at 59, 67, and 73 years of age succumbed to old age after follow-up periods of 22.0, 17.2, and 14.8 years, respectively. Another patient who presented at 75 years of age died from cerebral bleeding after 13.1 years of follow-up.

The overall survival of AIH patients was similar to that of the general population in Japan (Fig. 2A). The survival of AIH patients with definite AIH score was also similar to that of the general population (Fig. 2B), as were the cumulative survival curves of AIH patients with histological stage F3-F4 or grade 3 (A3) compared with F1-F2 or A0-A2, respectively (Fig. 2C,D). Among AIH patients, the liver-related death rates of AIH subgroups divided by sex, HLA-DR4 positivity, anti-HBc positivity, age at diagnosis, acute onset of disease, disease severity, presence of cirrhosis, or liver histology did not differ significantly. The incidence of relapse was significant on univariate analysis ($P = 0.001$) and the log-rank test ($P = 0.016$) (Table 3). The prognosis of two or more relapses was poorer than that of remission or a single relapse in both Fisher's exact test ($P < 0.001$) (Table 3) and log-rank test ($P = 0.003$) (Table 3 and Fig. 3A). On multivariate analysis, the prognosis of repeated relapses was selected as the only risk factor associated with liver-related death (hazard ratio, 12.8; 95% CI, 1.5-109.9; $P = 0.020$) (Table 3).

During the follow-up period, eight AIH patients developed hepatic malignancy (see Table 4) over a mean period of 14.5 years (range, 9-21 years). Six of these patients experienced multiple relapses, and four of them were biopsied two or more times. Histological stages had progressed in three patients, reaching F4 from F3 in two patients and F3 from F2 in one patient. One patient with A1F1 histology at AIH

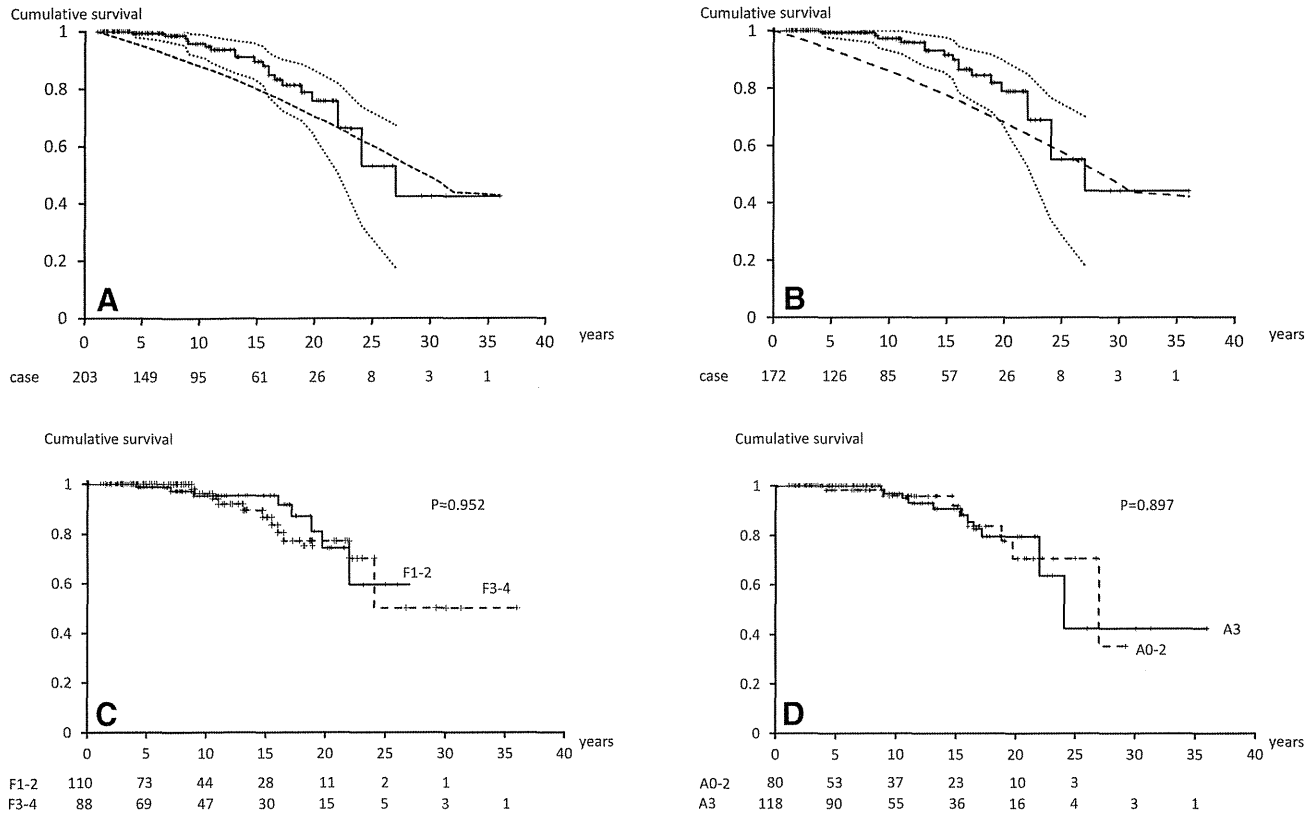

Fig. 2. (A) Cumulative overall survival curve (solid line) and its 95% CI (dotted lines) for patients with AIH. The estimated survival of the age- and sex-matched Japanese general population is represented by a broken line. The survival of patients is similar to that of the general population in Japan. (B) Cumulative overall survival curve (solid line) and its 95% CI (dotted lines) for patients with definite AIH. The estimated survival of the age- and sex-matched Japanese general population is represented by a broken line. The survival of patients is similar to that of the general population in Japan. (C) Cumulative survival curve of patients with histological stage 1 and 2 (F1 and F2) (solid line) and of patients with histological stage 3 and 4 (F3 and 4) (dotted lines). The survival curves of both groups of patients is similar. (D) Cumulative survival curve of patients with histological grade 3 (A3) (solid line) and of patients with histological grade 0-2 (A0-A2) (dotted lines). The survival curves of both groups of patients is similar.

diagnosis who did not undergo follow-up biopsy had cirrhosis at HCC development based on laboratory data and computed tomography findings. Another patient with cholangiocellular carcinoma (CCC) was diagnosed by computed tomography, angiography, and a serum carcinoembryonic antigen level of 310.9 (normal, <5.0). Her alpha-fetoprotein and PIVKA-2 levels were within the normal range. We had initially thought she had a metastatic tumor, but found no lesions other than in the liver. Therefore, we concluded that her liver tumor was CCC. An autopsy was not performed.

On univariate analysis, the incidences of relapse (seven of eight patients) and two or more relapses (six of eight patients) were significant risk factors associated with hepatic malignancy; P values were both <0.001 in Fisher's exact test and were 0.002 (Table 5) and 0.001 (Table 5 and Fig. 3B), respectively, in log-rank testing. Three patients (37.5%) were positive for anti-HBc. Accordingly, the risk of hepatic malignancy was found to be higher in anti-HBc positive patients than in anti-HBc negative ones (log-rank test, $P = 0.021$) (Table 5). On multivariate analysis, the prognosis of two or more relapses was identified as the only risk factor for development of hepatic malignancy (hazard ratio, 9.1; 95% CI, 1.8-45.5; $P = 0.007$) (Table 5).

The adverse effects of long-term immunosuppressive therapy included steroid-related osteoporosis in 25 (12.5%), diabetes mellitus in 20 (10.3%), fatty liver change in 19 (9.4%), cataract in five (2.5%), and compression fracture of spine, cerebral bleeding, gastric ulcer, and psychiatric problems in one (0.5%) each. These adverse effects were mild to moderate and controllable by medication. Only two patients experienced severe side effects (cerebral bleeding and necrosis of the femoral head in one patient each). All patients but one with diabetes mellitus were adequately managed with insulin injection therapy and/or antidiabetic medicine. The remaining patient was treated with prednisolone dose reduction and azathioprine administration.

Table 3. Comparison of Factors Between AIH Patients with and Without Liver-Related Death and Survival Analysis

Factors	Liver-Related Death				Cox Proportional Hazard Model	
	− (n = 196)	+ (n = 7)	P	Kaplan-Meier Log-Rank Test	HR (95% CI)	P
Background						
Sex, women/men	172/24	6/1	1	0.469		
HLA-DR4+/HLA-DR4−/ND	116/49/31	5/0/2	0.273	0.187		
Anti-HBc+/anti-HBc−/ND	24/163/9	2/5/0	0.398	0.524		
At diagnosis						
Age, years, median (range)	57 (12-86)	49 (33-58)	0.069	0.705†		
Acute onset	138	4	0.431	0.842		
Severe symptom*	68	2	1	0.243		
Liver cirrhosis	25	0	0.600	0.205		
Histology at diagnosis						
F3-F4/F1-F2/ND	83/108/5	5/2/0	0.305	0.587		
A2-A3/A0-A1/ND	162/29/5	5/2/0	0.571	0.542		
Treatment and response						
Relapse	42	6	0.001	0.016		
Relapse two or more times	21	6	<0.001	0.003	12.8 (1.5-109.9)	0.02

Abbreviations: HR, hazard ratio; ND, not determined.
Data are presented as no. of patients unless noted otherwise.
*Severe symptom: total bilirubin >5.0 mg/dL and/or prothrombin time <40%.
†Age >58 years.

Discussion

In this study, the overall survival of Japanese AIH patients diagnosed between 1974 and 2010 was found to be similar to that of the Japanese general population. The survival of AIH patients with definite AIH score was also similar to that of the general population, as were the cumulative survival curves of AIH patients with histologically severe stage or grade. These findings imply that the long-term prognosis of even severe AIH patients is good if adequate therapy is maintained over an extended period. Kanzler et al.[11] observed that the survival of German AIH patients followed for a long time did not differ from the age- and sex-matched normal German population. They described that long-term or life-long immunosuppressive therapy was needed in most cases and that only 6.8% of patients achieved sustained remission without the need of further immunosuppressive therapy. Similarly, only 6.4% of patients were successfully withdrawn from prednisolone (mean cessation time, 38.4 months; range, 29-52 months) with sustained remission in the present study. On the other hand, Hoeroldt et al.[16] reported the long-term mortality of AIH patients to be greater than that of the general population in England. This may be explained in part by genetic differences among races. Montano-Loza et al.[12] reported that multiple relapses are associated with a poorer prognosis than sustained remission or

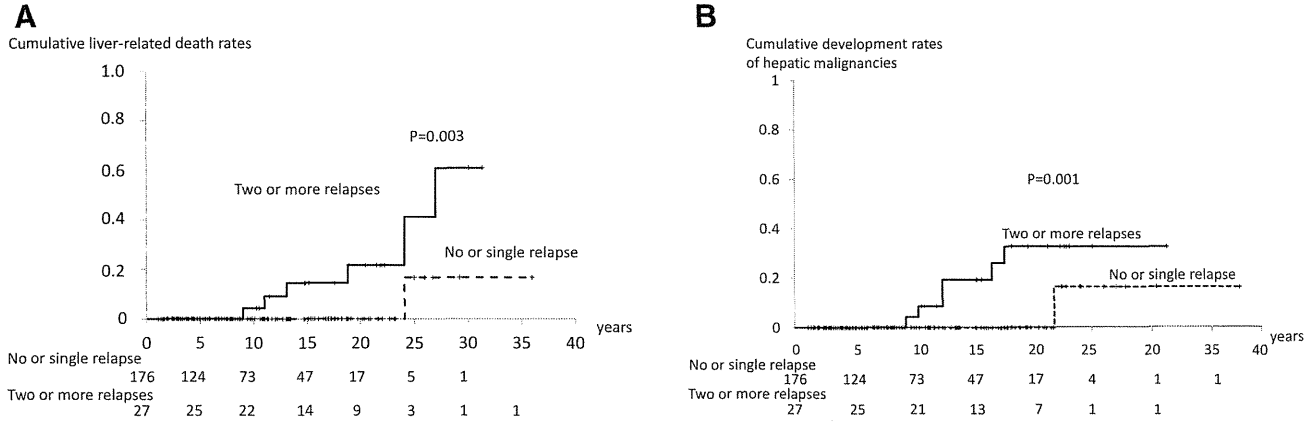

Fig. 3. (A) Cumulative liver-related death rates of patients who experienced no or a single relapse (broken line) and those with two or more relapses (solid line). The prognosis of patients with repeated relapses was poorer than those with remission or a single relapse ($P = 0.003$). (B) Cumulative hepatic malignancy rates of patients who experienced no or a single relapse (broken line) and those with two or more relapses (solid line). The development rate of hepatic malignancies was higher in patients with repeated relapses than in those with remission or a single relapse ($P = 0.001$).

Table 4. Cases of HCC or CCC Among AIH Patients

Patient Age/Sex	Histology at Presentation (at Diagnosis*)	No. of Relapses	Periods of HCC or CCC Development	Anti-HBc	Result
33/F	A3/F3 (F4)	4	12 years	+	Death
49/M	A3/F3 (F4)	7	9 years	+	Death
49/F	A3/F4 (LC†)	5	10 years	−	Death
56/F	A3/F3 (F3)	1	21 years	−	Death
60/F	A1/F3 (no LC†)	3	17 years (CCC)	−	Death
58/F	A1/F1 (LC†)	6	12 years	−	Death
56/	A3/F3 (F2)	2	14 years	+	Survival
63/F	A3/F4 (LC†)	0	21 years	−	Survival

Abbreviation: LC, liver cirrhosis.
*At diagnosis of HCC or CCC.
†Liver cirrhosis was diagnosed by laboratory findings and computed tomography.

a single relapse episode. Hoeroldt et al.[16] reported that more than four relapse episodes per decade were associated with a poor outcome. Similarly, our data revealed that repeated relapse episodes were the only factor affecting the long-term prognosis of AIH patients in Japan.

There is currently debate about the impact of cirrhosis on the natural history of AIH. Roberts et al.[17] reported similar outcomes in patients with and without cirrhosis at presentation. In contrast, other studies showed that cirrhosis at presentation was associated with a poorer prognosis.[16,18] Here, cirrhosis at presentation was not associated with mortality, but five of the seven patients who died with liver-related disease experienced repeated relapses, and all ultimately developed cirrhosis.

Conflicting data also exist on the influence of sex on long-term AIH outcome and survival. Whereas Al-Chalabi et al.[19] reported that male patients had better long-term survival rates and outcomes than female patients, Czaja and Donaldson[20] showed that sex did not influence the outcomes of their AIH cohort. Our data are consistent with the latter study.

Several studies from ethnically different countries have clarified strong genetic bases for both disease susceptibility and behavior.[21-27] In Japan, approximately 80% of AIH patients have the HLA-DR4 antigen, and most of them are HLA-DRB1*0405.[21,22,26,27] No patient or control case had the HLA-DR3 antigen in these studies. Caucasian AIH patients with HLA-DR4 have disease characteristics similar to those of Japanese patients with regard to age and effectiveness of treatment.[24-28] HLA-DR phenotypes were not associated with prognosis in this study ($P = 0.273$). We previously surveyed for susceptible or resistant genes in the HLA region[26] and whole genome[29] using genome-wide microsatellite analysis. However, no genes or genetic regions influencing the severity or prognosis of AIH were found in the present study (data not shown). Newly developed

Table 5. Comparison of Factors Between AIH Patients With and Without Occurrence of HCC and Survival Analysis

	HCC				Cox Proportional Hazard Model	
Factors	− (n = 195)	+ (n = 8)	P	Kaplan-Meier Log-Rank Test	HR (95% CI)	P
Background						
Sex, women/men	171/24	7/1	1	0.982		
HLA-DR4+/ HLA-DR4−/ND	115/49/31	6/0/2	0.255	0.220		
Anti-HBc+/anti-HBc−/ND	23/163/9	3/5/0	0.093	0.021		
At diagnosis						
Age, years, median (range)	57 (12-86)	53 (33-58)	0.136	0.306†		
Acute onset	136	6	1	0.545		
Severe symptom*	67	3	1	0.710		
Liver cirrhosis	24	1	1	0.640		
Histology at diagnosis						
F3-F4/F1-F2/ND	82/108/5	6/2/0	0.179	0.270		
A2-A3/A0-A1/ND	161/29/5	6/2/0	0.679	0.615		
Treatment and response						
Relapse	42	7	<0.001	0.002		
Relapse two or more times	21	6	<0.001	0.001	9.1 (1.8-45.5)	0.007

Abbreviations: HR, hazard ratio; ND, not determined.
Data are presented as number of patients unless noted otherwise.
*Severe symptom: total bilirubin >5.0 mg/dL and/or prothrombin time <40%.
†Age >58 years.

genome-wide association studies may be useful to detect other genes influencing the prognosis of AIH.

HCC has been reported in AIH with cirrhosis at the risks of 1.1%[30] and 1.9 %[31] per year. Among the seven AIH patients with HCC in our study, two had cirrhosis at presentation. In three patients without F4 histological stage, however, their liver histology or clinical stage progressed during relapses and reached cirrhosis at diagnosis of HCC. The other HCC patients were F3 and F2 stages. Based on this, cirrhosis appeared to be a risk factor for HCC development in our AIH patients. One patient with CCC in our study showed F3 in histology at AIH diagnosis in 1986. She experienced three relapses, but her laboratory data and computed tomography findings did not show liver cirrhosis at CCC diagnosis in 2003. Only three AIH cases complicated with CCC have been reported to date,[32-34] suggesting that CCC is a rare but possible complication in AIH patients.

Miyake et al.[9] described that the prognosis of Japanese AIH improved when serum ALT levels were persistently normal (<40 IU/L) during follow-up. Because our findings show that the survival of AIH patients with normal aminotransferase levels or having a single relapse is better than that of patients with multiple relapses, we must emphasize the importance of sustaining remission, especially after first relapse, by maintaining small but sufficient doses of immunosuppressive agents for an extended period.

Historically, immunosuppressive therapy was recommended to be withdrawn after remission was achieved.[1-3] However, more than half of patients discontinuing therapy relapse.[5-8] Muratori et al.[35] described that a small amount of immunosuppressive agents should be continued for an extended period. In most of our cohort, prednisolone monotherapy was continued indefinitely, even if remission was achieved, suggesting that long-term or life-long immunosuppressive therapy was safe, well tolerated, and effective. Severe side effects from corticosteroids were rare, but clinicians should be proactive; most patients in this study concurrently took bisphosphonate, vitamin D, and/or vitamin K supplements to prevent osteoporosis, as most of them were elderly women. It will be crucial to find the withdrawal point, if any, of immunosuppressive therapy and establish therapeutic guidelines for AIH.

In conclusion, repeated relapses were associated with a poorer long-term prognosis in Japanese AIH patients in our relatively large, multicenter, long-term study, which has been deemed necessary for this rare disease.[36] It is therefore preferential to reduce prednisolone doses carefully and/or maintain them for an indefinite amount of time, especially if a first relapse occurred during the drug's reduction or withdrawal.

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自己免疫性肝胆道疾患における最近の知見

自己免疫性肝炎の診断・治療における最近の知見

銭谷幹男*

要　旨

最近の我が国の全国集計により，自己免疫性肝炎の診断数は増加していることが明らかとなっている．一方，従来臨床的特徴とされたIgG上昇，血清自己抗体の高力価陽性所見に乏しい非定型とも言える症例の増加や，慢性肝炎像を伴わない急性発症型の存在は，薬物性肝障害との鑑別診断を含め，診断指針の再検討の必要性を示している．我が国で臨床的に使用されているウルソデオキシコール酸の意義についても明確な指針が求められている．

はじめに

自己免疫性肝炎（AIH）の成因は，多くの研究にもかかわらず現在なお不明であり，結果としてAIHと診断可能な特異的臨床指標は確立されていない．現状では既知の肝障害の要因を除外し，特異性はないが，AIHに高頻度で認められる血清IgG上昇，抗核抗体（ANA）をはじめとする血清自己抗体陽性所見，組織学的に形質細胞浸潤を伴う活動性の高い門脈域を中心とする炎症所見などから診断がなされている．国際診断スコア[1]はこれら診断に有用な諸所見を数量化して，AIHと診断可能な病態領域を囲い込むものである．我が国の診断指針にも記載されているように，このポイントは診断上参考にはなるが，ポイントが条件を満たしたことがAIHの診断を100%担保するものでもない．簡易型診断スコア[2]は，国際診断スコアが臨床上ベッドサイドで応用するには煩雑で，このスコアの項目を満たすために診断が遅れることを勘案して策定されたものである．したがって，簡易型診断スコアは早期の治療介入を容易にすることに重点が置かれ，抗ミトコンドリア抗体（AMA）による鑑別が排除されていることから，結果として原発性胆汁性肝硬変（PBC）で肝細胞障害が高度の症例も，AIH同様に副腎皮質ステロイド（CS）治療適応と診断されることとなる．いずれのスコアにおいても，組織学的所見，血清自己抗体，特にANA所見はスコアのポイント上重要な要素となっている．2009年に我が国で行われた全国集計により[3]，上記に示した重要な臨床所見の特異性が低下している事実が明らかにされた．この事実は，AIHの診断がより困難になっていることを示すものである．一方，C型肝炎ウイルス（HCV）の診断が確立したことに

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オーバーラップ症候群，
ウルソデオキシコール酸

図1　我が国の自己免疫性肝炎症例の年齢分布

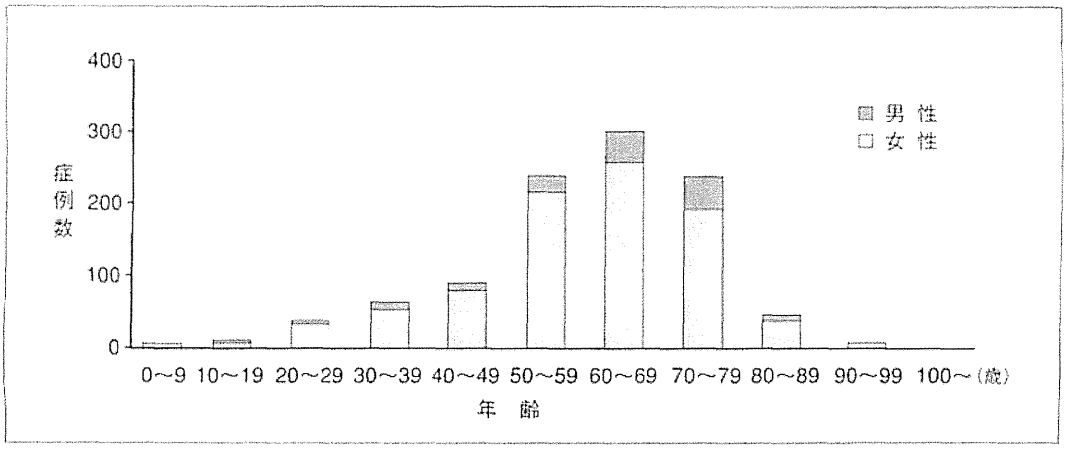
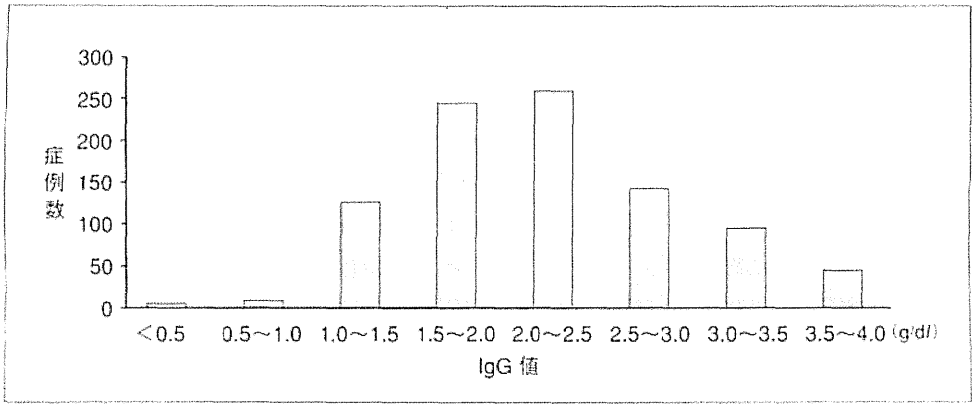


図2　診断時血清 IgG 値の分布



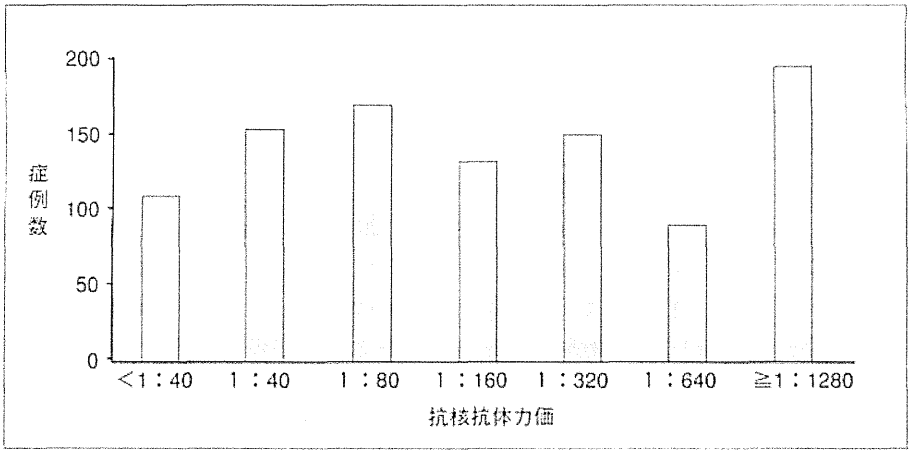
より，肝炎ウイルス感染によらない AIH を含む原因不明の肝細胞障害症例の診断数は増加しているという現状がある．

本稿では，最新の我が国における全国集計の結果を示し，治療を含めた現状での問題点を含めて概説する．

最新の全国集計による我が国の自己免疫性肝炎（AIH）の臨床像

図1～3に全国集計結果の概要を示した．図1で明らかなように，診断時年齢は 60 歳をピークとする一峰性を呈し，50 歳，70 歳代が次いで高率である．すなわち，我が国の AIH 症例は人口高齢化と同様に高齢化しているといえる．この事実は，高齢女性に原因不明の肝障害を認めた場合は AIH を念頭に置く必要性を示しているとも言える．なお，男女比は1：6で女性優位であることは従来と同様である．図2，3で明らかなように診断時の IgG 値，ANA 力価は以前の報告に比較して低値である．我が国の診断指針で示されている血清 IgG 値が 2.0g/dl 以上を満たさない症例が多く，かつ ANA が陰性の症例も少なからず認められる．血清 IgG に関しては，簡易型スコアで提唱されている基準値上限の 1.1 倍以上を当てはめてもこれを満たさない症例が少なからず存在し，かつ臨床的に診断され，治療されているという事実

図3 診断時血清抗核抗体力価の分布



である．IgG 値の変化の要因には測定系の変化も勘案されるが，従来には非定型とされている症例の増加に対しては新たな診断指針の提唱が不可欠である．

抗核抗体（ANA）測定について

AIH の診断に際し，ANA はげっ歯目の新鮮凍結組織を用いた間接蛍光抗体法（IF 法）で測定することが国際自己免疫性肝炎グループより提唱され，最近の米国肝臓学会のガイドラインでも同様の趣旨が記載されている．しかし，我が国でこの方法を用いて ANA を測定している施設はほとんどなく，多くは樹立化細胞株である Hep-2 細胞を用いた IF 法で測定されている．両者の間には感度に差異があり，また凍結組織，培養細胞の生育状態など，標準化には多くの問題がある．しかし筆者らの検討では，Hep-2 細胞を用いた検討により，少なくとも ANA 検出に関しては診断上大きな齟齬が認められないことが明らかとなっている．一方 ANA 測定に関しては，定量性，特異性が優れていることから酵素結合免疫吸着法（ELISA）も汎用されている．しかし，我が国で汎用されている ELISA 法は，Hep-2 細胞による測定に比較し，AIH での検出感度は明らかに劣るという問題がある．我が国で使用されている ANA-ELISA の抗原は，他の自己免疫疾患，特に全身性エリテマトーデス（SLE）などを対象に開発されたものであり，含有抗原には AIH における対応抗原が含まれていない可能性があるからである．事実，米国で汎用されている Hep-2 細胞核の抽出物を加えた ELISA 法は，我が国での ELISA に比較して AIH での ANA 検出感度は良好である．しかし凍結切片を用いた IF 法に比較すると，米国での ELISA も検出感度は十分とは言えない．したがって現状では，AIH の診断には既存の ELISA は不適であり，IF 法を用いることが必要であり，診断に当たって留意が必要である．

組織像について

AIH の肝組織の特徴は壊死・炎症反応で，インターフェイス肝炎を主体とし，浸潤細胞に形質細胞を含むことが特徴とされている．壊死・炎症が高度であることから，肝細胞ロゼット形成も高頻度で認められる．また，門脈域の炎症が高度の場合胆管障害も認められるが，胆管消失所見はまれである．しかし，いずれの所見も AIH に特異的とは言えないという問題がある．胆管障害が高度の場合，

PBC で肝細胞障害が高度の症例との鑑別は必ずしも容易でない．しかも，これら所見を記載する病理医の所見一致率，いわゆるκ値が低いことも示されている．国際診断スコア，簡易型スコアともに組織所見はスコア確定上重要であるが，組織所見を参考に，臨床所見と併せて診断を進めることが重要である．なお，簡易型スコアでは AIH の組織所見の特徴として emperipolesis が提唱された．この所見は我が国ではあまりなじみがなく，組織所見として記載される頻度もまれであった．emperipolesis 所見を除外して従来の AIH の組織所見に符合することをもっても，簡易型スコアは十分有効なのであるが，emperipolesis は肝細胞内に単核球が侵入するという，免疫学的肝細胞障害を示す所見でもあり，今後標本の見直しを含め十分な再評価が必要である．

急性型 AIH

組織学的所見では AIH の急性発症型という新たな問題も提起されている．従来 AIH は慢性肝炎に分類され，急性発症のほとんどは慢性状態の急性増悪と理解されていた．したがって，肝組織は急性発症であっても，慢性肝炎像を示す．しかし，慢性肝炎像を全く伴わず，急性肝炎として発症する AIH 群が存在することが明らかとなり，全国集計でも組織学的に急性肝炎と診断された症例は 10% を超えている．臨床的には，AIH の特徴とされる IgG 高値，自己抗体陽性所見を欠き，診断は困難である．さらに組織学的所見でも，従来報告されている AIH の所見は認められず，報告例からは形質細胞浸潤を伴う，小葉中心の壊死・炎症反応を示すとされている．現状での診断指針，診断スコアでは，これらの診断は困難である．急性型 AIH に対しても，CS が通常の AIH 同様に著効を示す．しかし，診断が遅れ，病態が進展し，肝の壊死が高度になれば肝不全となり，治療奏効は期待できない．したがって，急性型 AIH は迅速な診断が必要であり，この解決が大きな課題である．

オーバーラップ症候群

PBC の経過中に肝細胞障害が増悪し，ANA 陽性化や IgG 上昇が認められた場合，従来はいわゆるオーバーラップ症候群として取り扱われることが多かった．こうした病態をオーバーラップ症候群として別の病態に層別化する意義は，治療対応が異なるからである．しかし，診断上は PBC の肝炎型として取り扱うことが妥当であることが提唱され[7]，コンセンサスを得てきており，米国肝臓学会（AASLD）のガイドラインや我が国の検討[8]でも同様の提唱がなされている．AMA 陰性で，組織学的に PBC に矛盾しないが，肝障害に対し CS が有効であることから，autoimmune cholangitis との病名を付与された病態も，実際は AMA 陰性 PBC の肝炎増悪型ととらえて何ら矛盾はなく，autoimmune cholangitis の病名は当初の提唱された概念としては取り扱わないのが妥当である．我が国での全国集計から導かれた判別式[9]においてもこの事実は確認されている．一方 AIH と診断され，後に PBC 病態が顕性化する病態も報告されている．この病態は PBC で初期に肝細胞障害が顕性化したとも考えられ，実際は主たる病態は PBC とするのが多くの場合妥当である．

治療対応

CS の有効性は AIH の特徴でもあり，診断が確定されればその投与により，上昇していた血清 AST，ALT は速やかに改善し，多くの場合基準値以下になる．初期投与量に関してはプレドニゾロンで 0.5～1 mg kg が妥当である．欧米の成書では 60mg 日の記

載が多いが，体格が異なる我が国ではそれより少量で十分な効果が得られている．全国集計では，初期量 30～40mg 日で 90% 以上の症例で良好な効果が得られている．しかし少数ながら CS 抵抗性症例も存在し，その場合我が国では，保険収載はなされていないがアザチオプリン併用が多用されている．アザチオプリン使用に当たっては，代謝異常の遺伝子背景を有する場合があることで急激な白血球減少を見ることがあり，遺伝子検査，あるいは血中濃度測定が必要である．我が国では CS に加え，ウルソデオキシコール酸（UDCA）が少なからず処方されている．UDCA 併用は CS の減量に有効であることは確認されているが，併用，非併用で長期予後，あるいは再燃への影響はいまだ明確ではなく，今後の検討が必要である．さらに，我が国では臨床的活動性が低い，すなわち AST，ALT が低値の症例に対しては UDCA のみの治療も行われている．我が国では健診などにより，無症状症例が AST，ALT 上昇によって捕捉され，欧米では診断困難な AIH 初期あるいは従来指摘がない病態が軽微で推移する症例を扱っているとも考えられる．UDCA のみの治療で経過良好症例の組織学的変化の推移を含めた長期予後に関する検討も，今後の課題である．

おわりに

診断技術の進歩により AIH の診断数は増加しているが，同時に従来記載された AIH 典型症例の特徴を具備しない，いわゆる非定型 AIH の頻度も増加している．典型的 AIH の根底には多くの非定型症例が存在していることも十分あり得ることである．特異的臨床診断指標が確立していない AIH の診断は，より困難になっているとも言える．非定型でかつ軽微な病像を呈する症例の診断，治療方策，その予後に関する今後の検討が重要である．

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Sal-Like Protein 4 (SALL4), a Stem Cell Biomarker in Liver Cancers

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Liver cancers, including hepatocellular carcinomas (HCCs), cholangiocarcinomas (CCs), and fibrolamellar HCCs (FL-HCCs) are among the most common cancers worldwide and are associated with a poor prognosis. Investigations of genes important in liver cancers have focused on Sal-like protein 4 (SALL4), a member of a family of zinc finger transcription factors. It is a regulator of embryogenesis, organogenesis, pluripotency, can elicit reprogramming of somatic cells, and is a marker of stem cells. We found it expressed in normal murine hepatoblasts, normal human hepatic stem cells, hepatoblasts and biliary tree stem cells, but not in mature parenchymal cells of liver or biliary tree. It was strongly expressed in surgical specimens of human HCCs, CCs, a combined hepatocellular and cholangiocarcinoma, a FL-HCC, and in derivative, transplantable tumor lines in immune-compromised hosts. Bioinformatics analyses indicated that elevated expression of SALL4 in tumors is associated with poor survival of HCC patients. Experimental manipulation of SALL4's expression results in changes in proliferation versus differentiation in human HCC cell lines *in vitro* and *in vivo* in immune-compromised hosts. Virus-mediated gene transfer of SALL4 was used for gain- and loss-of-function analyses in the cell lines. Significant growth inhibition *in vitro* and *in vivo*, accompanied by an increase in differentiation occurred with down-regulation of SALL4. Overexpression of SALL4 resulted in increased cell proliferation *in vitro*, correlating with an increase in expression of cytokeratin19 (CK19), epithelial cell adhesion molecules (EpCAM), and adenosine triphosphate (ATP)-binding cassette-G2 (ABCG2). *Conclusion:* SALL4's expression is an indicator of stem cells, a prognostic marker in liver cancers, correlates with cell and tumor growth, with resistance to 5-FU, and its suppression results in differentiation and slowed tumor growth. SALL4 is a novel therapeutic target for liver cancers. (HEPATOLOGY 2013;57:1469-1483)

Liver cancers, comprised primarily of hepatocellular carcinomas (HCCs), cholangiocarcinomas (CCs), and fibrolamellar HCCs (FL-HCCs), are the fifth most common cancer and the third leading cause of cancer mortality in the world.[1] Cancers have a subpopulation of cancer stem cells (CSCs) or tumor-initiating cells (TICs), which have properties shared with normal stem cells.[2,3] CSCs and TICs have highly

Abbreviations: ABCG2, ATP-binding Cassette-G2; AFP, alpha-fetoprotein; ALB, albumin; BD, bile duct; CASP3, caspase-3; CC, cholangiocarcinoma; CK19, cytokeratin19; CSCs, cancer stem cells; DAPI, 4',6-diamidino-2-phenylindole; DP, ductal plate; EMT, epithelial-mesenchymal transition; EpCAM, epithelial cell adhesion molecules; FACS, fluorescent-activated cell sorter; FL-HCC, fibrolamellar hepatocellular carcinoma; 5-FU, 5-fluorouracil; hBTSCs, human biliary tree stem cells; HCC, hepatocellular carcinoma; HC-CC, combined hepatocellular and cholangiocarcinoma; hHBs, human hepatoblasts; hHpSCs, human hepatic stem cells; HNF4α, hepatocyte nuclear factor 4-alpha; PBGs, peribiliary glands; PT, portal tract; qRT-PCR, quantitative real-time polymerase chain reaction; SALL4, Sal-like protein 4; shRNA, short hairpin RNA; TICs, tumor-initiating cells; TTR, transthyretin; UGT2B7, UDP-glucuronosyltransferase-2B7.

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aggressive phenotypes in oncogenesis and are resistant to chemotherapies and radiation therapies. Expression of membrane pumps, adenosine triphosphate (ATP)-binding cassette-G2 (ABCG2), account for the resistance to chemotherapies and are responsible for elimination of DNA-binding dyes causing the cells to be displayed as a side fraction, a "side population (SP)."[4,5] Epithelial cell adhesion molecule (EpCAM), a key factor in the Wnt signaling pathway, was reported as a specific cell surface markers of human hepatic stem cells (hHpSCs), of some, but not all, subpopulations of human biliary tree stem cells (hBTSCs)[6-8] and liver TICs.[9] CD133 (prominin), CD90 (Thy-1), CD44 (hyaluronan receptor), and CD13 (alanine aminopeptidase) have also been found in liver TICs.[10-12] In parallel, CD133 and CD90 have been found on angioblasts or other mesenchymal cells tightly associated with hHpSCs,[13] and so some data discussing CD90 or CD133 may actually be interpreted as relevant to the mesenchymal cell components of the tumors. Several lines of evidence implicate genetic alternations during hepatocarcinogenesis, particularly the Wnt signaling pathway, p53 and alterations in matrix-degrading enzyme secretion.[14-20]

Sal-like Protein 4 (SALL4), a homolog of the *Drosophila* homeotic gene spalt, is a zinc finger transcription factor required for proliferation and maintenance of pluripotency through interactions with OCT3/4, SOX2, and NANOG. It is found at high levels in embryonic stem cells (ESCs),[21-26] and is one of the genes capable of eliciting reprogramming of somatic cells to become induced pluripotent stem cells (iPSCs).[27,28] Mutations in SALL4 cause Okihiro syndrome, known as an autosomal dominant disorder and characterized by multiple organ defects.[29] Recent studies have demonstrated that SALL4 is constitutively expressed in hematopoietic stem cells and a potent regulator of their expansion.[30,31] SALL4 transgenic mice exhibit symptoms like myelodysplastic syndrome (MDS) and subsequently develop acute myeloid leukemia (AML). Primary AML and MDS patients have higher SALL4 expression levels than that in controls, indicating that SALL4 plays a major role in leukemogenesis. Furthermore, SALL4 contributed to the maintenance of SP cells and chemosensitivity in leukemia by regulating the ABC drug transporter genes.[31-33] Solid tumors, such as germ cell tumors, breast, and alpha-fetoprotein (AFP)-producing gastric cancers also express SALL4.[34-37] Taken together, these data suggest that SALL4 is a novel stem cell marker, a gene involved in embryogenesis and organogenesis and a putative stem cell gene associated with CSCs. We now report that SALL4 expression occurs in diverse liver cancers including HCCs, CCs, and FL-HCCs, and that SALL4 increases growth and blocks differentiation in liver cancer cell lines.

Materials and Methods

Cell Proliferation and Chemoresistance Assays. Liver cancer cell lines were infected with a retroviruses or lentivirus at a multiplicity of infection of 40 in the presence of 10 μg/mL protamine sulfate. After infection, cells were cultured for 3 days. Cells then were collected and isolated using a MoFlo fluorescence-activated cell sorter (FACS) (DAKO, Glostrup, Denmark). Then 2×10^3 cells were seeded into 96-well plates and cultured in the presence or absence of 2 μg/mL 5-fluorouracil (5-FU) for 3 to 7 days. Cell proliferation was evaluated in triplicate using the Cell Counting Kit-8 (Dojindo Laboratory, Kumamoto, Japan). After incubation at 37°C for 2 hours, the absorbance at 450 nm was measured.

Immunohistochemistry. The tissues were embedded in paraffin and cut into 5-μm sections. After deparaffinization, antigen retrieval was performed with sodium citrate buffer for EpCAM, CK19, or ethylenediaminetetraacetic acid (EDTA) buffer (pH 8.0) for SALL4 in a steamer for 20 minutes. Endogenous peroxidases were blocked by incubation for 30 minutes in 0.3% H_2O_2. After blocking, primary antibodies (Supporting Table 3) were applied at 4°C overnight. The M.O.M immunodetection kit (Vector Laboratories, Burlingame, CA) was used for detecting primary mouse anti-human SALL4 antibody on mouse xenotransplant FL-HCC tumor to avoid the inability of the antimouse secondary antibody to endogenous mouse

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