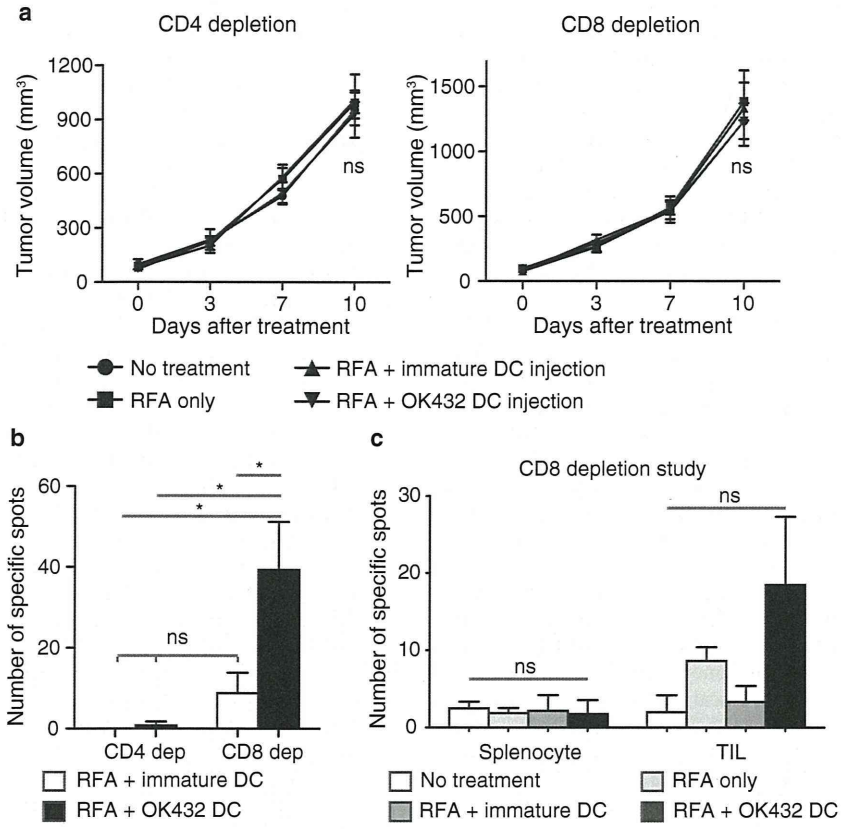

Fig. 5 The augmented antitumor effects depended on both CD4-positive and CD8-positive cells. **a** For in vivo CD4 or CD8 depletion, monoclonal antibodies specific to CD4 (GK1.5) or CD8 (2.43), respectively, were injected intraperitoneally at 1 day before and 3 days after RFA. Tumor volumes were compared among the four groups for 10 days after RFA. In each experiment, data were obtained from four mice per group and are presented as the mean ± SE. *ns* not significant. **b** The draining lymph nodes were harvested at 3 days after RFA and analyzed for their tumor specificities using the IFN-γ ELISPOT assay. Two mice were used in each group. Data are shown as the mean ± SE. $*P < 0.005$; *ns* not significant. **c** In the CD8 depletion study, splenocytes and tumor-infiltrating lymphocytes (TILs) were evaluated for their tumor specificities using the IFN-γ ELISPOT assay as described in Fig. 3. Four mice were used in each group. Data are shown as the mean ± SE. *ns* not significant

[32, 33]. However, in our experimental models, tumor-specific CD4-positive cells were not observed to contribute to the antitumor effect. Summarizing the above, in our study, the CD4-positive cells were required for the priming of the immune responses, and the CD8-positive cells acted as the effector cells after help from the CD4-positive cells.

In conclusion, we consider on the basis of our preclinical findings regarding combination therapy involving OK-432-stimulated DCs with RFA for the treatment of metastatic liver cancer that clinical trials can now proceed. It is anticipated that this combination therapy will be markedly superior to RFA single therapy.

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Conflict of interest The authors received financial support for this study from Chugai Pharmaceutical Co., Ltd.

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Original Article

Multicenter validation study of anti-programmed cell death-1 antibody as a serological marker for type 1 autoimmune hepatitis

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Aim: Recently, serum levels of anti-programmed cell death-1 (anti-PD-1) antibodies have been reported to be useful for the discrimination of type 1 autoimmune hepatitis (AIH) from drug-induced liver injury (DILI) and to be associated with clinical features of type 1 AIH. This multicenter study aimed to validate the usefulness of serum anti-PD-1 antibody as a serological marker for type 1 AIH.

Methods: Serum samples before the initiation of corticosteroid treatment were obtained from 71 type 1 AIH patients and 37 DILI patients. Serum levels of anti-PD-1 antibodies were measured by indirect enzyme-linked immunosorbent assay.

Results: Serum levels of anti-PD-1 antibodies were higher in type 1 AIH patients than in DILI patients ($P < 0.001$). The receiver–operator curve analysis showed that serum levels of anti-PD-1 antibodies were useful for the discrimination of type 1 AIH from DILI (area under the curve, 0.80). On the other hand, the multivariate Cox proportional hazard model showed that positivity for serum anti-PD-1 antibody, probable diagnosis based on the revised scoring system proposed by the International Autoimmune Hepatitis Group, and prothrombin activity of less than 60% were associated with the later normalization of serum transaminase levels. During the clinical course, the disease relapsed more frequently in patients positive for serum anti-PD-1 antibody (36% vs 11%).

Conclusion: This study suggests that serum anti-PD-1 antibody is useful for the diagnosis of type 1 AIH as an auxiliary diagnostic marker, and that serum levels of anti-PD-1 antibodies reflect clinical features of type 1 AIH.

Key words: autoantibody, autoimmune hepatitis, drug-induced liver injury, programmed cell death-1, validation

INTRODUCTION

AUTOIMMUNE HEPATITIS (AIH) is a progressive inflammatory liver disorder characterized by histological interface hepatitis, elevation of serum immunoglobulin G (IgG) level, circulating autoantibodies and good response to immunosuppressive treatment.[1,2] The

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pathogenesis of AIH has not been fully revealed yet. Elevated serum IgG levels and positivity for serum antinuclear antibody (ANA) are hallmarks for the diagnosis of type 1 AIH; however, the diagnosis has been made based on the scoring systems for lack of specific diagnostic markers for AIH.[1,2]

Recently, serum anti-programmed cell death-1 (anti-PD-1) antibody has been reported to be useful for the discrimination of type 1 AIH from drug-induced liver injury (DILI) as an auxiliary diagnostic marker and to be associated with clinical features of type 1 AIH.[3] PD-1 is a co-stimulatory molecule expressed on activated T and B cells and has inhibitory properties. Anti-PD-1 antibody enhances the proliferation of allogeneic T cells.[4] PD-1-deficient mice thymectomized 3 days after birth develop massive hepatic necrosis with the appearance of serum ANA,[5] and this hepatitis responds well to corticosteroid treatment.[6] In addition, a recent clinical trial using anti-PD-1 antibody as an immunotherapeutic agent for advanced cancer shows the development of hepatitis, which responds well to corticosteroid treatment, as an adverse event.[7] Dysfunction of PD-1 may be associated with the pathogenesis of AIH.

This study aimed to validate the usefulness of serum anti-PD-1 antibody for the diagnosis of type 1 AIH and to confirm the association of serum anti-PD-1 antibody with the disease severity, response to corticosteroid treatment and the disease relapse.

METHODS

Ethics

THIS RETROSPECTIVE VALIDATION 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.

Patients and serum samples

This study was carried out by the Intractable Hepato-Biliary Disease Study Group of Japan, sponsored by the Ministry of Health, Welfare and Labor of Japan. Serum samples before the initiation of corticosteroid treatment and clinical data were obtained from 71 type 1 AIH patients and 37 DILI patients, diagnosed in the following eight hospitals: Ehime University Hospital, Fukushima Medical University Hospital, Shinshu University Hospital, Teikyo University Hosiptial, Kyushu Medical Center, University of Fukui Hospital, Yamagata University Hospital, and Kochi Medical School Hospital.

For each patient, the following clinicopathological features were collected by the review of medical records: age, sex, laboratory data (white blood cell count, hemoglobin concentration, platelet count, bilirubin, aspartate aminotransferase, alanine aminotransferase [ALT], prothrombin activity, IgG, ANA) at the diagnosis, histological staging of liver fibrosis, initial dose of corticosteroid, timing of the normalization of serum ALT levels and timing of relapse. Clinical features of the study population are shown in Table 1. Collected serum samples were sent to Okayama University and stored at −30°C until use.

All type 1 AIH patients underwent liver biopsy. Type 1 AIH was diagnosed based on the revised scoring system proposed by the International Autoimmune Hepatitis Group.[1] DILI was diagnosed based on the diagnostic criteria of the Digestive Disease Week – Japan 2004 workshop,[8] the usefulness of which in the diagnosis of DILI has been confirmed by a study with a large sample size.[9]

Criteria for relapse in type 1 AIH

Relapse was defined as an increase in serum ALT levels to more than twofold of the upper normal limit (>60 IU/L), following the normalization of serum ALT levels (≤30 IU/L) with medical treatment.

Indirect enzyme-linked immunosorbent assay (ELISA)

Serum levels of anti-PD-1 antibodies were measured by indirect ELISA using the Protein Detector ELISA Kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) in Okayama University.[3] All serum samples were tested in duplicate.

Briefly, 96-well U-bottom microtiter plates (Greiner Bio-One, Baden, Germany) were coated with 100 μL of 1 μg/mL recombinant PD-1 (Abnova, Taipei, Taiwan) in phosphate-buffered saline (PBS) at room temperature for 1 h. Unbound antigen was removed, non-specific binding sites were blocked by incubation with 1% bovine serum albumin (BSA) in PBS, and the wells were incubated with 100 μL of human sera diluted 1:20 in PBS with 1% BSA for 1 h. Thereafter, the wells were incubated with anti-human IgG diluted 1:1000 in PBS with 1% BSA, covalently linked to alkaline phosphatase, and the reaction was visualized by adding 100 μL of a substrate buffer (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium). The optical densities at 630 nm (OD_{630nm}) were read with a Model 680 microplate reader (Bio-Rad, Richmond, CA, USA). In

Table 1 Clinical features of study population

	Type 1 autoimmune hepatitis	Drug-induced liver injury	*P*-value
Sample size, *n*	71	37	–
Age, years	58 (20–86)	53 (24–78)	0.029
Sex, female (%)	63 (89%)	23 (62%)	0.001
Revised scoring system proposed by the International Autoimmune Hepatitis Group			
Definite diagnosis	55 (77%)	–	–
Laboratory data			
WBC (/mm^3)	5300 (1600–9300)	5700 (1200–24500)	0.016
Hemoglobin (g/dL)	12.9 (8.7–16.1)	13.4 (9.0–16.6)	0.039
Platelet (×10^4/mm^3)	18.2 (7.4–46.0)	21.4 (4.4–32.0)	0.047
Bilirubin (mg/dL)	1.4 (0.5–24.9)	6.0 (0.3–26.7)	0.011
AST (IU/L)	288 (30–2466)	658 (99–13966)	0.003
ALT (IU/L)	337 (21–2377)	750 (54–4816)	<0.001
IgG (g/dL)	2.2 (1.0–4.9)	1.4 (0.8–2.4)	<0.001
Prothrombin activity (%)	78.8 (19.7–111.3)	75.4 (12.1–161.5)	0.25
ANA (%)			<0.001
<1:40	2 (3%)	25 (68%)	
≥1:40 and ≤1:80	28 (39%)	6 (16%)	
≥1:160	41 (58%)	6 (16%)	
Liver histology			
Acute hepatitis (%)	8 (11%)	–	–
Cirrhosis (%)	6 (8%)	–	–

ALT, alanine aminotransferase; ANA, antinuclear antibody; AST, aspartate aminotransferase; IgG, immunoglobulin G; WBC, white blood cell.

order to avoid inter-plate variability, we used a positive serum, assigned it 0.200 OD_{630nm}, and read the optical densities of all samples against this positive serum. Intra-assay variability was found to be 8.4%.

Previously, we showed that serum levels of anti-PD-1 antibodies in 62 healthy volunteers were a median of 0.033 (range, 0.002–0.144) OD_{630nm}.[3] According to our previous report,[3] the cut-off level in this study was represented by a mean absorbance +2 standard deviations in healthy volunteers (=0.086 OD_{630nm}).

Statistical analysis

Statistical analysis was performed using the SPSS statistical program (SPSS, Chicago, IL, USA).

Continuous variables were expressed as a median (range). Differences in continuous variables between two independent samples were evaluated by the Mann–Whitney *U*-test. Dichotomous variables were compared by the χ^2-test. Spearman's rank correlation coefficient was used to evaluate the consistency in the continuous variables between two independent samples. Univariate and multivariate Cox proportional hazard models were performed to identify factors associated with the later normalization of serum ALT levels. The variables, which showed $P < 0.1$ by univariate analysis, were included into the multivariate analysis. A cumulative incidence was analyzed using the Kaplan–Meier method, and the differences in the curves were evaluated using the log-rank test. The diagnostic accuracy of each factor was evaluated based on the area under the curve (AUC) using receiver-operator curve (ROC) analysis. The threshold of the reported *P*-values for significance was accepted as less than 0.05.

RESULTS

Anti-PD-1 antibody in type 1 AIH and DILI

SERUM LEVELS OF anti-PD-1 antibodies were significantly higher in type 1 AIH patients (0.071 [0.011–0.449] OD_{630nm}) than in DILI patients (0.023 [0.003–0.180] OD_{630nm}) ($P < 0.001$) (Fig. 1). When the cut-off level was represented according to the previous report (=0.086 OD_{630nm}),[3] positivity for serum anti-PD-1 antibody was shown in 38% (27/71) of type 1 AIH patients and 8% (3/37) of DILI patients ($P = 0.001$).

Two of three DILI patients positive for serum anti-PD-1 antibody had acute liver failure positive for serum ANA (Table 2).[10]

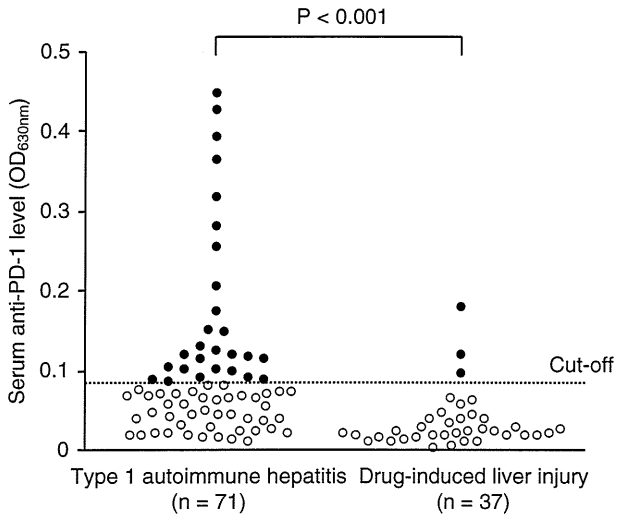

Figure 1 Serum levels of anti-programmed cell death-1 (anti-PD-1) antibodies in study populations. Closed circles show positivity for serum anti-PD-1 antibody. Open circles show negativity for serum anti-PD-1 antibody. According to the previous report,[3] the cut-off level was represented by a mean absorbance +2 standard deviations in healthy volunteers (=0.086 optical densities at 630 nm [OD_{630nm}]).

Anti-PD-1 antibody and clinical features of type 1 AIH

In type 1 AIH patients, serum levels of anti-PD-1 antibodies were inversely correlated with prothrombin activities ($\rho = -0.31$; $P = 0.008$), and prothrombin activities were lower in patients positive for serum anti-PD-1 antibody than in those negative for serum anti-PD-1 antibody (69.5% [19.7–102.6%] vs 86.1% [36.0–111.3%]; $P = 0.002$). Serum bilirubin levels tended to be correlated with serum levels of anti-PD-1 antibodies ($\rho = 0.23$; $P = 0.051$) and higher in patients positive for serum anti-PD-1 antibody (2.7 mg/dL [0.5–24.4] vs 1.2 mg/dL [0.5–24.9]; $P = 0.078$). But, age, sex, serum transaminase levels and serum ANA titers were not associated with positivity for serum anti-PD-1 antibody (Table 3). On the other hand, serum levels of anti-PD-1 antibodies were correlated with serum IgG levels ($\rho = 0.37$; $P = 0.002$), but serum IgG levels were not correlated with serum bilirubin levels ($\rho = -0.05$; $P = 0.67$) and prothrombin activities ($\rho = -0.18$; $P = 0.15$). In DILI patients, serum levels of anti-PD-1 antibodies were not correlated with serum IgG levels ($\rho = 0.18$; $P = 0.29$), serum bilirubin levels ($\rho = 0.19$; $P = 0.25$) and prothrombin activities ($\rho = -0.25$; $P = 0.14$).

Histologically, of 71 type 1 AIH patients, seven were diagnosed with acute hepatitis,[11] and the remaining 64 patients were diagnosed with chronic hepatitis. Serum levels of anti-PD-1 antibodies did not differ between patients with acute hepatitis and the others ($P = 0.62$). Positivity for serum anti-PD-1 antibody was shown in 38% (3/8) of patients with acute hepatitis and 38% (24/63) of patients with the chronic disease ($P = 0.97$). Of six cirrhotic patients, three (50%) were positive for serum anti-PD-1 antibody.

Table 2 Clinical features of three DILI patients positive for serum anti-PD-1 antibody

	Case 1	Case 2	Case 3
Age, years	74	38	71
Sex	Female	Female	Female
Causal drug	Allopurinol	Sairei-to	Phenytoin
Laboratory data			
Anti-PD-1 level (OD_{630nm})	0.096	0.180	0.120
WBC (/mm^3)	8300	9500	8800
Hemoglobin (g/dL)	10.3	12.2	10.1
Platelet (×10^4/mm^3)	10.2	30.9	31.8
Bilirubin (mg/dL)	6.2	20.6	7.0
AST (IU/L)	740	1253	204
ALT (IU/L)	340	1263	181
IgG (g/dL)	1.3	1.5	1.5
Prothrombin activity (%)	21	21	46
ANA titer	1:40	1:40	<1:40
Outcome	Died	Survived	Survived

ALT, alanine aminotransferase; ANA, antinuclear antibody; AST, aspartate aminotransferase; DILI, drug-induced liver injury; IgG, immunoglobulin G; OD_{630nm}, optical densities at 630 nm; PD-1, programmed cell death-1; WBC, white blood cell.

Table 3 Associations of positivity for serum anti-PD-1 antibody with clinical features in type 1 autoimmune hepatitis patients

	Positive for anti-PD-1	Negative for anti-PD-1	*P* value
Sample size, *n*	27	44	–
Age, years	59 (29–81)	58 (20–86)	0.70
Sex, female (%)	24 (89%)	39 (89%)	0.97
Revised scoring system proposed by the International Autoimmune Hepatitis Group			
Definite diagnosis	22 (81%)	33 (75%)	0.53
Laboratory data			
WBC (/mm^3)	5600 (1600–7200)	5200 (2300–9300)	0.83
Hemoglobin (g/dL)	13.0 (10.7–15.5)	12.9 (8.7–16.1)	0.43
Platelet (×10^4/mm^3)	15.7 (9.5–24.4)	18.6 (7.4–46.0)	0.054
Bilirubin (mg/dL)	2.7 (0.5–24.4)	1.2 (0.5–24.9)	0.078
AST (IU/L)	346 (30–2466)	255 (39–1561)	0.20
ALT (IU/L)	272 (21–2377)	360 (31–1355)	0.85
IgG (g/dL)	2.8 (1.3–4.9)	2.2 (1.0–4.0)	0.006
Prothrombin activity (%)	69.5 (19.7–102.6)	86.1 (36.0–111.3)	0.002
ANA (%)			0.90
<1:40	1 (4%)	1 (2%)	
≥1:40 and ≤1:80	10 (37%)	18 (41%)	
≥1:160	16 (59%)	25 (57%)	
Liver histology			
Acute hepatitis (%)	3 (11%)	5 (11%)	0.97
Cirrhosis (%)	3 (11%)	3 (7%)	0.53

ALT, alanine aminotransferase; ANA, antinuclear antibody; AST, aspartate aminotransferase; IgG, immunoglobulin G; WBC, white blood cell.

Anti-PD-1 antibody in the diagnosis of type 1 AIH

The ROC analysis showed that measurement of serum levels of anti-PD-1 antibodies was useful for the discrimination of type 1 AIH from DILI (Fig. 2; AUC, 0.80; 95% confidence interval [CI], 0.72–0.89; $P < 0.001$). The AUC of serum IgG levels and ANA titers for the discrimination of type 1 AIH from DILI was 0.86 (95% CI, 0.79–0.94; $P < 0.001$) and 0.83 (95% CI, 0.73–0.93; $P < 0.001$), respectively.

When patients positive for serum anti-PD-1 antibody 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 38%, 92%, 90% and 44%, respectively.

In type 1 AIH, five (21%) of 24 patients with serum IgG levels of less than 2 g/dL and nine (41%) of 22 patients with serum ANA titer of 1:40 or less were positive for serum anti-PD-1 antibody. Four (33%) of 12 patients showing serum IgG levels of less than 2 g/dL and serum ANA titer of 1:40 or less were positive for serum anti-PD-1 antibody.

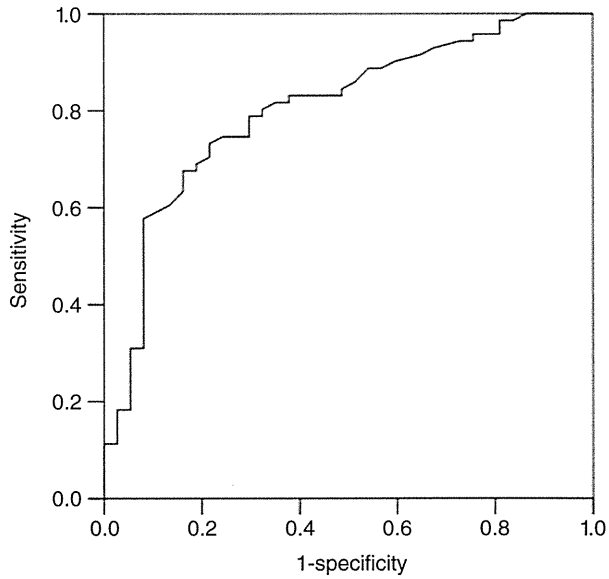

Figure 2 Receiver–operator curve of serum anti-programmed cell death-1 antibodies for the discrimination of type 1 autoimmune hepatitis from drug-induced liver injury. The area under the receiver–operator curve was 0.80 (95% confidence interval, 0.72–0.89; $P < 0.001$).

Anti-PD-1 antibody and initial response to corticosteroid treatment in type 1 AIH

Of 71 type 1 AIH patients, three were not treated with corticosteroid, and the other four were transferred to other hospitals before the normalization of serum ALT levels. So, the association of serum anti-PD-1 antibody with the normalization of serum ALT levels was evaluated in 64 patients treated with corticosteroid. Sixty-two patients (97%) achieved the normalization of serum ALT levels. Cumulative incidences of the normalization of serum ALT levels at 1, 3, 6 and 12 months from the initiation of corticosteroid treatment were 42%, 83%, 94% and 95%, respectively. Patients positive for serum anti-PD-1 antibody achieved later normalization of serum ALT levels than the others (Fig. 3, log-rank test; $P = 0.019$). On the other hand, of the 64 patients treated with corticosteroid, 13 patients initially received i.v. methylprednisolone pulse therapy (125–1000 mg/day for 3 days). Of the remaining 51 type 1 AIH patients initially treated without i.v. methylprednisolone pulse therapy, 16 patients were positive for serum anti-PD-1 antibody and achieved the later normalization of serum ALT levels than the others (log-rank test; $P = 0.048$) although the initial dose of prednisolone (PSL) was similar between those positive for serum anti-PD-1 antibody and the others (40 mg/day [20–60] vs 30 mg/day [15–60]; $P = 0.52$).

In the 64 patients, the univariate Cox proportional hazard model showed that positivity for serum anti-PD-1 antibody, probable diagnosis based on the revised scoring system proposed by the International Autoimmune Hepatitis Group, serum bilirubin levels of 10 mg/dL or more, and prothrombin activity of less than 60% were significantly associated with the later normalization of serum ALT levels. By the multivariate Cox proportional hazard model, positivity for serum anti-PD-1 antibody, probable diagnosis and prothrombin activity of less than 60% were shown to be significantly associated with the later normalization of serum ALT levels (Table 4).

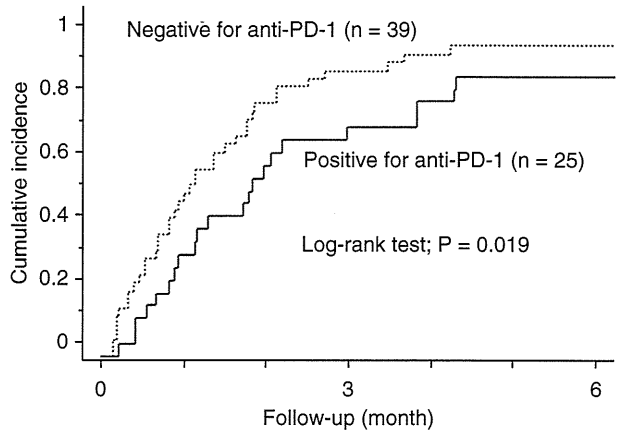

Figure 3 Cumulative incidences of the normalization of serum alanine aminotransferase levels after the initiation of prednisolone treatment. Anti-PD-1, anti-programmed cell death-1.

Table 4 Cox proportional hazard analysis for the factors associated with the later normalization of serum ALT levels

Variables	Univariate		Multivariate	
	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value
Positivity for anti-PD-1	1.86 (1.10–3.16)	0.022	1.79 (1.03–3.11)	0.038
Age, years	1.01 (0.99–1.02)	0.48	–	–
Sex, male	1.72 (0.73–4.01)	0.21	–	–
Revised scoring system proposed by the International Autoimmune Hepatitis Group				
Probable diagnosis	2.23 (1.20–4.14)	0.011	3.60 (1.84–7.04)	<0.001
Liver histology, acute hepatitis	1.27 (0.57–2.78)	0.57	–	–
Bilirubin, <10 mg/dL	0.53 (0.28–0.96)	0.045	0.99 (0.44–2.20)	0.97
ALT, <300 IU/L	0.90 (0.53–1.51)	0.68	–	–
IgG, <2 g/dL	1.44 (0.83–2.47)	0.19	–	–
Prothrombin activity <60%	2.22 (1.16–4.28)	0.017	3.13 (1.27–7.68)	0.013
ANA <1:80	0.89 (0.52–1.51)	0.66	–	–
Initial PSL dose <40 mg	0.72 (0.43–1.21)	0.22	–	–
Pulse steroid therapy, yes	1.69 (0.90–3.20)	0.11	–	–

ALT, alanine aminotransferase; ANA, antinuclear antibody; CI, confidence interval; IgG, immunoglobulin G; PD-1, programmed cell death-1; PSL, prednisolone.

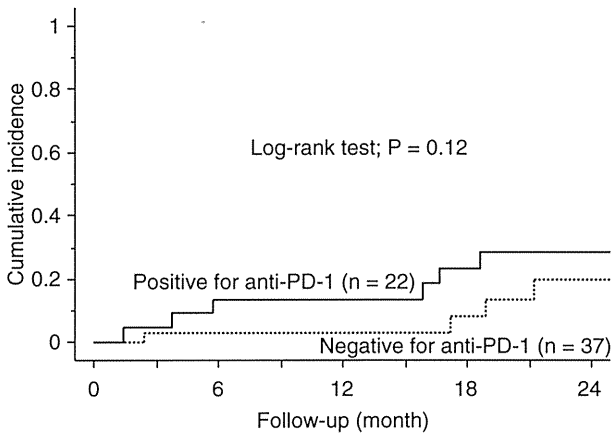

Figure 4 Cumulative incidences of the disease relapse after the normalization of serum alanine aminotransferase levels. Anti-PD-1, anti-programmed cell death-1.

Anti-PD-1 antibody and relapse in type 1 AIH

Of the 62 patients achieving the normalization of serum ALT levels with corticosteroid treatment, 59 were followed up during 1 month or more after the normalization of serum ALT levels. The median follow-up duration was 39 months (2–180). Of these 59 patients, the disease relapse was shown in 12 patients (20%) during the follow up. The disease relapse was shown in eight (36%) of 22 patients positive for serum anti-PD-1 antibody and four (11%) of 37 patients negative for serum anti-PD-1 antibody ($P = 0.018$). The Kaplan–Meier analysis showed that the disease relapse tended to be shown earlier in patients positive for serum anti-PD-1 antibody although the difference did not reach statistical significance (Fig. 4, log–rank test; $P = 0.12$). The duration from the normalization of serum ALT levels to the disease relapse was not different between eight patients positive for serum anti-PD-1 antibody and four patients negative for serum anti-PD-1 antibody (16.3 months [1.5–79.6] vs 18.0 months [2.4–21.2]; $P = 0.81$). Patients showing the disease relapse had higher serum levels of anti-PD-1 antibody (0.096 OD_{630nm} [0.044–0.427] vs 0.064 OD_{630nm} [0.013–0.449]; $P = 0.006$).

DISCUSSION

ELEVATION OF SERUM IgG level and positivity for serum ANA are hallmarks of AIH and main variables included in the scoring systems for the diagnosis of AIH.[1,2] However, almost 15–25% of patients, particularly acute cases, show normal IgG levels at presentation,[12] and 30% were negative for serum ANA.[13] A recent nationwide survey in Japan showed that 40% of type 1 AIH patients showed serum IgG level of 2 g/dL or less, and 10% were negative for serum ANA.[14] In patients showing normal IgG level and/or negativity for serum ANA, the diagnosis of AIH is not always easy. Thus, some markers useful for the diagnosis of AIH are desired.

The purpose of this study was to validate the usefulness of serum anti-PD-1 antibody as an auxiliary diagnostic marker for type 1 AIH. In this study, the prevalence of positivity for serum anti-PD-1 antibody in DILI patients was as low as that of the previous report.[3] On the other hand, the prevalence of positivity for serum anti-PD-1 antibody was not so high in type 1 AIH patients, but the ROC analysis showed that the AUC of serum anti-PD-1 antibody was almost equal to those of serum IgG and ANA. In addition, 21% of type 1 AIH patients showing serum IgG levels of less than 2 g/dL, 41% of those showing serum ANA titer of 1:40 or less, and 33% of those showing serum IgG levels of less than 2 g/dL and serum ANA titer of 1:40 or less were positive for serum anti-PD-1 antibody. Thus, serum anti-PD-1 antibody may be useful for the diagnosis of type 1 AIH as an auxiliary diagnostic marker.

Serum IgG levels have been shown to be associated with the prognosis of type 1 AIH.[15] So, serum IgG of type 1 AIH patients may contain some autoantibodies associated with the disease severity. This study indicated that serum levels of anti-PD-1 antibodies were correlated with the disease severity. This result is consistent with that of the previous report.[3] PD-1 blockade contributes to hyper-responsiveness of $CD8^+$ T cells to antigen and reduced ability of regulatory T cells.[16–18] Serum anti-PD-1 antibodies of type 1 AIH patients may aggravate inflammatory activity through reduced interaction between PD-1 expressed on T cells and its ligands, although further studies are needed. On the other hand, serum IgG levels were not correlated with the disease severity. Thus, not serum IgG but serum anti-PD-1 antibody may be useful as a marker reflecting the disease severity when deciding the treatment strategy.

The previous report has indicated that type 1 AIH patients positive for serum anti-PD-1 antibody achieve the later normalization of serum transaminase levels after the initiation of PSL treatment by the univariate analysis.[3] On the other hand, in this study, positivity for serum anti-PD-1 antibody was confirmed to be associated with the later normalization of serum transaminase levels by the multivariate analysis. Initial response to

corticosteroid treatment has been shown to be a predictive factor for liver-related death or liver transplantation.[19,20] In type 1 AIH patients positive for serum anti-PD-1 antibody, initial treatment should be introduced more carefully.

Repeated relapse is a risk factor for liver-related death or liver transplantation in type 1 AIH.[21,22] In order not to worsen the prognosis, the persistent normalization of serum transaminase levels is important.[23,24] This study indicated that positivity for serum anti-PD-1 antibody was associated with the disease relapse. So, in patients positive for serum anti-PD-1 antibody, the dose reduction of immunosuppressant and the termination of immunosuppressive treatment should be decided more carefully. On the other hand, because of the small sample size, the difference in a cumulative incidence of the disease relapse between patients positive for serum anti-PD-1 antibody and those negative for serum anti-PD-1 antibody did not reach statistical significance when using the Kaplan–Meier method. A further study with a larger sample size and/or longer follow up is needed.

In the previous report,[3] serum levels of anti-PD-1 antibodies were correlated with serum transaminase levels and serum ANA titers. In addition, patients with acute hepatitis showed higher serum levels of anti-PD-1 antibodies than those with the chronic disease. But, these findings were not confirmed by this study.

In conclusion, this study is the first to show that serum anti-PD-1 antibody is a valid auxiliary diagnostic marker for type 1 AIH. Especially, for the diagnosis of type 1 AIH showing normal IgG level and/or negativity for serum ANA, measurement of serum levels of anti-PD-1 antibodies will be useful. In addition, serum anti-PD-1 antibody is confirmed to be associated with the disease severity, response to corticosteroid treatment and the disease relapse. In patients positive for serum anti-PD-1 antibody, the dose reduction of immunosuppressant and the termination of immunosuppressive treatment should be decided more carefully. Hereafter, further studies are needed in order to investigate the functions of anti-PD-1 antibodies in sera of type 1 AIH patients.

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HEPATOLOGY PRACTICE VOL.3

C型肝炎の診療を極める

基本から最前線まで

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Ⅳ C型肝炎を理解するための最前線研究のトピックス

3 自然免疫

要点

- DAAを用いた新規治療においてもPEG-IFNα＋RBV併用療法に対してnull responseとなる，いわゆるIFN不応例における治療成績は十分ではない．
- HCVの排除にはRIG-I/IPS-1系を中心とした宿主自然免疫機構が重要であるが，IFN不応性には宿主自然免疫が密接に関与している．
- 宿主自然免疫系遺伝子の発現プロファイルを解析することで，IFN応答性を予測することが可能である．
- HCVは自己のNS3/4AプロテアーゼおよびNS4B蛋白により宿主自然免疫を攪乱する逃避機構を有している．
- 宿主自然免疫およびそれに対するHCV逃避機構は，HCVの持続感染を終息させるための新たな治療標的となり得る．

はじめに

近年C型肝炎ウイルス(hepatitis C virus：HCV)増殖に重要な働きをもつHCV蛋白を直接阻害する経口投与可能な新規抗ウイルス薬，いわゆるHCV直接阻害薬(direct acting anti-viral agent：DAA)の開発が急速に進んでいる．これらDAAのうち最も開発が進んでいるのは，HCVのNS3/4Aを標的としたプロテアーゼ阻害薬であり，genotype 1b型かつ高ウイルス症例のいわゆる難治性C型慢性肝炎に対して，ペグインターフェロンα(PEG-IFNα)およびリバビリン(RBV)と併用する3剤併用療法が臨床導入され飛躍的に治療成績が向上した．

しかし，未だHCVの持続感染を完全に制御することは困難で，疾患を完全に克服するには至っていない．特に，これら新規治療においてもPEG-IFNα＋RBV併用療法に対してnull responseとなる症例，すなわちインターフェロン(IFN)不応例における治療成績は十分ではない．したがって，IFNの応答性を規定する因子やその分子機構を理解しておくことは，最先端の臨床を実践するうえでも重要である．IFNの応答性にはウイルス側要因および宿主側要因の双方が関与すると考えられており，これまで種々の因子が同定されてきた．中でも宿主要因としては自然免疫系が宿主ゲノムの遺伝子多型とともに大きく関与することが明らかとなり重要である．本項ではC型肝炎における宿主自然免疫系の臨床的意義ついて概説する．

Ⅰ HCV感染と自然免疫

HCVが細胞に感染すると，まずHCV由来のRNAが細胞内のウイルスセンサーであるRIG-I(retinoic acid inducible gene I)によって探知され，そのシグナルがアダプター分子であるIPS-1(IFNβ promoter stimulator-1：別名MAVS, Cardif, VISA)を介して核に伝達されIFNβおよびIFNλが産生される．この宿主自然免疫の作

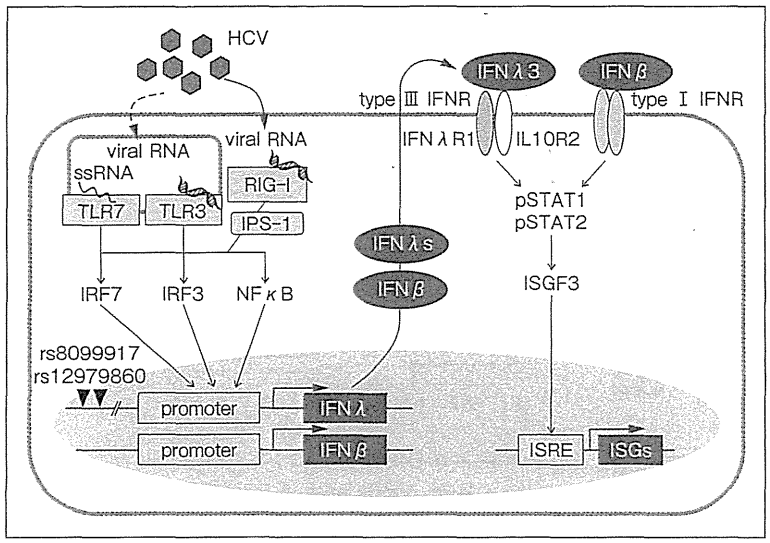

図1 図1 HCV感染と宿主自然免疫機構
HCVが細胞に感染すると細胞内ウイルスセンサーのRIG-IがHCV由来の核酸を探知し，アダプター分子IPS-1を介してシグナルが核に伝達され，IFNβおよびIFNλが誘導される．IFNβおよびIFNλは，それぞれの受容体を介してJak-STAT系を活性化し多様なIFN誘導遺伝子(ISG)の発現を誘導し宿主が抗ウイルス状態となる．

動がHCV感染に際して生体側で起こる最初の防御機構であるとされる．これらHCV感染により誘導されたIFNβおよびIFNλは，それぞれⅠ型およびⅢ型IFN受容体に結合しJak-STAT経路を介して多様なIFN誘導遺伝子(ISG)を誘導し，宿主の抗ウイルス状態を惹起すると考えられている(図1)．

一方，HCVのNS3/4Aセリンプロテアーゼは IPS-1をミトコンドリアとアンカリングしている部分で切断することが知られており，HCVはRIG-I/IPS-1系を標的とすることで巧みに宿主の自然免疫系から逃れている可能性が示唆されている(図2)．したがって，RIG-I/IPS-1系は宿主によるHCVの排除およびそれに対するHCVの抵抗性の双方に大きく関与していると考えられる．

Memo 細胞内ウイルスセンサーRIG-Iとアダプター分子IPS-1

RIG-Iは細胞質に存在するヘリカーゼであり，ウイルス由来の5′の三リン酸構造や3′のポリU配列を認識し，形質細胞様樹状細胞以外のほとんどの細胞においてウイルス感染センサーとして必須の役割を担っている．RIG-Iと構造上非常に類似したファミリー分子であるMDA5も同様の機能を有するウイルスセンサー分子であることが示されているが，ピコルナウイルスなどRIG-Iとは違ったウイルス種を認識しているとされる．RIG-IまたはMDA5がウイルス由来RNAを探知するとその三次構造が変化し，下流で機能するアダプター分子であるIPS-Iと会合する．IPS-1はミトコンドリアにアンカリングしている蛋白であり，RIG-IやMDA5が探知したウイルス感染シグナルを核に伝えるのに必須である．

Memo RIG-I/IPS-1系の制御機構

RIG-I/IPS-1系は，いろいろな宿主分子により制御を受けている．例えば，RIG-Iと同様の分子構造をもつRIG-Iファミリー分子のLGP2はIPS-1との結合に必要なCARDドメインを欠くため，シグナルを核に伝達することができずRIG-I/IPS-1系の制御に関与すると報告されている．また，ユビキチン様蛋白であるISG15はSTAT1やJak1と結合しIFNシグナル伝達を修飾する一方，RIG-IやIPS-1などと結合することにより(ISGylation)その機能を修飾していると考えられている．そして，ISG15とその結合蛋白とを解離させる特異的プロテアーゼであるUSP18はISGylationに対して抑制的に働くことが報告されている．さらに，最近このRIG-I/IPS-1系の特異的ユビキチンE3リガーゼであるRNF125が同定され，ubiqutin-proteasome pathwayによりRIG-I，MDA5およびIPS-1を分解し抑制的に調節していることが示されている．

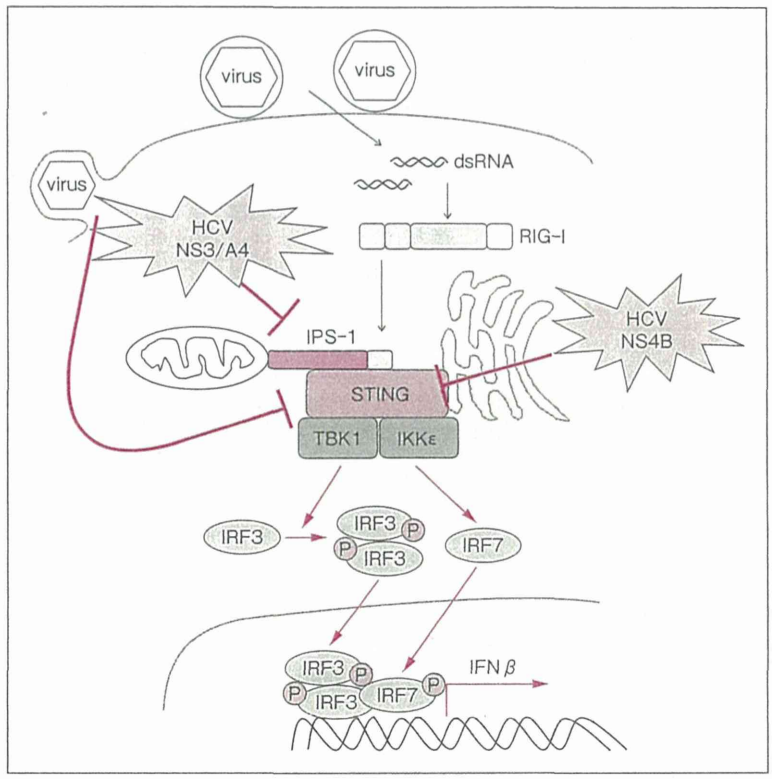

図2 HCV 蛋白による自然免疫逃避機構
HCV の NS3/4A セリンプロテアーゼは IPS-1 をミトコンドリアとアンカリングしている部分で切断することで宿主の自然免疫シグナルを遮断する．また，NS4B 蛋白は IPS-1 の下流分子である宿主 STING を標的として宿主のシグナル伝達を抑制する．HCV はこれら複数の HCV 蛋白を協調的に作用させて宿主自然免疫機構を攪乱し，宿主自然免疫から逃避している．

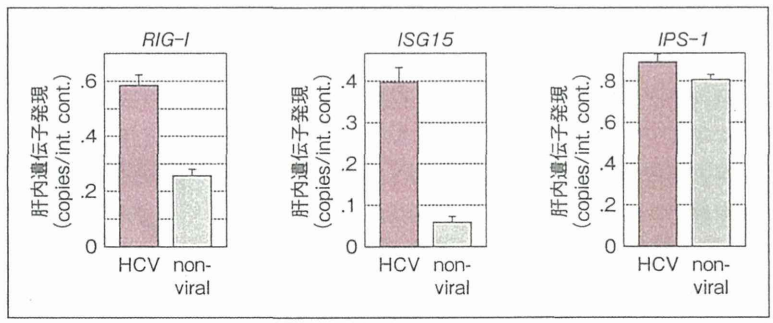

図3 C 型慢性肝炎および非ウイルス性肝疾患における自然免疫系分子の肝内遺伝子発現の比較
C 型慢性肝炎患者における *RIG-I* および *ISG15* の肝内遺伝子発現は非ウイルス性肝疾患患者に比し有意に高く，HCV 持続感染により誘導される内因性 IFN によるものと考えられる．一方，*IPS-1* は C 型慢性肝炎と非ウイルス性肝疾患の間で肝内発現量に有意差はなく，比較的 constitutive に発現している．（文献 1 を改変引用）

Ⅱ 自然免疫系遺伝子の肝内発現プロファイルと抗ウイルス効果

1 C 型慢性肝炎における自然免疫系遺伝子の発現

細胞内ウイルスセンサーである *RIG-I* やアダプター分子である *IPS-1* および IFN 誘導遺伝子である *ISG15* の mRNA の肝内発現を非ウイルス性肝疾患とで比較すると，C 型慢性肝炎患者における *RIG-I* および *ISG15* の肝内遺伝子発現は非ウイルス性肝疾患患者に比し有意に高かった（図3）[1]．すなわち，C 型慢性肝炎患者では，HCV 持続感染により内因性 IFN が誘導されているため，これらの自然免疫系遺伝子が肝内において高発現状態となっていることが示唆された．それに対して，*IPS-1* は C 型慢性肝炎と非ウイルス性肝疾患の間で肝内発現量に有意差はなく，比較的 constitutive に発現していると考えられた．

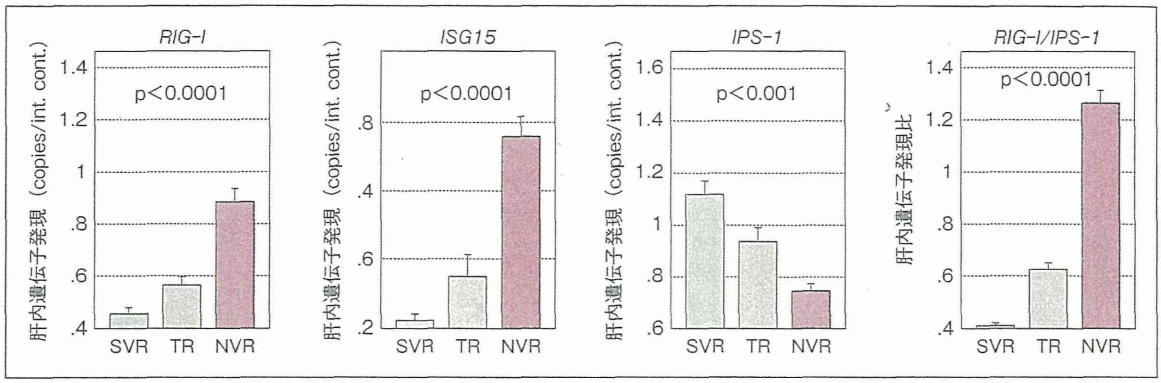

図4 PEG-IFN/RBV 併用療法の最終ウイルス学的治療効果と自然免疫系分子の肝内遺伝子発現

RIG-I および *ISG15* の肝内遺伝子発現は，治療中 HCV が減衰しない non-viral responder（NVR）ではウイルス学的著効例（sustained viral responder：SVR）群に比し有意に高発現しているのに対して，*IPS-1* の治療前肝内遺伝子発現は NVR 群で有意に低値で，*RIG-I/IPS-1* 比は NVR で有意に高い．これら自然免疫系遺伝子の治療前における肝内発現を定量することは IFN 応答性を予測するのに有用である．（文献 1 を改変引用）

2 治療前肝内遺伝子発現プロファイルと PEG-IFNα/RBV 併用療法の治療効果

一方，治療前の自然免疫系遺伝子の発現状態とウイルス学的治療効果を検討すると，*RIG-I* および *ISG15* の肝内遺伝子発現は，治療中 HCV が減衰しない non-viral responder（NVR）ではウイルス学的著効例（sustained viral responder：SVR）群に比し有意に高発現しているのに対して，*IPS-1* の治療前肝内遺伝子発現は NVR 群で有意に低値で，*RIG-I/IPS-1* 比は NVR で有意に高かった（図 4）[1]．

多変量解析では，*ISG15* 発現および *RIG-I/IPS-1* 比と血小板数が NVR に関与する独立因子として有意で，ROC 解析では *ISG15* 発現および *RIG-I/IPS-1* 比 の area under the curve は 0.9 以上となり，これらの遺伝子の治療前における肝内発現を定量することは PEG-IFNα/RBV 併用療法の最終治療効果を治療前に予測するのにきわめて有用と考えられた．

Ⅲ 自然免疫系遺伝子の経時的発現と治療効果

前述のように，*RIG-I* などの治療前における肝内遺伝子発現は，PEG-IFNα/RBV 併用療法不応例である NVR で高発現しているが，PEG-IFNα/RBV 投与による反応性はどのようになっているであろうか．末梢血単核球中における，*RIG-I*，*ISG15* および *IPS-1* の PEG-IFN/RBV 投与前後における経時的遺伝子発現動態を解析すると，*RIG-I* と *ISG15* の発現量は，PEG-IFN/RBV 投与の 8 時間後に治療前の 30～70 倍となり，治療により強く誘導された（図 5）[1]．さらに，この遺伝子誘導は最終的にウイルスが駆除された SVR 例で高い傾向を認め，外因性 IFN による遺伝子の誘導能と治療効果との間に関連があることが示唆された．一方，*IPS-1* の発現動態は PEG-IFN/RBV 投与により大きく影響は受けないことが観察され，治療中も constitutive に発現していることが示唆された．

以上より，NVR 例では治療前に内因性 IFN により自然免疫系がすでに up regulation されているため，治療である外因性 IFN に対する反応性が現弱していることが示唆され，IFN に対する不応性のメカニズムの本質に近い現象と考えられる．

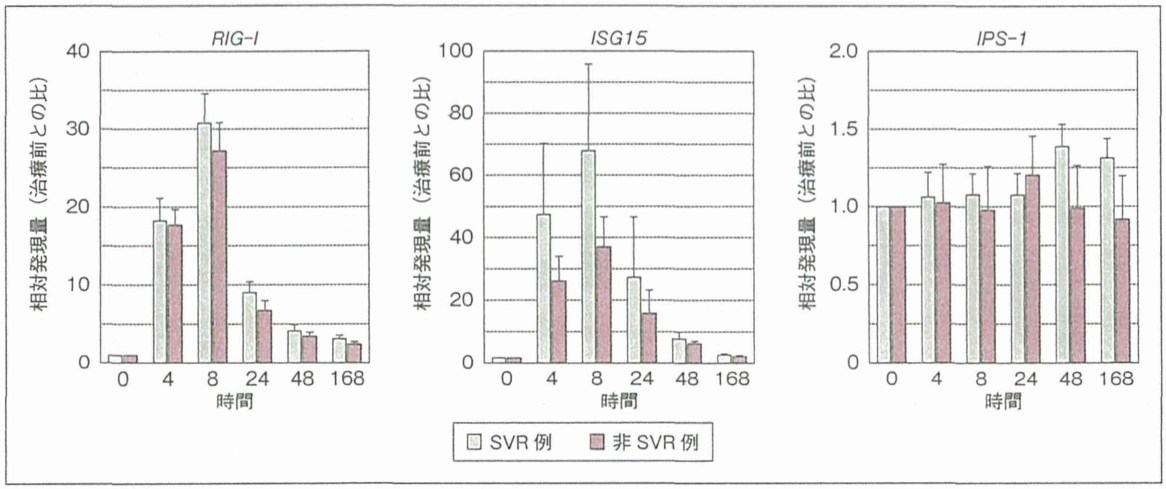

図5 PEG-IFN/RBV 併用療法治療中における自然免疫系遺伝子の末梢血単核球における発現動態と最終治療効果
末梢血単核球中における，*RIG-I* および *ISG15* の発現量は，PEG-IFN/RBV 投与により強く誘導された．しかし，ウイルスが駆除された SVR 例ではより高い誘導を認めたが，NVR 例では治療，すなわち外因性 IFN に対する反応性が減弱しており，これが治療不応性のメカニズムの一つと考えられた．一方，*IPS-1* の発現動態は PEG-IFN/RBV 投与により大きく影響は受けず治療中も constitutive に発現している．（文献 1 を改変引用）

Ⅳ 自然免疫系遺伝子発現プロファイルと *IL28B* 遺伝子多型との関連

最近 genome-wide association study（GWAS）を用いた宿主遺伝子の網羅的解析により，ヒト 19 番染色体上に存在し IFNλ をコードする *IL28B* 近傍の一遺伝子多型（SNP）と PEG-IFNα/RBV 併用療法における NVR との関連が明らかとなり，大きな注目を集めている[2~4]．この *IL28B* の SNP（rs8099917）と宿主自然免疫系の遺伝子発現の関連を genotype 1b の C 型慢性肝炎患者で検討すると[5]，NVR に関与する rs8099917 non-TT の症例では *RIG-I* および *ISG15* の肝内遺伝子発現は rs8099917 TT の症例に比し有意に高値であった．しかし，rs8099917 TT の症例でも NVR となった症例では，ウイルス反応が得られた症例に比し，これらの肝内遺伝子発現は高値で，同様に rs8099917 non-TT でも治療中ウイルス減衰がみられた症例では遺伝子発現が低値であった（図 6）．NVR に寄与する因子を検討した多変量解析では，これら自然免疫系遺伝子発現と年齢のみが有意な因子として抽出され，*IL28B* SNP は抽出されなかった．したがって，宿主自然免疫系は *IL28B* の SNP と密接に関連しつつも独立して IFN 不応性に関与している可能性が示唆された．

Ⅴ 宿主自然免疫に対する HCV の逃避機構

これまで述べたように，HCV の排除には RIG-I/IPS-1 系を中心とした宿主自然免疫とそれに引き続く IFN 応答が重要であり，*IL28B* SNP とともに密接に治療効果に関連している．これに対して，HCV は自己の NS3/4A セリンプロテアーゼで IPS-1 を特異的に切断することでこの宿主自然免疫機構を攪乱し，巧みな逃避機構を有していることが知られている．実際，前項に挙げた *IL28B* non-TT 症例においても IFN 感受性を示した症例では IPS-1 が HCV により切断されないことが関連し[5]，HCV 逃避機構は治療効果と関連していると考えられる．

最近，NS3/4A プロテアーゼとは別に HCV の NS4B 蛋白による自然免疫からの逃避機構が明らかとされ，その標的分子が IPS-1 のさらに

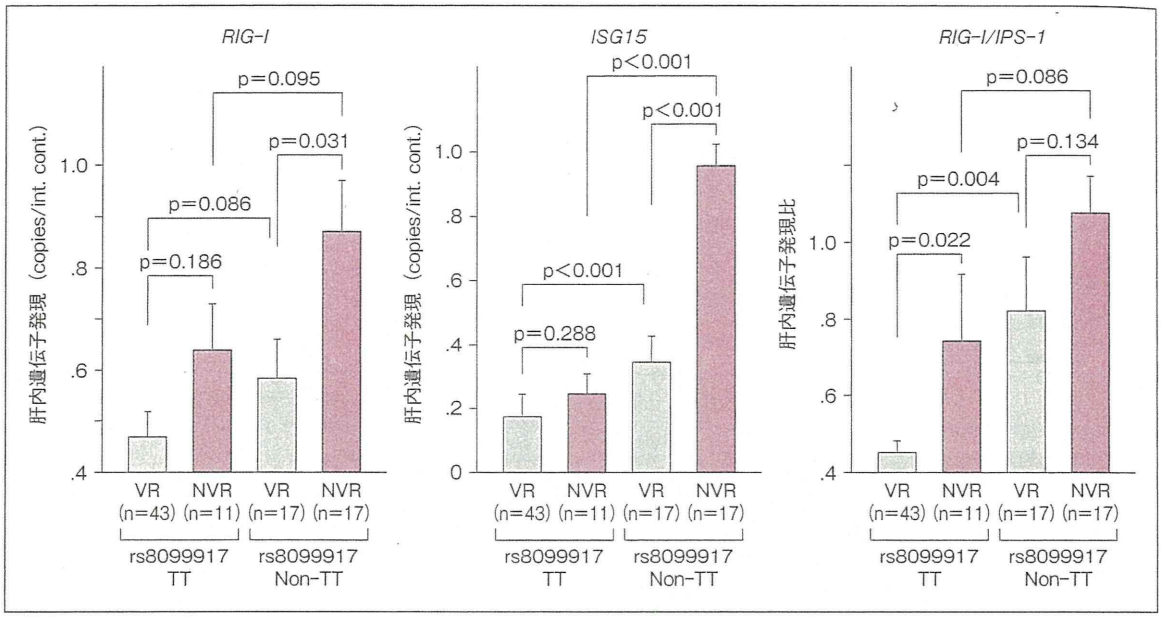

図6 自然免疫系遺伝子の肝内発現と *IL28B* 遺伝子多型およびウイルス学的治療効果
rs8099917 non-TT の症例では *RIG-I* および *ISG15* の肝内遺伝子発現は rs8099917 TT の症例に比し有意に高値である．しかし，rs8099917 TT の症例でも NVR となった症例では，これらの肝内遺伝子発現は高値で，同様に rs8099917 non-TT でも治療中ウイルス減衰がみられた症例では遺伝子発現が低値である．宿主自然免疫系は *IL28B* の SNP と密接に関連しつつも独立して IFN 不応性に関与している可能性がある．
（文献 5 を改変引用）

下流分子である STING であることが明らかとなった[6]（図 2）．さらに本研究では，NS4B 蛋白による宿主自然免疫抑制機構が NS3/4A プロテアーゼによる阻害機構と協調的に作用し RIG-I 依存性シグナルを遮断していることも明らかとされた．したがって，NS4B 蛋白が HCV の持続感染を終息させるための新たな標的となり得ることを示唆しており，今後の研究の発展が期待される．

おわりに

これまで述べてきたように，RIG-I/IPS-1 系の宿主自然免疫系は IFN 不応性の本質と密接に関与しており，これらの遺伝子発現プロファイルを解析することは治療効果の予測に有用である．一方，ウイルス側も複数の経路で宿主自然免疫機構を攪乱しシグナル伝達を遮断し，宿主自然免疫から巧みに逃避している．今後これら宿主自然免疫とウイルス逃避機構の全貌が明らかとなることで，これらを標的とした治療法が開発され，ウイルス持続感染の終息と肝炎の完全制圧が期待される．

（朝比奈靖浩）

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