Table 2 Information regarding candidate genes and genotyping of tag SNPs

Role	Gene product	Gene symbol	Location	Functional details	Tag SNP	Typing method (restriction enzyme)
Synthetic enzyme	CYP7A1	*CYP7A1*	8q11–q12	Rate–limiting enzyme determining total bile acid pool size	rs8192879	PCR–RFLP (*Hpy*188 I)
					rs11786580	PCR–RFLP (*Fnu*4H I)
					rs6997473	PCR–RFLP (*Mse* I)
					rs3747809	PCR-HRM
					rs8192875	PCR–RFLP (*Mse* I)
					rs1457043	PCR–RFLP (*Hpy*CH4 III)
					rs8192870	PCR-direct DNA sequencing
					rs3808607	PCR–RFLP (*Alw*26 I)
					rs3824260	PCR–RFLP (*Hpy*CH4 IV)
Activators	HNF4α	*HNF4A*	20q13.12	Orphan nuclear receptor activating *CYP7A1* expression as a transcription factor	rs2071197	PCR–RFLP (*Hpy*CH4 IV)
					rs3212180	PCR–RFLP (*Bsr* I)
					rs6017340	PCR–RFLP (*Bso*B I)
					rs6031587	PCR–RFLP (*Ava* II)
					rs11574736	PCR–RFLP (*Taq* I)
					rs6031590	PCR–RFLP (*Bst*U I)
					rs3746575	PCR–RFLP (*Hae* III)
	PGC-1α	*PPARGC1A*	4q15.1	Coactivator enhancing HNF4α activity	rs8192678	PCR–RFLP (*Msp* I)
					rs12374310	PCR–RFLP (*Hsp*I92 II)
					rs4235308	PCR–RFLP (*Hpy*CH4 IV)
Repressors	FXR	*NR1H4*	12q23.1	Bile acid–activated nuclear receptor repressing *CYP7A1* via induction of SHP and FGF19	rs12304867	PCR–RFLP (*Hinf* I)
					rs3789988	PCR–RFLP (*Ban* I)
					rs56163822	PCR–RFLP (*Fok* I)
					rs1327099	PCR-direct DNA sequencing
					rs12424084	PCR–RFLP (*Hsp*92 II)
					rs11110411	PCR-HRM
					rs17030285	PCR–RFLP (*Eco*O109 I)
					rs17030306	PCR–RFLP (*Fok* I)
					rs10860603	PCR–RFLP (*Hpy*CH4 IV)
					rs1030454	PCR–RFLP (*Bst*4C I)
					rs35735	PCR–RFLP (*Eco*91 I)
	SHP	*NR0B2*	1p36.1	Orphan nuclear receptor repressing *CYP7A1* expression	rs7504	PCR–RFLP (*Alw*21 I)
	GPS2	*GPS2*	17p13	Corepressor interacting with SHP	rs2292065	PCR–RFLP (*Pvu* II)
					rs2270981	PCR-HRM
					rs8610	PCR–RFLP (*Hinf* I)
	PXR	*NR1I2*	3q12–q13.3	Bile acid–activated nuclear receptor repressing *CYP7A1* expression	rs3814055	PCR-HRM
					rs2472677	PCR–RFLP (*Hpy*188 I)
					rs7643645	PCR–RFLP (*Bsr*D I)
					rs2472681	PCR–RFLP (*Hpy*188 III)
					rs2472682	PCR–RFLP (*Hsp*92 II)
					rs6785049	PCR–RFLP (*Hph* I)
					rs3814057	PCR–RFLP (*Dde* I)
	FGF19	*FGF19*	11q13	Hormone binding to and activating FGFR4	rs948992	PCR–RFLP (*Bts*C I)
					rs1789364	PCR–RFLP (*Fok* I)
	FGFR4	*FGFR4*	5q35	Receptor repressing *CYP7A1* via its downstream signals	rs351855	PCR–RFLP (*Bcn* I)
	KLB	*KLB*	4p14	Co-receptor working with FGFR4	rs17618244	PCR–RFLP (*Msp* I)
					rs4975017	PCR-HRM
	FOXO1	*FOXO1*	13q14.1	Insulin-activated transcription factor repressing *CYP7A1* expression	rs17592236	PCR–RFLP (*Ava* II)
					rs2755209	PCR-HRM
					rs12865518	PCR-HRM
					rs2995991	PCR–RFLP (*Tsp*R I)
					rs12585434	PCR–RFLP (*Bsl* I)
					rs2721044	PCR–RFLP (*Hinf* I)

PCR polymerase chain reaction, *RFLP* restriction fragment length polymorphism, *HRM* high resolution melting curve analysis

fragments carrying a G or T allele at the rs3808607 SNP site, a 384-bp fragment of the *CYP7A1* promoter region was amplified by PCR from genomic DNA of PBC patients with a G/G or T/T homozygous genotype using Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) and integrated into the pGL3-Basic plasmids (Promega, Madison, WI, USA) at the *Kpn* I-*Xho* I restriction site using a Rapid DNA Ligation Kit (Roche Diagnostics, Mannheim, Germany). Finally, direct-DNA sequencing was carried out in order to confirm the insertion of the *CYP7A1* promoter region into the reporter gene plasmid vectors.

HepG2 cells were cultured in Dulbecco's modified Eagle's medium (D-MEM, Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10 % fetal bovine serum (FBS, Life Technologies, Carlsbad, CA, USA). For transient transfection studies, 4.0×10^5 cells were subcultured in each well of a 12-well plate with 1 ml of D-MEM without FBS. When the cells had reached ~60 % confluence, transfection was performed with 1 μg of either pGL3-CYP7A1-G or pGL3-CYP7A1-T using 2.5 μl of X-tremeGENE™ HP transfection reagent (Roche Diagnostics). In addition, 100 ng of pRL-TK (Promega) was added to each transfection fluid as a transfection control for normalization. Forty hours after transfection, HepG2 cells were treated with 0, 25, and 50 μM of CDCA (Sigma-Aldrich, St. Louis, MO, USA). The cells were cultured for an additional 24 h and then lysed. Subsequently, luciferase assays were performed using the Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer's instructions. Firefly and Renilla luciferase intensities were measured by ARVO™ MX 1420 (PerkinElmer, Inc., Waltham, MA, USA). The relative intensity of the Firefly enzyme signal was normalized to that of the Renilla enzyme signal in order to adjust for variations in transfection efficiencies. All experiments were performed in triplicate.

Statistical analysis

Differences in age and the observation period between early- and late-stage PBC patients were evaluated using an unpaired Student's *t* test and Mann–Whitney *U* test, respectively. Likewise, differences in gender and the concomitance of autoimmune diseases were compared by a chi-square test or Fisher's exact test. The unpaired Student's *t* test was used for a comparison of reporter gene expressions. All statistical analyses were performed using the PASW 18 statistical software package (SPSS Japan Inc., Tokyo, Japan).

To determine whether each SNP was in Hardy–Weinberg equilibrium among PBC patients, a chi-square test with Yates' correction was performed using the SNPAlyze® 7.1 standard software package. The frequencies of allele, genotype, haplotype, and diplotype between subgroups of PBC patients were compared by a chi-square test or Fisher's exact test with odds ratio (OR) and 95 % confidence interval (CI) in three different inheritance models—the allele, the minor allele dominant, and the minor allele recessive—using the SNPAlyze® 7.1 standard software package. A P value of less than 0.05 was considered to be statistically significant.

Results

Comparison of PBC patient characteristics

The characteristics were compared between early and late stage PBC patients (Table 1). The mean age and observation period of patients in late stage were significantly older and longer, respectively, than those of patients in early stage ($P < 0.005$ and $P < 0.001$, respectively). These results indicate that some early stage patients might progress to late stage in the future. Meanwhile, there were no significant differences in sex, treatment, and the concordance of autoimmune diseases between the two stages.

Association of genes related to bile acid synthesis with PBC progression

The distributions of alleles and genotypes at 52 tag SNPs in 11 candidate genes were compared between early and late stage PBC patients (data not shown). One tag SNP, rs12304867, in *NR1H4* was not in Hardy–Weinberg equilibrium (data not shown), and was therefore excluded from the association study. Three genes, *CYP7A1, HNF4A*, and *PPARGC1A*, showed a significant association with PBC progression (Table 3).

With regard to *CYP7A1*, four tag SNPs (rs1457043, rs8192870, rs3808607, and rs3824260) showed significant differences in allele and/or genotype frequencies in three different inheritance models between early and late stage PBC patients. At rs1457043, the frequencies of a minor A allele in the allele model ($P = 0.025$, OR = 0.662) and its homozygous A/A genotype in the minor allele recessive model ($P = 0.007$, OR = 0.328) were lower in late stage PBC patients as compared to those in early stage patients (Table 3), indicating that the A allele and the A/A genotype of rs1457043 in *CYP7A1* had a protective effect against PBC progression. Conversely, a major G allele and its homozygous G/G genotype or heterozygous G/A genotype of rs1457043 implicated susceptibility to PBC progression. Likewise, the patients possessing either a major homozygous G/G genotype or heterozygous G/T genotype of rs3808607, or a major homozygous A/A genotype or

Table 3 Allele and genotype comparisons in three inheritance models between early and late stage PBC patients in tag SNPs associated with the progression

Gene symbol	Tag SNP (Major > Minor)	Genotype	Number of genotypes (%)		Inheritance model[a]	*P* value	OR	95 % CI
			Early stage	Late stage				
CYP7A1		MAF	0.44	0.34	Allele	0.025	0.662	0.460–0.952
	rs1457043	G/G	74 (32.5)	34 (39.1)				
	(G > A)	G/A	106 (46.5)	46 (52.9)	Dominant	0.268	0.749	0.449–1.250
		A/A	48 (21.1)	7 (8.0)	Recessive	0.007	0.328	0.142–0.757
		MAF	0.16	0.24	Allele	0.022	1.643	1.071–2.518
	rs8192870	C/C	159 (69.7)	49 (56.3)				
	(C > A)	C/A	64 (28.1)	34 (39.1)	Dominant	0.025	1.787	1.074–2.974
		A/A	5 (2.2)	4 (4.6)	Recessive	0.267	2.149	0.564–8.198
		MAF	0.50	0.43	Allele	0.084	0.734	0.516–1.043
	rs3808607	G/G	56 (24.6)	24 (27.6)				
	(G > T)	G/T	115 (50.4)	52 (59.8)	Dominant	0.581	0.855	0.489–1.494
		T/T	57 (25.0)	11 (12.6)	Recessive	0.017	0.434	0.216–0.874
		MAF	0.50	0.43	Allele	0.114	0.753	0.530–1.071
	rs3824260	A/A	60 (26.3)	23 (26.4)				
	(A > G)	A/G	110 (48.2)	54 (62.1)	Dominant	0.983	0.994	0.568–1.740
		G/G	58 (25.4)	10 (11.5)	Recessive	0.007	0.381	0.185–0.785
HNF4A		MAF	0.20	0.29	Allele	0.012	1.663	1.116–2.479
	rs6017340	C/C	145 (63.6)	46 (52.9)				
	(C > T)	C/T	75 (32.9)	31 (35.6)	Dominant	0.082	1.557	0.944–2.567
		T/T	8 (3.5)	10 (11.5)	Recessive	0.012	3.571	1.360–9.377
		MAF	0.43	0.32	Allele	0.012	0.624	0.431–0.903
	rs6031587	C/C	68 (29.8)	39 (44.8)				
	(C > T)	C/T	126 (55.3)	41 (47.1)	Dominant	0.012	0.523	0.314–0.870
		T/T	34 (14.9)	7 (8.0)	Recessive	0.105	0.499	0.213–1.173
PPARGC1A		MAF	0.44	0.53	Allele	0.054	1.411	0.994–2.003
	rs8192678	G/G	73 (32)	15 (17.2)				
	(G > A)	G/A	108 (47.4)	52 (59.8)	Dominant	0.009	2.261	1.214–4.211
		A/A	47 (20.6)	20 (23.0)	Recessive	0.645	1.150	0.635–2.081

MAF minor allele frequency, *OR* odds ratio, *CI* confidence interval

[a] Allele, allele model; Dominant, the minor allele dominant model; Recessive, the minor allele recessive model

heterozygous A/G genotype of rs3824260 were at risk for PBC progression. At the remaining tag SNP, rs8192870, the frequencies of a minor A allele in the allele model ($P = 0.022$, OR = 1.643) and its minor homozygous A/A genotype or heterozygous C/A genotype in the minor allele dominant model ($P = 0.025$, OR = 1.787) were increased in late stage patients (Table 3), indicating susceptibility to PBC progression. Taken together, the G allele and G/G or G/A genotype of rs1457043, A allele and A/A or C/A genotype of rs8192870, G/G or G/T genotype of rs3808607, or A/A or A/G genotype of rs3824260 showed a genetic risk factor for PBC progression.

With respect to *HNF4A*, at rs6017340, the frequencies of a minor T allele in the allele model ($P = 0.012$, OR = 1.663) and its homozygous T/T genotype in the minor allele recessive model ($P = 0.012$, OR = 3.571) were higher in late stage PBC patients as compared to those in early stage patients (Table 3), indicating that the T allele and the T/T genotype of rs6017340 in *HNF4A* conferred susceptibility to PBC progression. Whereas, at rs6031587, the frequencies of a minor T allele in the allele model ($P = 0.012$, OR = 0.624) and its homozygous T/T genotype or heterozygous C/T genotype in the minor allele dominant model ($P = 0.012$, OR = 0.523) were decreased in late stage patients (Table 3). Conversely, a major C allele and its homozygous C/C genotype of rs6031587 implicated susceptibility to PBC progression. Thus, the T allele and the T/T genotype of rs6017340 and the C allele and the C/C genotype of rs6031587 were considered to be genetic risk factors for PBC progression.

Table 4 Allele and genotype comparisons in three inheritance models between responders and non-responders to PBC treatment in tag SNPs associated with the progression

Gene symbol	Tag SNP (Major > Minor)	Genotype	Number of genotypes (%)		Inheritance model[a]	*P* value	OR	95 % CI
			Responder	Non-responder				
CYP7A1		MAF	0.44	0.29	Allele	0.011	0.520	0.312–0.867
	rs1457043	G/G	74 (32.5)	20 (48.8)				
	(G > A)	G/A	106 (46.5)	18 (43.9)	Dominant	0.044	0.505	0.258–0.988
		A/A	48 (21.1)	3 (7.3)	Recessive	0.049	0.296	0.088–1.001
		MAF	0.16	0.26	Allele	0.040	1.777	1.020–3.095
	rs8192870	C/C	159 (69.7)	23 (56.1)				
	(C > A)	C/A	64 (28.1)	15 (36.6)	Dominant	0.086	1.803	0.915–3.554
		A/A	5 (2.2)	3 (7.3)	Recessive	0.106	3.521	0.808–15.347
		MAF	0.50	0.38	Allele	0.038	0.603	0.372–0.976
	rs3808607	G/G	56 (24.6)	14 (34.1)				
	(G > T)	G/T	115 (50.4)	23 (56.1)	Dominant	0.198	0.628	0.308–1.280
		T/T	57 (25.0)	4 (9.8)	Recessive	0.041	0.324	0.111–0.950
		MAF	0.50	0.38	Allele	0.050	0.619	0.382–1.002
	rs3824260	A/A	60 (26.3)	14 (34.1)				
	(A > G)	A/G	110 (48.2)	23 (56.1)	Dominant	0.301	0.689	0.339–1.400
		G/G	58 (25.4)	4 (9.8)	Recessive	0.027	0.317	0.108–0.927
HNF4A		MAF	0.20	0.29	Allele	0.058	1.660	0.979–2.815
	rs6017340	C/C	145 (63.6)	22 (53.7)				
	(C > T)	C/T	75 (32.9)	14 (34.1)	Dominant	0.227	1.509	0.772–2.950
		T/T	8 (3.5)	5 (12.2)	Recessive	0.017	3.819	1.184–12.326
		MAF	0.43	0.27	Allele	0.008	0.495	0.294–0.835
	rs6031587	C/C	68 (29.8)	21 (51.2)				
	(C > T)	C/T	126 (55.3)	18 (43.9)	Dominant	0.007	0.405	0.206–0.795
		T/T	34 (14.9)	2 (4.9)	Recessive	0.131	0.293	0.068–1.269
PPARGC1A		MAF	0.44	0.46	Allele	0.117	0.687	0.426–1.101
	rs8192678	G/G	73 (32)	7 (17.1)				
	(G > A)	G/A	108 (47.4)	24 (58.5)	Dominant	0.054	2.288	0.968–5.405
		A/A	47 (20.6)	10 (24.4)	Recessive	0.586	1.242	0.569–2.715

MAF minor allele frequency, *OR* odds ratio, *CI* confidence interval

[a] Allele, allele model; Dominant, the minor allele dominant model; Recessive, the minor allele recessive model

Finally, the number of the patients possessing an A/A genotype or G/A genotype of rs8192678 in *PPARGC1A* was increased in late stage as compared to that in early stage ($P = 0.009$, OR = 2.261; Table 3), indicating that the patients possessing the A/A or G/A genotype at rs8192870 had a genetic risk for PBC progression.

Association of genes related to bile acid synthesis with response to PBC treatment

During the observation period, 41 of 269 patients who were initially diagnosed as early stage progressed to late stage. Since these 41 patients could be considered to be resistant to PBC treatment, we defined these 41 patients as non-responders and the remaining 228 patients as responders to PBC treatment, and investigated whether the SNPs associated with PBC progression were related to response to the treatment. The six SNPs (rs1457043, rs8192870, rs3808607, rs3824260, rs6017340, and rs6031587) of the seven SNPs associated with PBC progression were also significantly associated with response to PBC treatment (Table 4). In regard to all seven SNPs, some parts of patients with the risk genotype for PBC progression showed to be non-responders to the treatment.

Association of CYP7A1 and HNF4A haplotypes with PBC progression

Subsequently, ten haplotypes composed of four tag SNPs in *CYP7A1* and three haplotypes composed of two tag SNPs in *HNF4A*, which displayed significant association with PBC progression in the individual SNP study and

Table 5 *CYP7A1* haplotype comparison in three inheritance models between early and late stage PBC patients

Gene symbol	*rs1457043-rs8192870-rs3808607-rs3824260*	Number of haplotypes (%)		Allele model[a]			Dominant model[b]			Recessive model[b]		
		Early stage	Late stage	*P* value	OR	95 % CI	*P* value	OR	95 % CI	*P* value	OR	95 % CI
CYP7A1	A-C-T-G	192 (42.0)	59 (33.9)	0.060	0.705	0.490–1.016	0.397	0.803	0.483–1.334	0.015	0.366	0.158–0.847
	G-C-G-A	149 (32.9)	57 (32.8)	0.984	0.996	0.687–1.446	0.880	1.039	0.633–1.705	0.848	0.928	0.430–2.000
	G-A-G-A	70 (15.1)	42 (24.1)	0.010	1.755	1.141–2.699	0.013	1.904	1.142–3.174	0.223	2.699	0.660–11.039
	G-C-T-G	32 (7.1)	14 (8.1)	0.657	1.159	0.603–2.223	0.644	1.175	0.593–2.326	–	–	–
	Others	13 (3.0)	2 (1.2)	–	–	–	–	–	–	–	–	–

OR odds ratio, *CI* confidence interval

[a] Each haplotype was compared with other haplotypes combined

[b] Dominant model, the haplotype dominant model; Recessive model, the haplotype recessive model

Table 6 *HNF4A* haplotype comparison in three inheritance models between early and late stage PBC patients

Gene symbol	rs6017340-rs6031587	Number of haplotypes (%)		Allele model[a]			Dominant model[b]			Recessive model[b]		
		Early stage	Late stage	*P* value	OR	95 % CI	*P* value	OR	95 % CI	*P* value	OR	95 % CI
HNF4A	C-T	194 (42.5)	55 (31.6)	0.012	0.624	0.431–0.903	0.012	0.523	0.314–0.870	0.105	0.499	0.213–1.173
	C-C	171 (37.5)	68 (39.1)	0.715	1.069	0.747–1.530	0.767	1.080	0.648–1.801	0.758	1.116	0.554–2.250
	T-C	91 (20.0)	51 (29.3)	0.012	1.663	1.116–2.479	0.082	1.557	0.944–2.567	0.006	3.571	1.360–9.379

OR odds ratio, *CI* confidence interval

[a] Each haplotype was compared with other haplotypes combined

[b] Dominant model, the haplotype dominant model; Recessive model, the haplotype recessive model

were located within the same LD block (supplementary figure), were constructed and identified using the SNPAlyze® 7.1 standard software package. The frequencies of haplotypes and diplotypes of *CYP7A1* and *HNF4A* were compared between early and late stage PBC patients (Tables 5, 6, respectively).

With respect to *CYP7A1* haplotypes, A-C-T-G and G-A-G-A haplotypes were significantly associated with PBC progression (Table 5). The frequency of the A-C-T-G homozygous diplotype (A-C-T-G/A-C-T-G) in the recessive model was decreased in late stage PBC patients as compared to that in early stage patients ($P = 0.015$, OR = 0.366), indicating that the A-C-T-G homozygous diplotype of *CYP7A1* conferred protection against PBC progression. On the other hand, the frequencies of the G-A-G-A haplotype in the allele model ($P = 0.010$, OR = 1.755) and its homozygous or heterozygous diplotype (G-A-G-A/any) in the dominant model ($P = 0.013$, OR = 1.904) were increased in late stage patients, indicating that the G-A-G-A haplotype and its homozygous or heterozygous diplotype of *CYP7A1* conferred susceptibility to PBC progression.

In the analysis of *HNF4A* haplotypes, C-T and T-C haplotypes were significantly associated with PBC progression (Table 6). The frequencies of the C-T haplotype in the allele model ($P = 0.012$, OR = 0.624) and its homozygous or heterozygous diplotype (C-T/any) in the dominant model ($P = 0.012$, OR = 0.523) were decreased in late stage patients in comparison to that in early stage patients, indicating that the C-T haplotype and its homozygous or heterozygous diplotype of *HNF4A* conferred protection against PBC progression. Meanwhile, the frequencies of the T-C haplotype in the allele model ($P = 0.012$, OR = 1.663) and its homozygous diplotype (T-C/T-C) in the recessive model ($P = 0.006$, OR = 3.571) were increased in late stage patients, indicating that the T-C haplotype and its homozygous diplotype of *HNF4A* conferred susceptibility to PBC progression.

A functional SNP in CYP7A1 affects its expression in cholestasis

In order to investigate the transcriptional activity of the *CYP7A1* promoter carrying the G allele of rs3808607 as compared to that of another *CYP7A1* promoter carrying the T allele at the same SNP site, a dual luciferase assay was performed using HepG2 cells maintained under normal or cholestatic conditions. Under normal conditions (0 μM CDCA) of bile acid homeostasis, the relative luciferase

intensity of the *CYP7A1* promoter carrying the G allele at rs3808607 was higher than that of the promoter carrying the T allele (Fig. 1). These results supported previously published data [29].

By contrast, under cholestatic conditions (25 and 50 μM CDCA), the *CYP7A1* promoters carrying the G or T allele of rs3808607 tended towards decreased relative luciferase intensities as compared to those obtained under normal conditions in statistical analysis which did not reach statistical significance (Fig. 1). This decrease in transcription activities under cholestatic conditions might reflect diminution of *CYP7A1* expression by the negative feedback regulation mechanism. Interestingly, the relative luciferase intensities obtained with the promoter carrying the G allele were significantly higher than those obtained with the promoter carrying the T allele at both 25 and 50 μM concentrations of CDCA ($P = 0.003$ and $P = 0.007$, respectively).

Discussion

In this study, we demonstrated an association of polymorphisms of the genes *CYP7A1*, *HNF4A*, and *PPARGC1A* with PBC progression. The functions of the progression-associated genes are related to activators of bile acid synthesis in hepatocytes. On the other hand, there were no associations between PBC progression and polymorphisms of other genes that encode repressors of bile acid synthesis via negative feedback regulation. In addition to the association, a reporter gene assay showed that rs3808607, one of the progression-associated SNPs in *CYP7A1*, differently modulated *CYP7A1* promoter activity under normal and cholestatic conditions in vitro. In some PBC patients, the increase of synthetic bile acids may affect the response to UDCA as well as PBC progression. However, the reproducibility of this association in other groups of Japanese PBC patients as well as in other ethnicities remains to be investigated.

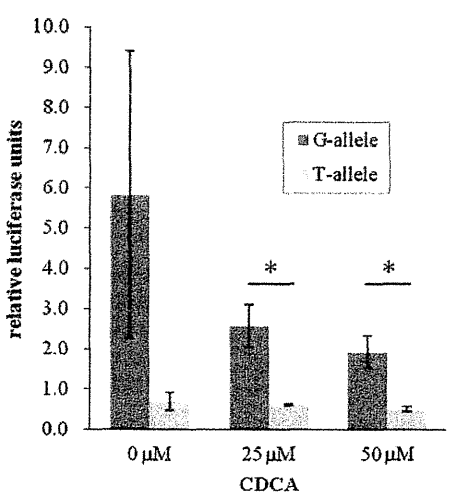

Fig. 1 Transcriptional activities of the *CYP7A1* promoter carrying either the G- or the T-allele at rs3808607 at 0, 25, and 50 μM concentrations of CDCA. Firefly luciferase signals were normalized to those of Renilla luciferase. Data shown represent mean ± standard deviation ($n = 3$). *$P < 0.01$

CYP7A1 is the first and rate-limiting enzyme in the classical bile acid synthetic pathway, and also plays a role in cholesterol catabolism and intestinal lipid absorption. Previous studies have demonstrated associations between genetic variants of *CYP7A1* and clinical phenotypes, including blood cholesterol levels and response to cholesterol-lowering drugs [30]. In addition, *CYP7A1* expression is negatively regulated in the liver of patients with cholestatic liver diseases including PBC [19, 20, 31] via the negative feedback regulation mechanism [15]. Thus, it is reasonable to speculate that the genetic variants of *CYP7A1* that were associated with PBC progression in this study, e.g., the G/G or G/A genotype of rs1457043, A/A or C/A genotype of rs8192870, G/G or G/T genotype of rs3808607, and A/A or A/G genotype of rs3824260, may enhance the expression and activities of CYP7A1, thereby leading to the accumulation of synthetic bile acids in hepatocytes. In particular, with respect to rs3808607 located within the *CYP7A1* promoter region, the transcriptional activity of the *CYP7A1* promoter carrying the G allele, which was the risk allele for PBC progression, was persistently higher under both normal and cholestatic conditions as compared to that of the promoter carrying the T allele. Although the activity of the *CYP7A1* promoter at rs3808607 has already been reported only under normal conditions [29], here we assessed the transcriptional activity of this promoter under not only normal conditions, but also experimental cholestatic conditions. Taken together, the data suggest that the genetic variants of *CYP7A1*, including rs3808607, may accelerate bile acid synthesis, thereby resulting in the accumulation of bile acids in hepatocytes, although it is unknown how much negative feedback regulation contributes to reduction of bile acid synthesis in bile acid homeostasis. The persistent accumulation of bile acids may attribute to the predisposition of PBC progression at any stage.

Another possible mechanism connecting progression-associated genes to PBC progression is the resistance to UDCA treatment. We also demonstrated that progression-associated SNPs of *CYP7A1* and *HNF4A* also showed association with response to PBC treatment, mainly to UDCA. This fact suggests that some parts of progressions we observed in patients who progressed from early to late stages during the observation period may be attributed to UDCA resistance. Under cholestatic conditions, in addition to negative feedback regulation by cholestasis as a

normally pathophysiological mechanism, UDCA also represses *CYP7A1* expression [21]. However, because the dual luciferase assay in this study revealed the higher transcriptional activities of the *CYP7A1* promoter carrying the risk-associated G allele in the experimental cholestatic conditions, UDCA may reduce the repression of *CYP7A1* expression in PBC patients bearing this allele at rs3808607. Furthermore, UDCA may also diminish a decrease in the proportion of potentially toxic hydrophobic bile acids, such as CDCA, to the total of biliary bile acids [32] due to the accumulation of synthetic endogenous bile acids. Thus, overexpression of *CYP7A1* and elevation of the proportion of hydrophobic bile acids in PBC patients with the genetic variants of *CYP7A1* may decrease therapeutic effects of UDCA, thereby resulting in the acceleration of PBC progression.

The *CYP7A1* promoter is activated by interaction of the orphan nuclear receptor HNF4α with PGC-1α, a versatile coactivator that also engages with other nuclear receptors, such as nuclear respiratory factor-1, peroxisome proliferator-activated receptor (PPAR) α and γ [33]. We identified an association between PBC progression and G1444A polymorphism of rs8192678, located in exon 8 of *PPARGC1A*, that leads to an amino acid change (Gly482Ser). A previous report has shown that the amino acid sequences around 482Ser are highly conserved among mammals, and that Gly482 has impaired coactivator activity towards the mitochondrial transcription factor A promoter and the PPAR responsive element in the reporter gene assay [26]. The fact that the frequency of 482Ser was increased in late stage PBC patients supports our hypothesis that the possession of the risk A allele of rs8192678 in *PPARGC1A*, which encodes for 482Ser, may accelerate *CYP7A1* transcriptional activities, resulting in the predisposition to PBC progression.

The mean age and the observation period of patients in the late stage group were higher and longer, respectively, than those in the early stage group. There is a possibility that some patients in the early stage group would progress to late stage in the future. This study, however, is a part of PBC cohort study to be continued. Thus, in the future study, we will get the conclusive results by adjusting age and observation period between the two groups.

In conclusion, we demonstrated the association of *CYP7A1* and its transcriptional activators, i.e., HNF4α with PBC progression. In addition, we demonstrated that one SNP in *CYP7A1* affected the expression of *CYP7A1* in both normal and cholestatic conditions in vitro. Bile acid derivatives are anticipated to exert therapeutic effects on cholestasis, and clinical trials have been conducted using these therapeutic agents to treat cholestatic liver diseases [34]. In addition to these bile acid derivatives, regulation of *CYP7A1* expression is considered to be an attractive therapeutic target. Thus, the genes identified in this study may not only modulate the therapeutic effect of certain drugs, but may also indicate susceptibility to PBC progression. Specifically, the tag SNPs that are associated with PBC progression to late stage have the potential to serve as new genetic biomarkers for PBC progression in Japanese patients.

Acknowledgments This study was supported by a Grant-in-Aid for Clinical Research from the National Hospital organization to Minoru Nakamura, by a Grant-in-Aid for Scientific Research from the Japan Society for the promotion of Science to Minoru Nakamura (#20590800, #23591006) and to Katsuhisa Omagari (#20590545), by the President's Discretionary Fund of Nagasaki University, Japan to Kazuhiro Tsukamoto, and by a research grant from the Non Profit Organization Aimed to Support Community Medicine Research in Nagasaki, Japan to Kazuhiro Tsukamoto. We thank Drs. Hiromi Ishibashi, Atsumasa Komori, Seigo Abiru, Shinya Nagaoka (NHO Nagasaki Medical Center); Makoto Nakamuta, Nobuyoshi Fukushima (NHO Kyushu Medical Center); Hajime Ota (NHO Kanazawa Medical Center); Tatsuji Komatsu (NHO Yokohama Medical Center); Jinya Ishida (NHO Nishisaitama Hospital), Hirotsugu Kouno (NHO Kure Medical Center); Michiyasu Yagura (NHO Tokyo Hospital); Masakazu Kobayashi (NHO Matsumoto Medical Center); Toyokichi Muro (NHO Oita Medical Center); Naohiko Masaki (National Center for Global Health and Medicine); Keiichi Hirata (NHO National Disaster Medical Center); Yukio Watanabe (NHO Sagamihara Hospital); Masaaki Shimada (NHO Nagoya Medical Center); Toshiki Komeda (NHO Kyoto Medical Center); Kazuhiro Sugi (NHO Kumamoto Medical Center); Eiichi Takesaki (NHO Higashihiroshima Medical Center); Yukio Ohara (NHO Hokkaido Medical Center); Hiroshi Mano (NHO Sendai Medical Center); Haruhiro Yamashita (NHO Okayama Medical Center); Michiaki Koga (NHO Ureshino Medical Center); Masahiko Takahashi (NHO Tokyo Medical Center); Tetsuo Yamamoto (NHO Yonago Medical Center); Fujio Makita (NHO Nishigunma Hospital); Hideo Nishimura (NHO Asahikawa Medical Center); Hitoshi Takagi (NHO Takasaki General Medical Center); Noboru Hirashima (NHO Higashinagoya Hospital); and Kaname Yoshizawa (NHO Shinshu Ueda Medical Center) for obtaining informed consents and collecting serum and DNA samples from PBC patients.

Conflict of interest The authors declare that they have no conflict of interest.

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日消誌　2013；110：8—15

今月のテーマ　PBC診療の最前線

原発性胆汁性肝硬変（PBC）全国調査にみる本邦PBCの病態と予後の変遷

廣原淳子[1)]　仲野俊成[2)]　關　壽人
岡崎和一[1)]　中沼安二[3)]　坪内博仁[4)]

要旨：厚生労働科学研究費補助金　難治性疾患克服研究事業「難治性の肝・胆道疾患に関する調査研究」班では約30年間にわたり原発性胆汁性肝硬変（PBC）全国調査を継続実施してきた．有病者数は年々増加し，男女比は1：6.8，最頻年齢は50～60歳代で，診断時年齢は年々高齢化傾向にある．無症候性PBCの10年生存率は93.3%で，無症状のまま経過する予後良好な群が80%程度を占めるのに対し，症候性PBCの10年生存率は65.5%である．無症候性，症候性PBCとも有意に生命予後は改善しているが，診断時発黄例（総ビリルビン値5mg/dl以上）や組織学的病期進行例では，予後改善は認められていない．PBCでの発癌率は男性例に有意に高く，女性例では組織学的病期の進展が発癌の危険因子である．

索引用語：原発性胆汁性肝硬変，疫学，予後，肝細胞癌

はじめに

原発性胆汁性肝硬変（primary biliary cirrhosis；PBC）は，その病因・病態に自己免疫学的機序が想定される慢性進行性の胆汁うっ滞性肝疾患である．厚生省「難治性の肝炎調査研究」班（現：厚生労働科学研究費補助金　難治性疾患克服研究事業「難治性の肝・胆道疾患に関する調査研究」班：研究代表者　坪内博仁）の原発性胆汁性肝硬変分科会（現：分科会会長　中沼安二）は，1980年以降2～3年に1回の頻度でPBC全国調査を継続的に実施してきた．その集計解析は，当時PBC分科会会長であった故市田文弘新潟大学名誉教授[1)]と故佐々木博富山医科薬科大学名誉教授[1)]から故井上恭一関西医科大学名誉教授[2)]へと引き継がれ，現在は筆者らが担当している．

当初調査の対象は全国における主要医療機関221施設であったが，14回に及ぶ登録症例の追跡調査を経て，現在では500施設を超える状況にある．調査方法はこれら登録施設へのアンケート調査によるが，登録されたPBC症例の臨床的データおよび予後に関して診断時から継続的な追跡調査を行い，得られた資料はすべてデータベース化

1）関西医科大学内科学第三講座　2）関西医科大学大学情報センター医療情報部　3）金沢大学大学院医学系研究科形態機能病理学　4）鹿児島大学医学部医歯学総合研究科消化器疾患・生活習慣病学

National surveys of primary biliary cirrhosis (PBC) in Japan

Junko HIROHARA[1)], Toshiaki NAKANO[2)], Toshihito SEKI, Kazuichi OKAZAKI[1)], Yasuni NAKANUMA[3)] and Hirohito TSUBOUCHI[4)]

1) Third Department of Internal Medicine, Kansai Medical University, 2) University Information Center, Kansai Medical University, 3) Department of Human Pathology, Kanazawa University Graduate School of Medicine, 4) Digestive and Lifestyle Diseases, Department of Human and Environmental Sciences, Kagoshima University Graduate School of Medical and Dental Science

Corresponding author：廣原　淳子（hirohara@kouri.kmu.ac.jp）

し一元管理を行っている．なお本研究は「疫学研究に関する倫理指針」（文部科学省・厚生労働省告示第2号，平成14年6月17日付）告示を遵守し，「医療・介護関係事業者における個人情報の適切な取扱いのためのガイドライン」（厚生労働省，平成16年12月24日付）に則り，厳格な個人情報管理システムを構築し，登録症例の個人情報漏洩などについて十分な配慮をしたうえで実施されている．

これまでその成果は，脳死肝移植レシピエント選択における医学的緊急性評価点の判断基準[3]，厚生労働省難病情報センターホームページ[4]，同研究班による「PBCの診療ガイドライン」[5]刊行時の疫学的資料として提供されており，また研究班内で共同利用を図ることで，PBCの病因，病態の解明，診断および治療指針の策定などに寄与しており，その解析結果は随時公表されてきた[1)2)6)~8)]．

本稿では，第1回（1980年実施）から第14回（2009年実施）全国調査まで，約30年に及ぶ全登録症例7376例の集積データをもとに，本邦におけるPBCの疫学的動向について概説し，最近増加傾向にある肝細胞癌の発生についても言及する．

I　諸外国におけるPBCの発生状況

諸外国の報告では，罹患率（発生数）は0.7～49人/人口100万人，有病率（患者数）は6.7～402人/人口100万人とされているが，国および地域により大きな相違がある[9)10)]．イギリス，スウェーデンなど北半球では罹患率および有病率ともに高率である一方，オーストラリアでは低率であり，アフリカの一部やインドにはほとんどみられない．また同じ国内においても地域差がある．これらの違いは医療側の診断能力の問題，または遺伝学的因子，感染因子，生体外異物による環境因子など，PBCの病因因子が関与している可能性もあるが明らかではない．

II　本邦におけるPBC患者の推定数

本邦におけるPBC患者の発生状況を知るうえでの統計資料は，①厚生労働省患者調査による総患者数（調査日当日の医療施設での受療者数から推定），②厚生労働省「特定疾患の疫学に関する研究班」の全国疫学調査による受療患者数（調査年度における病院施設での受療患者数から推定），③厚生労働省特定疾患医療受給者証交付件数による医療受給者数（受給対象となる症候性PBCの患者数），④厚生労働省「難治性の肝・胆道疾患に関する調査研究班」全国調査（調査期間における専門施設での登録患者），⑤人口動態調査や病理剖検輯報による死亡者数，などがある．

1989年より症候性PBCは，厚生労働省特定疾患として公費負担が認められた．2010年度（平成22年度）特定疾患医療受給者証交付件数による医療受給者数は17298人であり[4]，全体の約70％程度を占めると考えられる無症候性PBCを含めると[8]，PBC患者総数は57660人，年間推定発生数は約500人と推計される．日本の総人口を1億3千万人（平成22年度国勢調査）とすると，有病率は450人/人口100万人，20歳以上（1億5百万人）のみを対象とすると550人/人口100万人程度となり，諸外国と比較しても高率の部類に相当する．しかし厚生労働省患者調査による総患者数や厚生労働省「特定疾患の疫学に関する研究班」と「難治性の肝・胆道疾患に関する調査研究班」の共同で実施された，全国疫学調査による受療患者数に基づく推計患者数[11)]と，同時期の特定疾患医療受給者証交付件数による医療受給者数に基づく推計患者数[4)]には乖離があり，本邦におけるPBCの患者実数はこれよりやや少ないものと考えられる．

これらの中で，④の筆者らが携わってきた厚生労働省「難治性の肝・胆道疾患に関する調査研究班」による全国調査は，全国の専門施設を対象として登録症例の追跡予後調査を継続的に実施して得られた統計資料であり，本邦のPBC全症例を把握しているわけではないが，疫学的動向を把握するために有用とされている．

III　全国調査にみるPBCの疫学的動向

専門施設を対象とする全国調査における発生数は，調査開始当初はわずか10名程度であったが，PBCの疾患概念および診断基準が確立し本症に関する知識が医療者に浸透するに従い，また診断

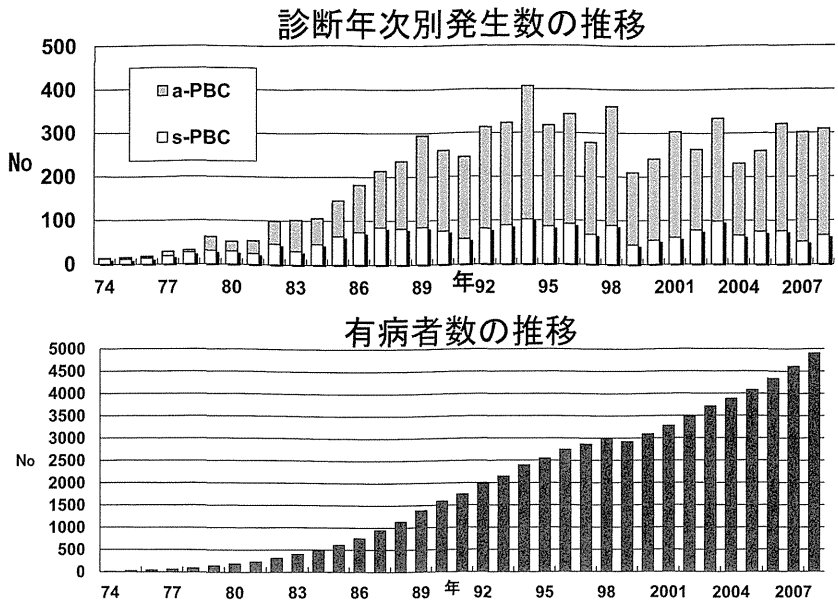

Figure 1. 全国調査における診断年次別発生数と有病者数の推移：PBC の発生数は，調査開始後漸増傾向にあったが，1990 年代以降は年間 250 ～ 300 名前後で推移している．近年は無症候性 PBC 例の占める割合が増加しており，近年新たに診断される症例のうち約 80% 以上は無症候性 PBC である．また有病者数は年々増加傾向にある（文献 8 より引用）．

根拠のひとつである抗ミトコンドリア抗体測定が簡便に行えるようになった時期に相応して，PBC と診断される症例は漸増してきたが，1990 年代以降は年間 250～300 名前後で推移している（Figure 1）．皮膚掻痒感，黄疸，食道胃静脈瘤，腹水，肝性脳症など肝障害に基づく自他覚症状を有する場合は症候性（symptomatic）PBC（s-PBC），これらの症状を欠く場合は無症候性（asymptomatic）PBC（a-PBC）と呼ばれる．近年は検診時や他疾患受療時に肝機能検査値異常を契機として診断される無症候性 PBC 例が増加しており，近年新たに診断される症例のうち約 80% 以上は無症候性 PBC である（Figure 1）．有病者数は年々増加しているが，無症候性 PBC 例が増加していることおよび予後が改善していることが，有病者数の増加に影響しているものと考えられる．男女比は 1：6.8 で女性に多く，最頻年齢は 50～60 歳代（診断時平均年齢は 56 歳；男性 59 歳，女性 55 歳）であり，幼小児期での発症はないとされている．男女比に大きな変化は認められないが，診断時年齢は年々高齢化する傾向にある．

IV 全国調査にみる治療の変遷と予後との関連

全国調査において年次別肝疾患関連死亡数は 2000 年以降明らかに減少している．PBC の主な死因は肝不全と消化管出血であることに変わりはないが，1990 年代以降は，原疾患以外の死因による死亡数が増加し，2000 年代になると肝不全，消化管出血による死亡数は減少傾向となっている（Figure 2）．最近の傾向としては長期生存例の増加により少数例ではあるが，肝細胞癌を合併し癌死に至る症例もある．

本邦における治療の変遷を Figure 2 にまとめて示すが，1987 年 Poupon らにより PBC に対してウルソデオキシコール酸（ursodeoxycholic acid；UDCA）の有用性が報告[12]されて以降，比較的早い段階から 80% 以上の症例に UDCA が投与されるようになっており，また 1996 年に本邦

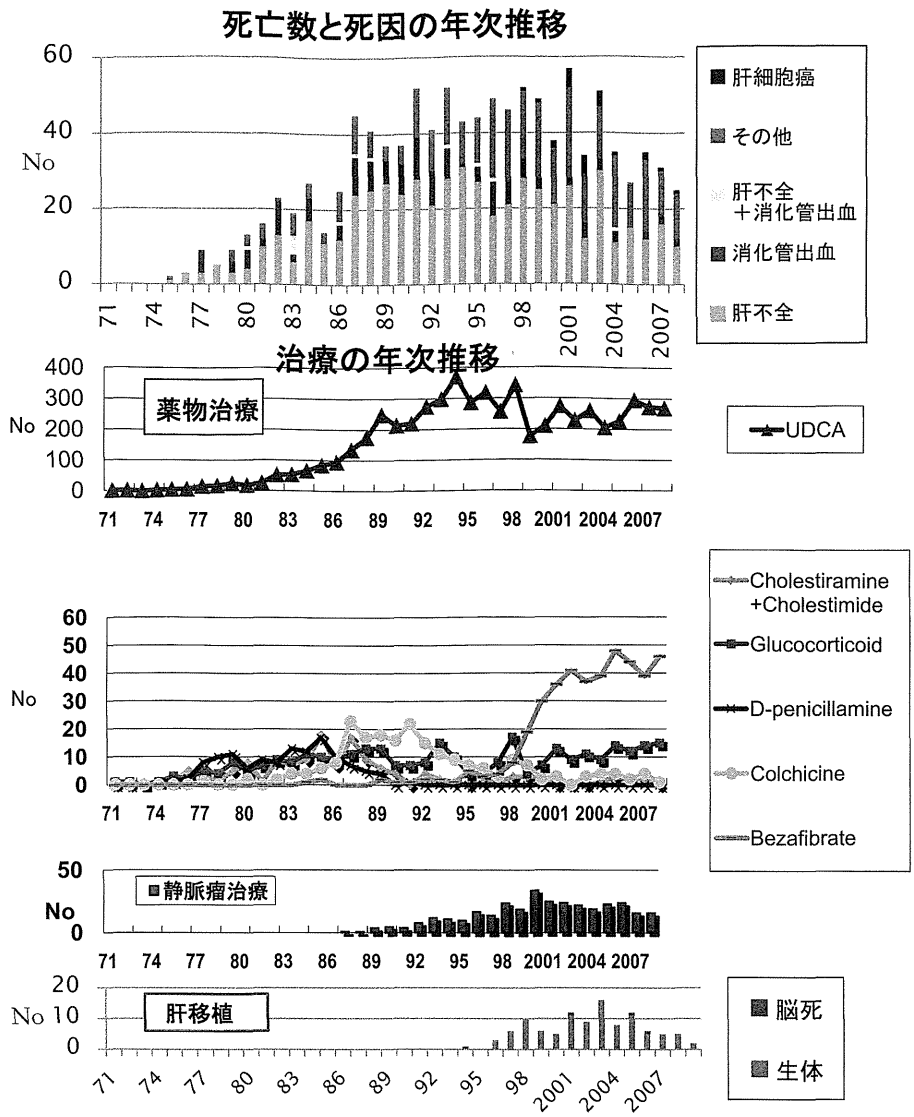

Figure 2. 全国調査における死亡数，死因の年次推移と治療の年次推移：全国調査において1114例の死亡例が報告されているが，年次別にみて肝疾患関連死亡数は2000年以降明らかに減少している．PBCの主な死因は肝不全と消化管出血であることに変わりはないが，1990年代以降は，原疾患以外の死因による死亡数が増加し2000年代になると肝不全，消化管出血による死亡数は減少傾向にある．1980年代後半からはウルソデオキシコール酸（ursodeoxycholic acid；UDCA）投与例が急増し，食道胃静脈瘤治療例も増加しており，1990年代半ばからは肝移植例も報告されるようになった（文献14より引用）．

からその有用性が発信されたベザフィブラート[13]はUDCA抵抗性の症例に対して投与されるようになったため，投与症例も最近増加していることがわかる．第14回調査ではUDCA単独投与例は61.1%，ベザフィブラートとの併用例16.5%，ステロイドとの併用例は3.3%であり，UDCA投与例は全体の80%以上となっている[8]．また1980年代後半からは食道胃静脈瘤治療例数が増加しており，1990年代半ばからは少数ながらも肝移植例も報告され，これまでに120例に及ぶが，その

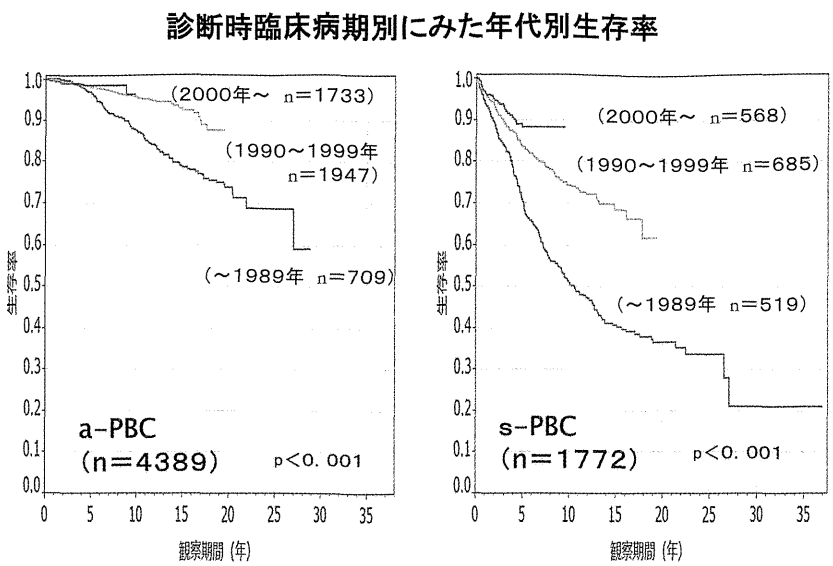

Figure 3. 全国調査における診断時臨床病期別にみた年代別生存率：全国調査登録症例（対象：登録症例中予後が明らかな6161例：男性759例，女性5402例，平均観察期間80.4カ月）について診断日をもとに，1989年までに診断された群（1228例），1990～1999年に診断された群（2632例），2000年以降に診断された群（2301例）に分けて年代別に解析したところ，生存率は年代を経る毎に有意に改善していた．診断時の臨床病期別にみても，無症候性PBC（a-PBC 4389例），症候性PBC（s-PBC 1772例）とも年代別にみて最近の症例群では有意に生存率が高く（$p<0.001$），予後は改善していることが明らかである（文献14より引用）．

大部分は生体部分肝移植である．静脈瘤治療の普及と消化管出血による死亡数の減少，肝移植例の増加と肝不全死亡数の減少は，各々呼応した動向を示している．

V 全国調査にみる予後の変遷

予後は診断時臨床病期により異なり，無症候性PBCの5年生存率は97.7%，10年生存率は93.3%，20年生存率は82.1%で，症候性PBCでは各々79.5%，65.5%，50.2%である[8]．

無症候性PBCでは，病期が進行せず予後良好な経過をたどる群と，徐々に進行する群が存在する．診断時無症候性であった症例の約80%は無症状のまま経過し，その大部分は15年以上生存し生命予後は一般集団と変わらないとされる[8]．一方，無症候性PBCの約20%は症候性に移行する．診断時無症候性PBCについて年代別に検討したところ，無症候性PBCのまま推移する予後良好な群の占める比率は，年代を経る毎に増加している[14]．

約30年に及ぶ全国調査登録症例の予後を年代別に解析したところ，予後は明らかに改善傾向にある．5年生存率では1989年までに診断された群85.4%，1990年から1999年に診断された症例群94.2%，2000年以降に診断された群95.8%と，年代を経る毎に有意に改善していた[14]．診断時の臨床病期別にみても，無症候性PBC，症候性PBCとも年代別にみて有意に予後は改善している（Figure 3）．本邦のPBCの80%以上の症例で投与されているUDCAは，ランダム化比較試験を含む多くの臨床試験で，組織学的進行を遅延し肝移植までの期間や死亡までの期間を延長することが確認されている[15]．予後良好な群の占める割合が無症候性PBCの大部分を占めるようになったことや，UDCAをはじめとする内科的治療が，各臨床病期において生命予後の改善に寄与しているものと推測される．しかしながら，症候性PBC

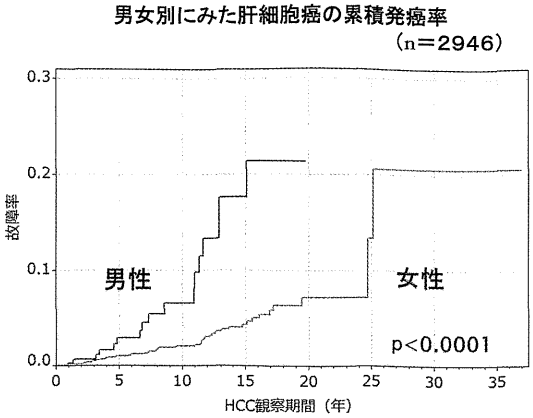

Figure 4. 全国調査における男女別にみた肝細胞癌の累積発癌率：全国調査登録症例のうち（対象：登録症例中肝細胞癌の発生の有無に関する情報が明らかな2946例，男性370例，女性2576例，平均観察期間80カ月），経過中肝細胞癌の発生したのは71例（男性19例，女性52例：B型，C型肝炎ウイルスマーカー陽性例は除外）であった．累積発癌率はPBC診断後10年で2.5％（男性6.5%，女性2.0%）であり，男性例での発癌率が有意に高い（$p<0.0001$）（文献17より引用）．

のうち診断時すでに総ビリルビン値5mg/dl以上の発黄例，また組織学的病期Scheuer分類[16]のIII，IV期の組織学的進行例では，各年代的にみても有意な予後改善は認められず[14]，これらの群では現行の内科的治療効果が乏しいと判断され肝移植が唯一の治療となる．

VI　悪性腫瘍の合併と肝発癌

第14回全国調査において，PBCの診断時に合併する悪性腫瘍は3.1%であった．その内訳は肝臓24%，胃17%，大腸11%，乳腺10%，子宮5%，甲状腺5%，血液疾患5%，卵巣3%，肺2%，副腎1%，その他17%となっている[8]．

従来PBCにおける肝細胞癌の発生はウイルス性肝炎と異なり比較的まれとされてきたが，長期生存例が漸増している中，経過中肝細胞癌を発症する症例が集積されつつある．第1回～第14回全国調査登録症例のうち，経過中に肝細胞癌の発生を確認した71例（B型，C型肝炎ウイルスマーカー陽性例は除外）について検討したところ，累積発癌率はPBC診断後10年で2.5%（男性

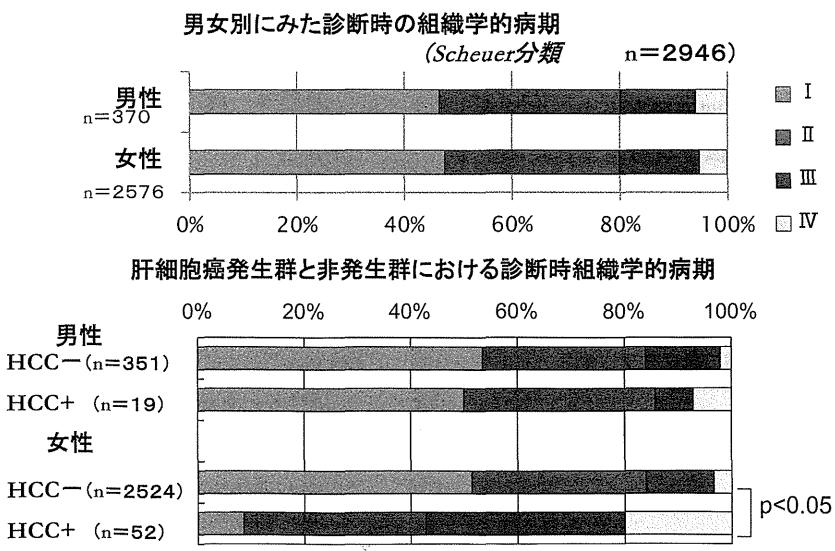

Figure 5. 全国調査における男女別にみた診断時の組織学的病期と肝発癌：全国調査登録症例のうち（対象：登録症例中肝細胞癌：hepatocellular caricinoma；HCCの発生の有無に関する情報が明らかな2946例，男性370例，女性2576例，平均観察期間80カ月），診断時の組織学的病期に性差は認められないが，肝細胞癌発生群と非発生群別に検討すると，女性例ではScheuer分類III期＋IV期の占める比率が肝細胞癌発生群は57.1%であるのに対して，非発生群は16.0%と有意に高い結果が得られた（$p<0.05$）（文献17より引用）．

6.5%，女性2.0%）と明らかな性差が認められ(Figure 4)，発癌した症例群は予後不良であった．診断時組織学的病期について検討すると，診断時に性差は認められないが，女性例では肝細胞癌発生群は非発生群と比較してIII期とIV期の比率が有意に高かった（Figure 5）．発癌の背景因子について多変量解析で検討したところ，女性例では組織学的病期進展が発癌の危険因子として選択されたが，男性例では他の要因の関与が示唆された[17]．Shibuyaら[18]は多変量解析を用いた報告で高齢，男性，輸血歴を発癌の危険因子として，また別の報告では組織学的進展が危険因子としてあげられており[19]，これらの危険因子を有するPBCにおいては定期的に肝細胞癌のスクリーニング検査を行う必要性があると考えられる．

おわりに

厚生労働省難治性疾患克服研究事業「難治性の肝・胆道疾患に関する調査研究」班による，PBC全国調査の約30年に及ぶ長期追跡症例の検討から，本邦における疫学的動向と予後の変遷について概説した．おおむねPBCの予後は改善しているが，病期進展例では最近の治療を駆使しても有意な予後改善は得られず，これらの群では肝移植に頼らざるを得ない状況にある．最近，Nakamuraら[20]によりゲノムワイド関連解析を用いて日本人のPBCの疾患感受性遺伝子が同定されたことを受け，新しい観点からの治療法の開発が今後大いに期待されるところである．

謝辞：本研究は，厚生労働科学研究費補助金　難治性疾患克服研究事業「難治性の肝・胆道疾患に関する調査研究」の助成金によりました．PBC全国調査にご協力いただきました全国登録516施設に深甚の謝意を表します．

本論文内容に関連する著者の利益相反
：なし

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論文受領，2012年10月18日
受理，2012年10月19日

Liver INTERNATIONAL
OFFICIAL JOURNAL OF THE INTERNATIONAL ASSOCIATION FOR THE STUDY OF THE LIVER

Liver International ISSN 1478-3223

CIRRHOSIS AND LIVER FAILURE

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

Yoshihiro Aiba[1], Kenichi Harada[2], Atsumasa Komori[1,3], Masahiro Ito[1,3], Shinji Shimoda[4], Hitomi Nakamura[1], Shinya Nagaoka[1], Seigo Abiru[1], Kiyoshi Migita[1,3], Hiromi Ishibashi[5], Yasuni Nakanuma[2], Nao Nishida[6], Minae Kawashima[6], Katsushi Tokunaga[6], Hiroshi Yatsuhashi[1,3] and Minoru Nakamura[1,3,7]

1 Clinical Research Center, National Hospital Organization Nagasaki Medical Center, Omura, Japan
2 Department of Human Pathology, Kanazawa University Graduate School of Medicine, Kanazawa, Japan
3 Department of Hepatology, Nagasaki University Graduate School of Biomedical Sciences, Omura, Japan
4 Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan
5 International University of Health and Welfare/Fukuoka Sanno Hospital, Fukuoka, Japan
6 Department of Human Genetics, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
7 Headquarters of PBC Research in the National Hospital Organization Study Group for Liver Disease in Japan (NHOSLJ), Omura, Japan

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

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

Correspondence
Minoru Nakamura, MD, PhD, Headquarters of PBC Research in NHOSLJ, Clinical Research Center, National Hospital Organization Nagasaki Medical Center and Department of Hepatology, Nagasaki University Graduate School of Biomedical Sciences, Kubara 2-1001-1, Omura, Nagasaki 856-8562, Japan
Tel: +81 957 52 3121
Fax: +81 957 53 6675
e-mail: nakamuram@nmc.hosp.go.jp

Received 15 February 2013
Accepted 24 July 2013

DOI:10.1111/liv.12296

Abstract

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

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

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

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

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

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

Materials and methods

Subjects

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

Enzyme-linked immunosorbent assay (ELISA)

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

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

Histological examination of liver biopsy samples

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

RNA extraction and quantification of mRNA

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

Ethics board

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

Statistical analysis

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

Results

Patient demographics and clinical characteristics

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

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

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

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

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

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

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

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

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

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

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

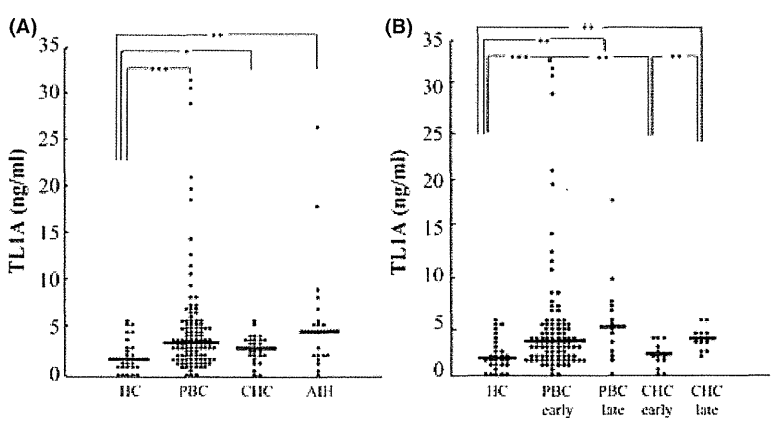

Fig. 1. Serum TL1A levels in PBC, CHC and AIH patients. (A) Serum TL1A levels were significantly higher in patients with PBC (n = 110, 4.9 ± 5.0 ng/ml), CHC (n = 26, 3.0 ± 1.5 ng/ml) and AIH (n = 19, 5.9 ± 6.4 ng/ml) as compared with healthy controls (n = 29, 2.2 ± 1.7 ng/ml). (B) Serum TL1A levels were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