icant elevation of serum G-CSF were also noticed. Furthermore, both serum white blood cell counts and G-CSF levels decreased after resection of the tumor. In light of these, we could be sure that this case was G-CSF-producing HCC.

The prognosis of G-CSF-producing tumors is generally considered to be poor and depends largely on the primary disease (8). Such a prognosis was also noted in the previous two cases of G-CSF-producing HCC. In support of this, G-CSF was demonstrated to stimulate growth of a non hematopoietic malignant cell line in vivo (9) and is considered to be an autocrine growth factor in rapid tumor proliferation and metastasis (10-13).

We also performed immunohistochemical staining for the G-CSF receptor, which yielded negative findings. This is the first case of G-CSF-producing HCC in which immunohistochemical staining for G-CSF receptor was performed. Although the localization of the tumor and absence of liver cirrhosis in this patient may have contributed to a better prognosis, we can also speculate that the absence of G-CSF receptors in the patient's tumor caused a deficiency or absence of autocrine growth, which led to a more favorable prognosis. In a similar manner, G-CSF-receptor-positive groups of oral and mesopharyngeal squamous cell carcinomas had a significantly lower disease-free and overall survival rate than G-CSF-receptor-negative groups (14).

Another difference between our case and previous ones lies in the degree of tumor cell differentiation seen in histopathology; our case showed moderate differentiation, but the others were poorly differentiated (Table 1). It may be likely that local expression and interactions between G-CSF and G-CSF receptors induce differentiation of HCC cells into an immature phenotype.

Lastly, serum values of IL-6 were elevated both in our case and in case 2 (6). It has been reported that co-production of G-CSF and IL-6 is associated with the production of IL-1, a known as inflammatory cytokine, in G-CSF producing cancer cell lines (15). High levels of serum IL-6 and CRP in the present case may have been responsible for the chief complaint of fever. IL-6 is considered to act as an endogenous pyrogen (16, 17) that regulates the synthesis of acute phase proteins, including CRP (18, 19). However, we were unable to clarify the production of IL-6 (IL-6 [R-49L]: sc-90110, mouse monoclonal antibody, Santa Cruz Biotechnology, Inc., Santa, Cruz, CA, USA) or expression of IL-6 receptor (gp130 [AN-H2], sc-9994, mouse monoclonal antibody, Santa Cruz Biotechnology, Inc.) immunohistochemically in our case. It is possible that the antibodies used were not sensitive enough for this tumor.

In conclusion, clinicians should consider G-CSF-producing HCC when encountering patients with leukocytosis and a hepatic tumor, and radical surgery may provide a more favorable prognosis in such instances. Further cases are needed to clarify the clinical findings of G-CSF-producing HCC.

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Research Article

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Association analysis of *cytotoxic T-lymphocyte antigen 4* gene polymorphisms with primary biliary cirrhosis in Japanese patients

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Background & Aims: Primary biliary cirrhosis (PBC) is an organ-specific autoimmune disease of still unidentified genetic etiology that is characterized by chronic inflammation of the liver. Since *cytotoxic T-lymphocyte antigen 4* (*CTLA4*) polymorphisms have recently been linked with PBC susceptibility in studies on Caucasians, we investigated the genetic association between *CTLA4* polymorphisms and PBC in a Japanese population.

Methods: Five single nucleotide polymorphisms (SNPs) in the *CTLA4* gene (rs733618, rs5742909, rs231775, rs3087243, and rs231725) were genotyped in 308 patients with PBC and 268 healthy controls using a TaqMan assay.

Results: One *CTLA4* gene SNP (rs231725) was significantly associated with susceptibility to anti-mitochondrial antibody (AMA)-positive PBC, but clinical significance disappeared after correction for multiple testing. Moreover, *CTLA4* gene SNPs did not influence AMA development or disease progression to orthotopic liver transplantation in our Japanese cohort. In haplotype analyses, one haplotype [haplotype 1 (CGGA)] at rs5742909, rs231775, rs3087243, and rs231725, was significantly associated with susceptibility to both AMA-positive PBC and overall PBC.

Conclusions: This study showed that *CTLA4* gene polymorphisms had a modest, but significant association with susceptibility to PBC in the Japanese population. The connection between genetic variants and the function of the *CTLA4* gene remains to be addressed in future investigations.

Keywords: Primary biliary cirrhosis; Single nucleotide polymorphisms; Cytotoxic T-lymphocyte antigen 4; Genetic susceptibility.

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Abbreviations: PBC, primary biliary cirrhosis; AMA, anti-mitochondrial antibody; CTLA4, cytotoxic T-lymphocyte antigen 4; OLT, orthotopic liver transplantation; SNPs, single nucleotide polymorphisms; UTR, untranslated region; LD, linkage disequilibrium; HWE, Hardy–Weinberg equilibrium; *pc*, corrected *p*; OR, odds ratio; CI, confidence interval; sCTLA4, soluble isoform of CTLA4.

Introduction

Primary biliary cirrhosis (PBC) is a liver-specific autoimmune disease characterized by female preponderance and the destruction of intrahepatic bile ducts that often results in cirrhosis and hepatic failure [1]. The etiology of PBC has yet to be conclusively elucidated, although genetic factors are considered to play a prominent role in family and population studies [2–5]. Prior reports have shown the HLA-*DRB1*08* allele to be a weak and regional determinant of PBC susceptibility [6–8]. However, HLA alone does not explain the entire genetic predisposition to PBC, mainly because at least 80–90% of patients with the disease do not carry the most common HLA susceptibility alleles. In this regard, other non-HLA genes are thus being considered to contribute to disease development [9,10].

PBC displays immunologically characteristic features like biliary lymphocytic infiltrates, anti-mitochondrial antibodies (AMA) against the inner lipoyl domain of the E2 subunits of the pyruvate dehydrogenase complex, and elevated serum levels of IFN-γ and TNF-α. The serologic hallmark of PBC is the presence of AMA [11,12], which are found in 95% of patients with PBC [13] and have a specificity of 98% for the disease [12]. Auto-reactive $CD4^+$ and $CD8^+$ T cells are also found in high concentrations in the portal triads of patients with PBC, often surrounding and infiltrating necrotic bile ducts [14–16]. A recent study suggested that a reduction in the number of $CD4^+CD25^+$ regulatory T cells in livers affected with PBC contributed to disease progression [17]. Accumulating data such as these, support a direct role of T-lymphocytes in the pathogenesis of PBC.

The cytotoxic T-lymphocyte antigen 4 (CTLA4) is an inhibitory receptor expressed on the cell surface of activated memory T cells and $CD4^+CD25^+$ regulatory T cells that acts largely as a negative regulator of T-cell responses. Since the potential inhibitory functions of CTLA4 [18] may also trigger a breakdown of immunological self-tolerance, polymorphisms affecting these processes could have significant effects on susceptibility to autoimmunity.

The *CTLA4* gene is a primary candidate for genetic susceptibility to autoimmune diseases, including type 1 diabetes, auto-

immune hepatitis [19,20], and autoimmune pancreatitis [21]. In particular, two single nucleotide polymorphisms (SNPs), rs231775 (49AG) and rs3087243 (CT60), have been widely studied in PBC [22–24]. Although early studies found an association between SNP 49G coding and PBC [22–24], ensuing reports showed negative relationships with susceptibility [25–30] or a positive association with liver damage [31]. A recent investigation reported that rs231725 in the 3′ flanking region of *CTLA4* is associated with AMA-positive PBC in Caucasians [27]. In addition to *CTLA4* polymorphisms, HLA class II, IL12A, IL12RB, and several other candidate SNPs were disclosed as predisposition genes for PBC by a high-density genome-wide association study [9]. Since these SNPs have not been extensively examined in a large Japanese population, the present study sought to evaluate the involvement of *CTLA4* SNPs and haplotype SNPs in susceptibility to PBC and disease progression in Japanese patients.

Patients and methods

Subjects

We analyzed a total of 576 subjects (308 PBC patients and 268 healthy controls) collected from two different regions of Japan (Table 1). Cohort 1 consisted of 198 patients clinically diagnosed with PBC (173 women, median age 58 years old) and 170 healthy subjects who were seen at Shinshu University Hospital, Matsumoto, Japan. Cohort 2 consisted of 110 patients clinically diagnosed with PBC (92 women, median age 61 years old) and 98 healthy subjects from the National Hospital Organization Nagasaki Medical Center, Omura, Japan. The racial background of all subjects was Japanese. Control subjects were volunteers from hospital staff who had indicated the absence of any major illnesses in a standard questionnaire. The diagnosis of PBC was based on criteria from the American Association for the Study of Liver Diseases [32]. Serum AMA, specific for the pyruvate dehydrogenase complex-E2 component, was measured by the enzyme-linked immunosorbent assay as reported previously [33]. An index of greater than seven was considered a positive result. All patients were negative for hepatitis B surface antigen, antibody to hepatitis C virus, and antibody to human immunodeficiency virus. To evaluate associations between SNPs and disease progression, patients were classified into two stages based on their most recent follow-up [34]: early stage patients were histologically in Scheuer stage I or II [35,36] or of unknown histological stage without liver cirrhosis, and late stage patients were histologically in Scheuer stage III or IV or clinically diagnosed with liver cirrhosis or hepatic failure. All participants provided informed written consent for this study, which had been approved by the institutional ethics committee.

CTLA4 SNP genotyping

Genomic DNA from patients and controls was isolated by phenolic extraction of sodium dodecyl sulfate-lysed and proteinase K-treated cells, as described previously [37,38], and adjusted to 10–15 ng/μl.

The five *CTLA4* gene SNPs examined in this study (rs733618, rs5742909, rs231775, rs3087243, and rs231725) were genotyped using the 5′ nuclease (TaqMan) assay using primer, probes, and reaction conditions as recommended by the manufacturer (Applied Biosystems, Tokyo, Japan). These SNPs were selected based on previous reports [21–23,26,27], and were all located in the *CTLA4* gene; SNPs rs733618 and rs5742909 were in the promoter region, SNP rs231775 in exon 1, and SNPs rs3087243 and rs231725 in the 3′ untranslated region (UTR). Polymerase chain reaction was performed with a TaqMan Assay for Real-Time PCR (7500 Real-Time PCR System; Applied Biosystems) following the manufacturer's instructions.

Haplotype–genotype estimation

The R package "haploview" [39] was used to evaluate the haplotype structure of the five examined *CTLA4* SNPs. Pairwise linkage disequilibrium (LD) patterns and haplotype frequency analysis for all SNPs in patients and controls were assessed by the block definition by Gabriel et al. [40].

Table 1. Demographic and clinical data of patients with PBC at study onset.

Characteristics	Cohort 1 Shinshu $n = 198$	Cohort 2 Nagasaki $n = 110$	Combined $n = 308$
Age, years[a]	58 (30–83)	61 (34–85)	58 (30–88)
Female/Male	173/25	92/18	265/43
Disease progression			
Early stage, *n*/Late stage, *n*	149/49	74/36	223/85
Orthotopic liver transplantation, *n* (%)	15 (7.6)	2 (1.8)	17 (5.5)
AMA positive, *n* (%)	171 (86.4)	102 (92.8)	273 (88.6)

PBC, primary biliary cirrhosis; AMA, anti-mitochondrial antibody specific for the pyruvate dehydrogenase complex-E2 component.
[a] Median (range).

Statistical analysis

The Hardy–Weinberg equilibrium (HWE) test was done for each SNP between control and patient groups. The significance of allele distribution between PBC patients and healthy controls was assessed using the χ^2-test with the use 2×2 or 2×3 comparisons. Fisher's exact probability test was used for groups with fewer than 5 samples. A p value of less than 0.05 was considered statistically significant; p values were corrected using Bonferroni's correction by multiplying by the number of different alleles observed in each locus (pc).

Results

In total, five SNPs located in the *CTLA4* gene were genotyped in 198 patients with PBC and 170 healthy controls in cohort 1 and 110 patients with PBC and 98 healthy controls in cohort 2 (Table 2). Hardy–Weinberg equilibrium (HWE) was observed for all 5 of the examined SNPs in both control groups, and the minor allele frequencies of all SNPs were greater than 5%. In cohort 1, one SNP (rs733618) differed significantly from HWE ($p = 0.03$) (Table 2), and the frequency of the minor A allele at rs231775 was significantly decreased (33.9% vs. 41.5%, odds ratio (OR) 0.72, 95% confidence interval (95% CI) 0.53–0.99, $p = 0.042$, $pc = 0.209$) in 171 AMA-positive PBC patients compared with controls. Positivity for the major G allele (A/G + G/G) at rs231775 was significantly higher in patients with AMA-positive PBC than in healthy subjects (88.3% vs. 79.1%, OR 1.96, 95% CI 1.08–3.53, $p = 0.026$, $pc = 0.128$). Additionally, the allele frequency (61.7% vs. 53.2%, OR 1.41, 95% CI 1.04–1.92, $p = 0.025$, $pc = 0.127$) and allele carrier frequency (86.0% vs. 75.9%, OR 1.96, 95% CI 1.12–3.41, $p = 0.018$, $pc = 0.089$) of the major A allele at rs231725 were significantly increased in AMA-positive PBC patients compared with healthy controls. However, these statistical significances disappeared after correction for multiple testing. No significant differences were observed among the 5 SNPs in cohort 2. The allele frequency (60.3% vs. 53.4%, OR 1.33, 95% CI 1.04–1.69, $p = 0.022$) of the major A allele at rs231725 was significantly increased in combined analysis (cohorts 1 and 2) of 273 AMA-positive PBC patients compared with 268 healthy controls (Table 3), but statistical significance was lost after correction for multiple testing ($pc = 0.110$) (Table 3).

Pairwise LD mapping confirmed that all alleles were in strong LD with an index of >0.8. A strong LD was detected in the same block for PBC patients and controls. We next evaluated haplotype association among AMA-positive PBC patients and healthy subjects in a combined analysis. To estimate haplotype frequencies and analyze haplotype association with PBC, we selected tag SNPs

Table 2. Allele frequencies of SNPs in the *CTLA4* gene in PBC patients and controls.

SNP No.	dbSNP	Allele major/minor	Position (bp)	Gene location	Cohort 1 (Shinshu)				Cohort 2 (Nagasaki)			
					Patients (n = 198)		Controls (n = 170)		Patients (n = 110)		Controls (n = 98)	
					MAF (%)	HWE p value	MAF (%)	HWE p value	MAF (%)	HWE p value	MAF (%)	HWE p value
1	rs733618	T/C	204439189	Promoter	44.4	0.030	39.1	0.071	39.5	0.570	43.4	0.366
2	rs5742909	C/T	204440592	Promoter	9.1	0.347	11.2	0.295	13.2	0.828	13.8	0.514
3	rs231775	G/A	204440959	Exon 1	35.4	0.784	41.5	0.089	39.5	0.334	41.8	0.827
4	rs3087243	G/A	204447164	3′ UTR	26.3	0.994	30.3	0.709	26.4	0.125	31.1	0.316
5	rs231725	A/G	204448920	3′ UTR	39.9	1.000	46.8	0.288	41.8	0.586	46.4	1.000

MAF, minor allele frequency; HWE, Hardy–Weinberg equilibrium; UTR, untranslated region.

Table 3. Allele frequencies of 5 SNPs in 273 AMA$^+$ patients with PBC and 268 healthy subjects.

SNP No.	Allele	Patients*	Controls*	p	pc	OR	95% CI
1	C	43.2	40.7	0.395	1.975	1.11	0.87–1.41
	T	56.8	59.3				
2	C	89.6	87.9	0.380	1.900	1.18	0.81–1.73
	T	10.4	12.1				
3	G	63.9	58.4	0.062	0.310	1.26	0.99–1.61
	A	36.1	41.6				
4	G	74.4	69.4	0.070	0.350	1.28	0.98–1.67
	A	25.6	30.6				
5	A	60.3	53.4	0.022	0.110	1.33	1.04–1.69
	G	39.7	56.6				

AMA, anti-mitochondrial antibodies; PBC, primary biliary cirrhosis; OR, odds ratio; pc, corrected p value; 95% CI, 95% confidence interval; *, frequency (%).
p value was calculated by a χ^2-test 2 × 2 contingency table (df = 1).

Table 4. *CTLA4* haplotypes in 273 AMA$^+$ patients with PBC and 268 healthy subjects.

Haplotype	SNP No.				Patients* (n = 546)	Controls* (n = 536)	p	OR	95% CI
	2	3	4	5					
1	C	G	G	A	59.7	51.9	0.0095	1.37	1.08–1.75
2	C	A	A	G	25.5	29.4	0.1464	0.82	0.62–1.07
3	T	A	G	G	10.3	11.8	0.4186	0.85	0.58–1.25
4	C	G	G	G	3.8	5.4	0.2153	0.70	0.39–1.23

PBC, primary biliary cirrhosis; OR, odds ratio; 95% CI, 95% confidence interval; *, proportion of indicated haplotype (%).
Values for n indicate two times the number of individuals since each person carries two haplotypes.
p value was calculated by a χ^2-test 2 × 2 contingency table (df = 1).

using the Tagger algorithm from the Haploview program. Four tag SNPs (SNPs 2–5: rs5742909, rs231775, rs3087243, and rs231725) were selected to capture most of the allelic diversity in the two cohorts. The four estimated haplotypes showed a frequency of >5% in 11 haplotypes created by expectation–maximization algorithms (Table 4). Haplotype 1 (CGGA) was significantly associated with AMA-positive PBC susceptibility (59.7% vs. 51.9%, OR 1.37, 95% CI 1.08–1.75, p = 0.0095). No other haplotypes were associated with either susceptibility or resistance to PBC.

Evaluation of the 5 *CTLA4* SNPs between AMA-positive and AMA-negative subgroups revealed neither significant allelic associations (Table 5) nor significant haplotype associations (Table 6), even when compared with early or late stages (Tables 5 and 6). Moreover, a comparison of 17 orthotopic liver transplantation (OLT) PBC cases and 291 non-OLT cases revealed no significant differences in allele frequencies (Table 5). In haplotype analysis, no statistical associations were found with OLT (Table 6).

Discussion

This study revealed that haplotype 1 (CGGA) was significantly associated with disease susceptibility in 273 AMA-positive PBC patients, as well as overall in all 308 PBC patients (p = 0.012) (data not shown). This finding is in agreement with the Caucasian study by Juran et al. [27], and thus constitutes a promising susceptibility gene candidate. However, since the precise function of *CTLA4* SNPs remains undefined, we cannot exclude the possibility that these SNPs may only be a linkage marker for a yet unidentified SNP within the *CTLA4* gene. Sequencing of the entire gene and assessing the functional role of these SNPs will be required.

SNP rs231775 associated with PBC is commonly referred to as 49AG in several studies [23,24,27,31,41]. Our finding corroborated a previous report [31], in which 49AG was not associated with susceptibility to PBC but there was a discrepancy in associ-

Table 5. Allele frequencies of *CTLA4* SNPs in AMA, histological or clinical disease progression, and OLT states.

SNP No.	Allele	AMA^{+}* (n = 273)	AMA^{-}* (n = 35)	p	Early* (n = 223)	Late* (n = 85)	p	Non-OLT* (n = 291)	OLT* (n = 17)	p
1	C	43.2	38.6	0.459	44.4	38.2	0.167	42.6	44.1	0.863
	T	56.8	61.4		55.6	61.8		57.4	55.9	
2	C	89.6	88.6	0.800	90.0	89.2	0.783	89.3	91.2	0.736
	T	10.4	11.4		10.0	10.8		10.7	8.8	
3	G	63.9	57.1	0.267	63.9	61.2	0.531	62.9	67.6	0.576
	A	36.1	42.9		36.1	38.8		37.1	32.4	
4	G	74.4	68.6	0.300	74.7	71.2	0.380	73.7	73.5	0.981
	A	25.6	31.4		25.3	28.8		26.3	26.5	
5	A	60.3	52.9	0.235	60.8	55.9	0.270	59.5	58.8	0.942
	G	39.7	47.1		39.2	44.1		40.5	41.2	

PBC, primary biliary cirrhosis; AMA, anti-mitochondrial antibodies; OLT, orthotopic liver transplantation; SNP, single nucleotide polymorphism; *, frequency (%).
p value was calculated by a χ^2-test 2 × 2 contingency table (df = 1).

Table 6. Comparison of *CTLA4* haplotype frequencies in AMA, histological or clinical disease progression, and OLT states.

Haplotype	SNPs No.				AMA^{+}* (n = 546)	AMA^{-}* (n = 70)	p	Early* (n = 446)	Late* (n = 170)	p	Non-OLT* (n = 582)	OLT* (n = 34)	p
	2	3	4	5									
1	C	G	G	A	60.1	52.8	0.245	60.5	55.9	0.292	59.3	58.8	0.959
2	C	A	A	G	25.5	30.0	0.415	25.1	28.2	0.430	26.1	23.5	0.738
3	T	A	G	G	10.3	10.0	0.947	10.3	10.0	0.909	10.3	8.8	0.781
4	C	G	G	G	3.5	4.3	0.720	3.1	4.7	0.346	3.4	5.9	0.458

PBC, primary biliary cirrhosis; AMA, anti-mitochondrial antibodies; OLT, orthotopic liver transplantation; SNP, single nucleotide polymorphism; *, proportion of indicated haplotype (%).
Values for n indicate two times the number of individuals since each person carries two haplotypes. p value was calculated by a χ^2-test 2 × 2 contingency table (df = 1).

ation with liver damage that might have arisen from the number of cases analyzed. 49AG also appears to affect cell surface expression of CTLA4 by CTLA4-driven down-regulation in response to T-cell activation [42]. This coding polymorphism is located in a signal peptide that is cleaved from the functional protein, and has been shown to affect glycosylation of the autoimmune susceptibility G allele, resulting in diminished processing efficiency and thus decreased trafficking to the cell surface [43]. It will be necessary to confirm the functional difference between patients with these SNPs and T-cell activation in a future study.

The rs3087243 SNP, also referred to as CT60, is located in the 3′ UTR of the *CTLA4* gene and reported to influence the production of the soluble isoform of CTLA4 (sCTLA4). The sCTLA4 mRNA encoded by the +CT60G-allele is produced at a reduced rate compared with that encoded by the A allele. As sCTLA4, which is secreted by resting T cells, is a suppressor of T-cell activation, it is conceivable that carriers of the +CT60G-allele allele may be more susceptible to autoimmune diseases [44]. Although studies from Canada and Italy found an association between PBC and the CT60 SNP [29,41], other studies have since failed to confirm this association [27,28], including ours.

In haplotype analysis, haplotype 1 contained all of the known SNP risk alleles that have been functionally determined in other disease studies. These include the C allele at −318, which has been found to affect the expression of CTLA4 mRNA cell surface expression [45], the minor G allele at 49AG, reported to reduce cell surface expression of CTLA4 [42], and the G allele of CT60, which affects the expression of the soluble form of the CTLA4 molecule, indicating the possibility that this haplotype might contribute to PBC susceptibility in the Japanese population.

Lastly, Juran et al. have suggested that CTLA4 plays a role in influencing AMA development as well as progression to OLT in PBC based on their haplotype analyses [27]. Our data revealed no statistical significance in regards to AMA development or disease progression to cirrhosis or OLT, possibly due to the number of patients showing AMA negativity and proceeding to OLT being too small to evaluate. Another consideration is that disease progression in Japanese patients might have a stronger association with positivity for anti-gp210 antibodies as a risk factor of progression to hepatic failure than *CTLA4* polymorphisms [46]. Further longitudinal follow-up studies in larger cohorts are required to resolve this critical question.

In conclusion, we found that *CTLA4* gene polymorphisms had a modest, but significant, association with susceptibility to PBC in the Japanese population and may share a common susceptibility haplotype with Caucasians. The connection between genetic variants and the function of the *CTLA4* gene remains to be addressed in future investigations.

Conflict of Interest

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A2BP1 as a novel susceptible gene for primary biliary cirrhosis in Japanese patients

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ABSTRACT

Primary biliary cirrhosis (PBC) is a complex autoimmune liver disease with an etiology that remains to be conclusively elucidated. As such, we screened the human genome for genes that might influence PBC susceptibility or resistance using 400 microsatellite markers. A strong candidate gene indicated by susceptibility microsatellite markers was further evaluated by association analysis using single nucleotide polymorphisms (SNPs). A total of 126 patients with PBC and 95 healthy Japanese controls were enrolled. Four candidate susceptible regions and seven candidate protective regions were statistically associated with PBC. Because the D16S423 marker on chromosome 16p showed the strongest evidence of linkage, the protein-coding gene *ataxin 2-binding protein 1* (*A2BP1*) lying 27 kb on the centromeric side of D16S423 was targeted as a candidate susceptible gene. Seven SNPs (rs17139207, rs12926282, rs17139244, rs6500742, rs4146812, rs4124065, and rs889699) in the *A2BP1* gene were genotyped in patients and controls. The rs17139244 SNP was found to be weakly associated with PBC in an additive model. The genotype frequency of the major C allele at rs6500742 was significantly associated with PBC, compared with healthy controls. This study showed a total of 11 candidate PBC susceptibility or resistance regions. In particular, the *A2BP1* gene might play a pivotal role for susceptibility to PBC.

1. Introduction

Primary biliary cirrhosis (PBC) is a chronic, slowly progressive, cholestatic autoimmune liver disease of unclear etiology. It is characterized by a T-lymphocyte–mediated attack on the small intralobular bile ducts, which commonly shows nonsuppurative intrahepatic portal tract inflammation, bile duct destruction, and eventual bile duct disappearance in histopathology. The sustained loss of intralobular bile ducts causes symptoms of cholestasis, and eventually results in cirrhosis and hepatic failure [1,2].

The exact etiology and pathogenesis of PBC is unclear, although susceptibility to this disease likely depends on a complex interaction of immunologic, genetic, and environmental factors [2,3]. Several earlier studies have reported a genetic predisposition to the pathogenesis of PBC; the incidence of PBC is higher in families and concordant twins of patients than in the general population [3–6]; in monozygotic twins, the rate of concordance is 63% and the age of disease onset is virtually identical, but natural history and disease severity are different [5].

Among the genes reported for their genetic contributions to PBC, human leukocyte antigen (HLA) has consistently been associated with disease susceptibility [7–13]. The signal transducer and activator of transcription 4 (*STAT4*) gene [7], cytotoxic T-lymphocyte antigen 4 (*CTLA4*) gene [7–10], multidrug resistance protein 3 (*MDR3*) gene [11], vitamin D receptor gene [14], and tumor necrosis factor–α (*TNF-α*) [10] gene have also been implicated with the onset or progression of PBC. However, no studies exist that comprehensively investigate the relation of genetic factors with PBC at the genome-wide level in the Japanese population.

In this study, we screened for candidate genes influencing PBC in Japanese patients using a genome-wide DNA microsatellite association study and analyzed single nucleotide polymorphisms (SNPs) of a strong candidate gene related to PBC susceptibility to shed light on the genetic etiology of PBC.

2. Subjects and methods

2.1. Subjects

Between December 1985 and April 2008, 126 patients clinically diagnosed with PBC (15 men and 111 women, median age 57 years) and 95 healthy Japanese controls (35 men and 60 women, median

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Table 1
Demographic and clinical characteristics of 126 patients with PBC

Characteristics	
Age, years[a]	57 (30–83)
Female/male	111/15
Biopsy at diagnosis, *n* (%)	65 (51.6)
Stage 1/2/3/4	49/8/4/4
Liver transplant, *n* (%)	4 (3.2)
AMA-positive, *n* (%)	106 (84.1)
ANA-positive, *n* (%)	100 (79.4)
Median values (normal values)[a]	
AST (12–37 IU/l)	35 (12–687)
ALT (7–45 IU/l)	32 (8–885)
ALP (124–367 mg/dl)	403 (131–2306)
γ-GTP (8–50 mg/dl)	104 (10–1029)
Total bilirubin (0.3–1.2 mg/dl)	0.73 (0.30–6.04)
IgM (35–220 mg dl)	232 (56–1390)
IgG (870–1700 mg dl)	1530 (764–3410)

Abbreviations: PBC, primary biliary cirrhosis; AMA, antimitochondrial antibody specific for the pyruvate dehydrogenase complex-E_2 component; ANA, anti-nuclear antibody; AST, aspartate amino transferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γ-GTP, γ-glutamyltransferase.
[a]Median (range).

age 51 years) were enrolled in this study. All subjects who visited the outpatient clinic at Shinshu University Hospital or affiliated hospitals were residents of Nagano Prefecture, Japan, and their ethnic background was Japanese. All control subjects had indicated the absence of major illness on a standard questionnaire, and were volunteers from hospital staff whose ethnic backgrounds were also all Japanese.

The diagnosis of PBC could be established when two of the following three criteria were met: biochemical evidence of cholestasis with elevation of alkaline phosphatase activity; presence of antimitochondrial antibody (AMA) specific for the pyruvate dehydrogenase complex-E_2 component antigen; and histopathologic evidence of nonsuppurative cholangitis and destruction of small or medium-sized bile ducts if a biopsy was performed [15]. Serum AMA was measured by enzyme-linked immunosorbent assay (ELISA). An index value of greater than 7 was considered a positive result. Liver samples were obtained from 65 patients (51.6%) and assessed using the Scheuer's classification [16,17] by investigators blinded to the results of other experiments. The numbers of histologically diagnosed PBC patients as Scheuer stage I, II, III, and IV were 49, 8, 4, and 4, respectively. These 65 patients were classified as having either early stage (Scheuer stage I or II) or late stage (Scheuer stage III or IV) PBC. Of all patients, 4 cases underwent orthotopic liver transplantation (OLT). We excluded patients with overlap syndrome from our cohort [18]. Clinical characteristics of the patients in this study are shown in Table 1.

Informed consent was obtained from all study participants. Our registry and present study conform to the ethical guidelines of the 1975 Declaration of Helsinki and have been approved by the Ethics Committee of Shinshu University School of Medicine.

2.2. Preparation of genomic DNA

Genomic DNA from patients and controls was isolated by phenolic extraction of sodium dodecyl sulfate–lysed and proteinase K-treated cells, as described previously [19–23].

2.3. Microsatellite typing

A total genome scan was carried out using 400 microsatellite markers (ABI Linkage Mapping Set v 2.5 – MD10; Applied Biosystems, Foster City, CA) with an average heterozygosity of 79% and an average intermarker distance of 9.4 ± 2.9 cM (mean ± SD). The entire marker set consisted of 28 panels, each containing markers pooled according to size and fluorescent tag (6-FAM, VIC, NED). The markers were amplified by polymerase chain reaction (PCR) in 10-μl reactions containing 40 ng of genomic DNA, according to the manufacturer's protocol. After PCR, pooled panels were analyzed using an ABI 3130 DNA Analyzer. Semi-automated genotyping was performed using GeneMapper v 3.5 software (Applied Biosystems).

2.4. SNP genotyping

One locus located on chromosome 16p13.3 (microsatellite marker D16S423) was identified as particularly associated with PBC. A protein-coding gene, *ataxin 2-binding protein 1* (*A2BP1*), was located 27 kb on the centromeric side of the nearest D16S423 microsatellite marker and therefore further investigated as a candidate susceptible gene for PBC.

Seven SNPs (rs17139207, rs12926282, rs17139244, rs6500742, rs4146812, rs4124065, and rs889699) distributed within the *A2BP1* gene were selected from the National Center for Biotechnology Information dbSNP database (build 36) and the SNP database from Applied Biosystems. All seven polymorphisms were typed by TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA) using the Applied Biosystems 7500 Real-time PCR system, following the manufacturer's instructions.

2.5. Statistical analysis

The phenotypic frequency at polymorphic sites among the 400 microsatellite markers was estimated by direct counting. The significance of differences in the distribution of alleles between patients with PBC and healthy control subjects was determined by the χ^2 test. Fisher's exact probability test was used for comparisons with fewer than five samples. A p value of less than 0.05 was considered statistically significant. p Values were corrected by multiplying by the number of different alleles observed in each locus (p_c). The strength of association with PBC was estimated by calculating the odds ratios (OR) and 95% confidence intervals (CI). The Hardy–Weinberg proportion (HWP) for multiple alleles was calculated using the Markov chain method within the GENEPOP software package (http://genepop.curtin.edu.au/) [24,25].

3. Results

To identify the genetic intervals that might contain susceptible or protective loci for PBC, 126 Japanese patients with PBC and 95 healthy Japanese controls were enrolled for an association anal-

Table 2
Statistically significant alleles associated with susceptibility to PBC

Chromosome	Marker	Significant allele	PBC % (n = 126)	Control % (n = 95)	OR	χ^2	p	PC
1p31	D1S207	156	16.7	4.2	4.55	8.38	0.00380	0.04181
12q24.2	D12S86	145	85.7	68.4	2.77	9.53	0.00203	0.00283
16p13.3	D16S423	135	59.5	33.7	2.90	14.48	0.00014	0.00170
21q21	D21S1256	102	78.6	56.8	2.78	12.01	0.00053	0.00637
16p13.3	D16S0602i	210	36.8	22.9	1.96	4.91	0.02665	NC

Abbreviations: OR, odds ratio; *Pc*, corrected P:NC, not calculated.

Table 3
Statistically significant alleles associated with protection against PBC

Chromosome	Marker	Significant allele	PBC % (n = 126)	Control % (n = 95)	OR	χ^2	p	p_c
1p13	D1S2726	292	13.5	28.4	0.39	7.57	0.00593	0.04747
3p14.2	D3S1300	232	19.1	39.0	0.37	10.73	0.00105	0.01263
5p13.3	D5S426	285	36.5	57.9	0.42	9.98	0.00158	0.01738
10p15	D10S249	119	15.9	36.8	0.32	12.74	0.00036	0.00394
14q12	D14S275	149	4.8	15.8	0.27	7.66	0.00565	0.03953
20p12	D20S186	137	3.2	15.8	0.17	10.97	0.00093	0.01205
Xp22.3	DXS1060	246	15.1	19.0	0.30	10.43	0.00124	0.01236

Abbreviations: OR, odds ratio; p_c, corrected p value.

ysis using 400 microsatellite markers dispersed throughout the human genome. As shown in Table 2, the alleles in four microsatellites (*D1S207*, *D12S86*, *D16S423*, and *D21S1256*) were positively associated with PBC susceptibility. Conversely, the alleles in seven microsatellite markers were negatively associated with disease (Table 3).

3.1. Candidate genes associated with D16S423 on chromosome 16p13.3

Among the susceptible and protective markers, allele 135 of the D16S423 marker on chromosome 16p13.3 showed the strongest association with PBC (59.5% vs 33.7%, OR =2.90, $p_c = 0.0017$) (Table 2). We therefore chose D16S423 as a marker of interest for further analysis. To validate its association with PBC, we investigated the association with PBC of an additional marker, D16S0602i, which is closely situated to D16S423. D16S0602i was extracted from the Gene Diversity DataBase System, Japan Biological Informatics Consortium (http://jbirc.jbic.or.jp/gdbs/top.jsp). The allele 210 of D16S0602i also showed a significant association with PBC (36.8% vs 22.9%, OR =1.96, $p = 0.026\,65$) (Table 2). We next used the National Center for Biotechnology Information Map Viewer from the National Institutes of Health (http://www.ncbi.nlm.nih.gov/mapview/) to predict novel susceptibility genes associated with both significant markers. This revealed the *A2BP1* gene, located within a 100-kb span of the two markers, to be a promising candidate gene for susceptibility to PBC.

3.2. A2BP1 SNP analysis in patients with PBC and healthy subjects

In total, seven SNPs in the *A2BP1* gene were genotyped in 126 PBC patients and 95 healthy controls (Table 4). We observed that the genotype distribution of all SNPs exhibited Hardy–Weinberg equilibrium, and that the minor allele frequencies of all SNPs were greater than 5% in patients and controls (Table 4). The major A allele at rs17139244 was significantly increased in an additive model (A/A+A/G vs G/G) (89.7% vs 78.9%, OR 2.318, 95% CI 1.100–4.883, $p = 0.027$) in patients compared with controls (Table 5). However, the significance of this allele disappeared after correction for multiple testing. In contrast, the genotype frequency of the major C allele at rs6500742 was significantly increased (62.3% vs 51.6%, OR 1.55, 95% CI 1.05–2.27, $p = 0.024$) in patients compared with controls as well as in an additive model (C/C+C/T vs T/T) (86.5% vs 71.6%, OR 2.546, 95% CI 1.306–4.961, $p = 0.006$, p_c=0.042) (Table 5).

Evaluation of the seven SNPs between early and late stage PBC subgroups revealed no significant allelic associations. Moreover, a comparison of four OLT PBC cases and 122 non-OLT cases revealed no significant differences in allele frequencies (data not shown).

Table 4
Allele frequencies of 6 SNPs on the A2BP1 gene in PBC patients and controls

DbSNP	Alleles	Position	Patients (n = 126)		Controls (n = 95)	
	Major/minor	(Bp)	MAF (%)	HWE p value	MAF (%)	HWE p value
rs17139207	A/G	6,026,273	9.9	0.200	7.4	1.000
rs12926282	C/A	6,034,558	23.4	0.718	18.4	0.686
rs17139244	A/G	6,045,214	29.8	0.532	40.0	0.058
rs6500742	C/T	6,049,222	37.7	0.916	48.4	0.073
rs4146812	G/A	6,055,827	27.0	0.806	20.0	1.000
rs4124065	T/G	6,068,401	25.4	1.000	25.3	1.000
rs889699	A/G	6,074,528	34.9	0.494	35.8	0.489

Abbreviations: MAF, minor allele frequency; HWE, Hardy–Weinberg equilibrium.

4. Discussion

The present study is a case-control association analysis to identify candidate regions of PBC pathogenesis susceptibility in Japanese patients using a previously reported [22] method of genome-wide microsatellite analysis. Our results showed that at least four susceptible and 7 protective markers were significantly associated with PBC patients. The four susceptible regions for PBC differed from those for autoimmune hepatitis in an earlier report (D11S902, D18S464) [22], suggesting a separate genetic etiology between the two. A recent report by Hirschfield et al. [7] showed that HLA class II, IL12A, and IL12RB2 loci had a strong association with PBC susceptibility, and that STAT4 and CTLA4 loci had a modest association with PBC in a genome-wide association analysis using 373,400 SNPs. A possible reason for the differing findings found here may be that the dense SNP marker sets offered more precise information than the lower resolution microsatellite scan [26]. Here, a total of 400 microsatellite markers were used throughout the whole human genome. With an intermarker distance of 9.4 cM, the number of microsatellite markers in this study was not sufficient to supersede information provided by 373,400 SNPs, considering that approximately 300,000 microsatellite markers are needed for whole genome association studies of complex diseases if charting at 100-kb intervals [27]. Thus, many regions associated with the pathogenesis of PBC might have remained undetected in our study. Nonetheless, four statistically significant susceptible regions and seven protective regions were identified, which imply that new or previously unidentified genes responsible for the pathogenesis of PBC exist within the proximity of the corresponding microsatellite markers because of the average length of linkage disequilibrium between the markers [27–29]. Several candidate genes were considered within a 100-kb perimeter of each marker for further analysis. Two candidate PBC susceptibility protein coding genes (*latrophilin 2* and *A2BP1*) and eight protective genes (*KCNA2, KCNA3, FHIT, similar to hCG1745223, RAI14, TTC23L, ZMYND11*, and *DIP2C*) were located within this 100-kb range. However, no previously reported linked genes, including *STAT4* [7], *MDR3* [11], *CTLA4* [8], *vitamin D receptor* [14], and *TNF-α* [10], were found to be related to PBC susceptibility.

Based on our findings, we sought to determine whether the *ataxin-2 binding protein 1* (*A2BP1*) gene had a significant association with susceptibility to PBC by SNP analysis. The *A2BP1* gene was 27 kb on the centromeric side of the D16S423 microsatellite marker, which had shown the strongest significance to PBC. This gene has been described as a protein-coding gene of 1,691,715 bps in size

Table 5
A2BP1 polymorphisms in patients with PBC and healthy subjects

DbSNP	Alleles	PBC (n = 126)			Controls (n = 95)			p^a	Additive model	
	(1/2)	1/1	1/2	2/2	1/1	1/2	2/2		p	OR (95% CI)
rs17139207	A/G	104	19	3	81	14	0	0.644	NC	NC
rs12926282	C/A	75	43	8	62	31	2	0.466	0.240	0.317 (0.071–1.423)
rs17139244	A/G	64	49	13	39	36	20	0.071	0.027	2.318 (1.100–4.883)
rs6500742	C/T	48	61	17	30	38	27	0.024	0.006	2.546 (1.306–4.961)
rs4146812	G/A	66	52	8	61	30	4	0.303	0.069	0.648 (0.190–2.208)
rs4124065	T/G	70	8	48	53	6	36	0.961	0.992	0.994 (0.332–2.976)
rs889699	A/G	51	62	13	37	48	10	0.974	0.960	1.023 (0.427–2.448)

Abbreviations: A2BP1, ataxin 2-binding protein 1; PBC, primary biliary cirrhosis; OR, odds ratio; 95% CI, 95% confidence interval; NC, not calculated.
[a] p Value in genotype frequency caluculated by χ^2 test of a 2×3 contingency table (df =2). p Value in additive model, which was compared 1/1+ 1/2 vs 2/2, was calculated by χ^2 test of a 2×2 contingency table (df = 1). If there were fewer than five data samples, Fisher's exact probability test was used.

that consists of 16 exons and is located on chromosome 16p13.3. The *A2BP1* gene encodes a ribonucleoprotein motif that is highly conserved among RNA-binding proteins [30,31]. This protein binds to the C-terminus of ataxin-2, which in turn signals encoding of many transcripts by alternative splicing [32]. A2BP1 is predominantly expressed in muscle and the brain [30]. *A2BP1* gene polymorphisms have been reported to be associated with autism in a subset of patients [33], as well as with smoking cessation [34], early-stage non–small-cell lung cancer [35], and osteoarthritis of the hand [36]. However, there have been no reports of any association between the *A2BP1* gene and PBC to date. The *A2BP1* gene was also reported to be a novel transcriptional regulator that mediates the neuron-specific splicing pattern of calcitonin-calcitonin gene-related peptide (CGRP) pre-mRNA [37]. CGRP is a neuropeptide produced in the neural body of dorsal root ganglion cells that is released from sensory nerve endings. One of the isoforms of CGRP, αCGRP, is produced mainly in the nervous system by the tissue-specific alternative splicing of the primary RNA transcript of the calcitonin-CGRP gene [38]. αCGRP was also reported to suppress the production of TNF-α and IL-12 in bone marrow–derived dendritic cells stimulated with lipopolysaccharide in *an vivo* study [39]. Therefore, we cannot exclude the possible involvement of *A2BP1* SNPs in the pathogenesis of PBC, as previous reports have implicated TNF-α and IL-12 SNPs with PBC as well [7,10].

Overall, our work with genome-wide microsatellite analysis yielded a combination of 10 candidate genes potentially associated with PBC by analysis of linkage disequilibrium (LD). However, our study was preliminary in nature because the numbers of cases and controls were limited, as well as the number of microsatellite markers. To overcome type I error, further studies are needed to analyze the relationship between gene polymorphisms and the expression and functions of these gene products in a second cohort or larger test group.

In conclusion, we identified four candidate PBC susceptibility and seven candidate PBC resistance regions by genome-wide microsatellite analysis, which include two candidate susceptible genes and eight resistant genes to PBC by statistical LD analysis. Among these, *A2BP1* gene polymorphisms might be associated with the onset of PBC. As the direct functional effects of the *A2BP1* gene are still uncertain, further investigation is needed to clarify the interaction of this gene with PBC pathogenesis.

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INTERNAL MEDICINE

□ CASE REPORT □

A Patient with Advanced Hepatocellular Carcinoma Treated with Sorafenib Tosylate Showed Massive Tumor Lysis with Avoidance of Tumor Lysis syndrome

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Abstract

A 33-year-old man presented with pain and palsy of the leg in 2008 for treatment of hepatocellular carcinoma with huge distant metastases. The patient's tumors had slowly enlarged despite several treatments. Oral administration of sorafenib at 800 mg/day with careful observation was commenced in 2009. Laboratory investigations on day 7 showed massive tumor lysis. An abdominal CT showed multiple low density areas and tumor markers decreased, indicating extended tumor necrosis. In conclusion, clinicians should bear in mind not only the published adverse effects, but also massive tumor lysis, when treating patients with large tumor burden by sorafenib.

Key words: hepatocellular carcinoma, sorafenib, tumor lysis, tumor lysis syndrome

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Introduction

Sorafenib tosylate was approved by the Food and Drug Administration (FDA) in 2007 to treat hepatocellular carcinoma (HCC) and could be prescribed in Japan from 2009 to treat unresectable HCC. It is an oral multitargeted kinase inhibitor that inhibits tumor cell proliferation and angiogenesis (1, 2). Sorafenib is believed to mark the beginning of a new era in advanced HCC therapy since it is the first approved molecular targeted agent for HCC and can be prescribed in outpatient clinics. However, the most frequently reported adverse events of sorafenib have been observed in 80% of patients (1). We report a case of HCC who avoided tumor lysis syndrome (TLS) following massive tumor lysis from treatment with sorafenib.

Case Report

A 33-year-old man presented with pain and palsy of the left leg at our hospital in 2008 for further examination and treatment of huge masses in the pelvic cavity (70 mm in diameter) and liver (110 mm in diameter) that had been earlier detected with ultrasonography, computed tomography (CT) and magnetic resonance imagining (MRI). A specimen taken from a direct tumor biopsy from the sacral bone histologically showed well-differentiated hepatocellular carcinoma (HCC) with monotonous tumor cell proliferation (Fig. 1a) and immunohistologically stained for hepatocyte paraffin 1 (Fig. 1b). Laboratory tests were normal except for levels of serum HCC tumor makers, such as alpha-fetoprotein (AFP) at 2,580 ng/ml, AFP like the Lens culinaris agglutinin-reactive fraction (AFP-L3) at less than 0.5% of total AFP, and prothrombin induced by vitamin K absence or antago-

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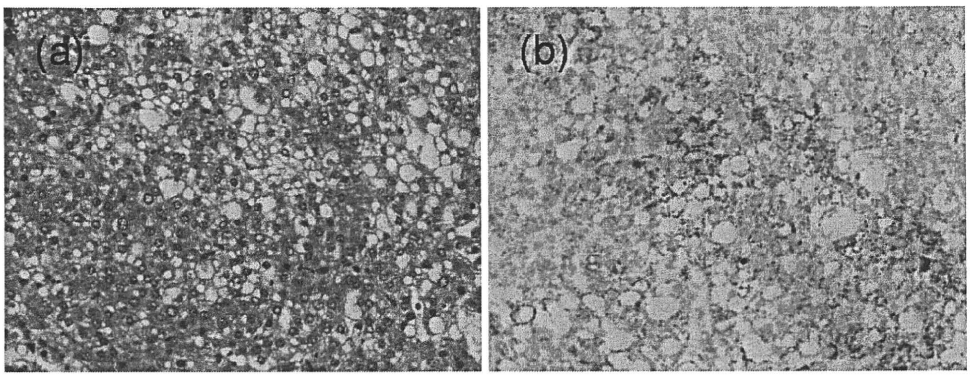

Figure 1. a) Microscopic examination revealed that the tumor was a well-differentiated hepatocellular carcinoma with monotonous tumor cell proliferation. Hematoxylin and Eosin staining ×100. b) Cells were immunohistologically positive to antibodies against hepatocyte paraffin 1 (Hep Par 1) ×100.

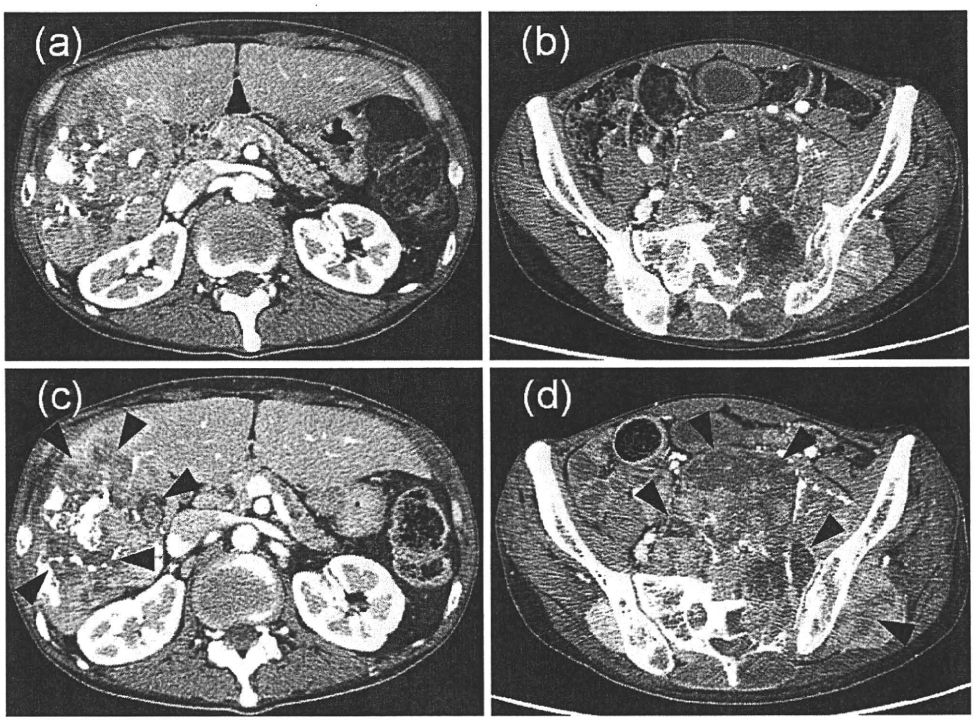

Figure 2. a) Contrast-enhanced computed tomography (CT) right before treatment with sorafenib depicted a maximum 78×63 mm-wide tumor on the right robe in the liver with several small intrahepatic metastases. b) It also showed a maximum 102×110 mm-wide tumor in the pelvic cavity directly infiltrating out of the sacral bone. c) Contrast-enhanced CT on day 14 depicted multiple low density areas in the liver (black arrowheads). d) It also showed multiple low density areas in the pelvic cavity (black arrowheads), indicating extended tumor necrosis by sorafenib.

nist II (PIVKA II) at 781 mAU/mL. The patient's tumors had slowly become enlarged over 14 months despite several treatments with transarterial chemoembolization and transarterial infusion chemotherapy for both hepatic and pelvic masses. He also suffered from complications of severe cancer pain that were managed by local radiation therapies and several analgesic agents, including morphine.

Oral administration of sorafenib at 800 mg/day with careful observation was commenced in 2009 at an outpatient clinic visit after sorafenib became approved for unresectable HCC in Japan. An abdominal CT right before treatment showed a maximum 78×63 mm-wide tumor (Fig. 2a) with several small intrahepatic metastases and a huge maximum 102×110 mm-wide tumor directly infiltrating out of the sacral bone (Fig. 2b). Pretreatment laboratory tests were as follows: aspartate aminotransferase (AST), 102 IU/L; alanine aminotransferase (ALT), 28 IU/L; lactate dehydrogenase (LDH), 377 IU/L; uric acid (UA), 5.8 mg/dL; phosphorus (P), 3.9 mg/dL; creatinine (Cre), 0.39 mg/dL; potassium (K), 4.3 mmol/L; calcium (Ca), 10.0 mg/dL; AFP, 91,260

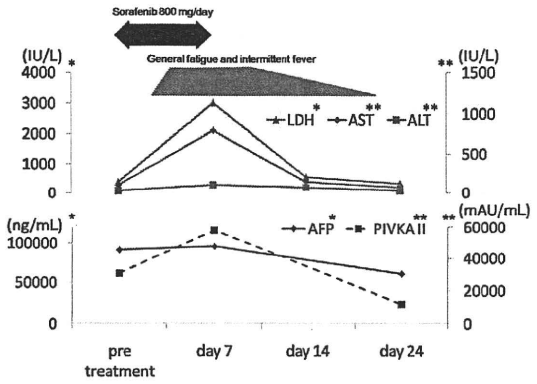

Figure 3. Clinical course from the beginning of treatment with sorafenib. On day 4 of taking sorafenib, the patient started complaining of general fatigue and showed intermittent fever. Sorafenib treatment was discontinued because levels of lactate dehydrogenase (LDH, red line) and aspartate aminotransferase (AST, blue line) were elevated on day 7, accompanied by a discrepancy in the level of alanine aminotransferase (ALT, green line), which was indicative of massive tumor lysis. On day 24, levels of both alpha-fetoprotein (AFP, black line) and prothrombin induced by vitamin K absence or antagonist II (PIVKA II, broken black line) were decreased, indicating extended tumor necrosis by sorafenib. The patient's clinical symptoms gradually improved and the elevated levels of AST and LDH returned to pretreatment levels.

ng/mL (AFP-L3 less than 0.5%); and PIVKA II, 30,533 mAU/mL. On day 4 after taking sorafenib, the patient started complaining of general fatigue and showed intermittent fever. Laboratory investigations on day 7 were extremely exacerbated and were as follows: AST, 788 IU/L; ALT, 105 IU/L; LDH, 3,016 IU/L; UA, 4.4 mg/dL; P, 3.9 mg/dL; Cre, 0.38 mg/dL; K, 4.9 mmol/L; and Ca, 8.9 mg/dL. Sorafenib treatment was discontinued due to concerns of tumor lysis syndrome (TLS) development despite laboratory findings not entirely meeting the diagnostic criteria of TLS (3, 4). An abdominal CT on day 14 showed no interval changes in the size of the tumors, but multiple low density areas were evident (Fig. 2c in the liver; Fig. 2d in the pelvic cavity. Black arrowheads indicate multiple low density areas.). Furthermore, on day 24, levels of AFP and PIVKA II were decreased to 60,810 ng/mL (AFP-L3 less than 0.5%) and 11,701 mAU/mL, respectively, indicating extended tumor necrosis by sorafenib. The patient's clinical symptoms gradually improved and the elevated levels of AST and LDH returned to pretreatment levels after a 17-day interruption of sorafenib treatment (Fig. 3). He was then started on oral administration of sorafenib at a reduced dose of 400 mg/day on day 30 and showed no tumor lysis in blood examination, and has since received extended does of 400 mg/800 mg sorafenib on alternate days from day 38 and has been alive to date (day 171) despite showing tumor progression.

Discussion

Sorafenib increases the rate of tumor apoptosis by inhibition of the serine-threonine kinases Raf-1 and B-Raf and the receptor tyrosine kinase activity of vascular endothelial growth factor receptors (VEGFRs)-1 (flt-1), -2 (KDR/flk-1), and -3 (flt-4) and platelet-derived growth factor receptor (PDGFR)-β (2). Cellular signaling mediated by Raf-1 and vascular endothelial growth factors (VEGFs) has been implicated in the molecular pathogenesis of HCC (5). One isoform of VEGF, VEGF-A, was reported to be expressed and related to angiogenesis along with VEGFR-1 and -2 in relatively well-differentiated HCC (6). Another VEGF isoform, VEGF-C, was found to be related to HCC disease progression together with VEGFR-2 and -3 (7). Clinically, the multicenter European randomized SHARP trial demonstrated a statistically significant survival benefit and disease-control rate according to the Response Evaluation Criteria in Solid Tumors (RECIST) for sorafenib in patients with advanced HCC in 2008 (1). In the present case of histologically well-differentiated HCC with distant metastases, the antitumor effect of sorafenib was clearly observed and resulted in massive tumor lysis.

The most frequently reported adverse events of sorafenib observed in 80% of patients include dermatologic events, such as hand-foot skin reactions, constitutional symptoms, such as weight loss, and gastrointestinal events like diarrhea (1). The present case showed none of these side effects although he only received sorafenib treatment for 7 days. TLS is an oncologic emergency that is caused by the release of intracellular tumor components due to massive tumor lysis, especially in cases that show a large tumor burden, a high proliferative tumor rate, or high sensitivity to cytotoxic therapy (3, 4). In general, TLS is less likely to occur in patients with solid tumors than in those with hematological malignancies (8) because the former tend to be more resistant to cytotoxic therapy. However, with the advent of multi-targeted kinase inhibitors that show effectiveness against solid tumors, an increasing number of reports on TLS in these cases are surfacing (9-11). Recently, a 55-year-old man with HCC who had shown a good initial response to sorafenib later experienced TLS and died after 30 days of continuous treatment (12). Careful observation, especially in the initial period after sorafenib commencement, and earlier discontinuation of the drug may have prevented TLS development. Administration with sorafenib was halted at 7 days in the present case but it still yielded positive results without the onset TLS, indicating that further studies are required to determine the optimal dosage and treatment course with sorafenib in unresectable HCC patients such as the current case.

In conclusion, clinicians should bear in mind not only the published major adverse effects (1), but also the possibility of TLS brought on by massive tumor lysis, when starting treatment of advanced HCC patients with large tumor bur-

den using oral administration of sorafenib.

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Authors' disclosures of potential conflicts of interest

The authors indicated no potential conflicts of interest.

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Review Article

Guidelines for the treatment of chronic hepatitis and cirrhosis due to hepatitis B virus infection for the fiscal year 2008 in Japan

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In the 2008 guidelines for the treatment of patients with cirrhosis, who are infected with hepatitis B virus (HBV), the main goal is to normalize levels of alanine and aspartate aminotransferases by eliminating HBV or reducing viral loads. In patients with compensated cirrhosis, the clearance of HBV from serum is aimed for by entecavir, as the main resort, for histological improvement toward the prevention of hepatocellular carcinoma (HCC). In patients with decompensated cirrhosis, by contrast, meticulous therapeutic strategies are adopted for the reversal to compensation, toward the eventual goal of decreasing the risk of HCC. For maintaining liver function and preventing HCC, branched chain amino acids and nutrient supplements are applied, in addition to conventional liver supportive therapies. For patients with chronic hepatitis B, separate guidelines are applied to those younger than 35 years and those aged 35 years or older. Even for patients with chronic hepatitis who are negative for hepatitis e antigen (HBeAg), but who harbor HBV DNA in titers of 7 log copies/mL or more, a "drug-free state" is aimed for by sequential treatment with interferon (IFN) plus entecavir as the first line. For patients with chronic hepatitis B aged 35 years or older, who are HBeAg-negative and carry HBV DNA in titers of less than 7 log copies/mL, long-term IFN for 24–48 weeks is adopted anew. To HBeAg-negative patients who have either or both platelet counts of less than $150 \times 10^3/mm^3$ and less than 7 log copies of HBV DNA, also, long-term IFN for 24–48 weeks is indicated.

Key words: chronic hepatitis, cirrhosis, hepatitis B virus, hepatocellular carcinoma, interferon, liver supportive therapies, nucleos(t)ide analogs

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INTRODUCTION

SINCE THE FISCAL year 2002, guidelines for the treatment of patients with viral hepatitis have been compiled annually by the Study Group for the Standardization of Treatment of Viral Hepatitis Including Cirrhosis, under the auspice of the Ministry of Health, Labor and Welfare of Japan, supported by enduring efforts of many specialists recruited from all over the nation. Guidelines have been improved every year with many supplementary issues, which had surfaced as our understanding of many facets of viral hepatitis deepened and treatment options widened increasingly with time. For the fiscal year 2008, guidelines have been worked out for a comprehensive standardization of the treatment of chronic hepatitis and cirrhosis due to hepatitis B virus (HBV) and hepatitis C virus (HCV) infections in Japan. These guidelines have been observed by more than 70% of practicing hepatologists treating patients with viral liver disease in Japan. It is hoped that these guidelines will continue being widely accepted and implemented to help as many patients as possible who are suffering from sequelae of persistent hepatitis virus infections.

Here, we relate excerpts of the 2008 guidelines for the treatment of patients with liver disease due to HBV, covering a wide range from those with chronic hepatitis to those with decompensated cirrhosis. The 2008 guidelines for the treatment of liver disease due to HCV are reported in an accompanying paper.

GUIDELINES FOR THE TREATMENT OF PATIENTS WITH CHRONIC HEPATITIS B

PATIENTS WITH CHRONIC hepatitis B can stabilize the activity of liver disease in their natural course, after they have seroconverted from hepatitis B e antigen (HBeAg) to the corresponding antibody (anti-HBe), accompanied by decrease in HBV DNA titers. For that reason, treatment guidelines were constructed separately for the patients younger than 35 years and those aged 35 years or older.

GUIDELINES FOR THE TREATMENT OF PATIENTS WITH CHRONIC HEPATITIS B YOUNGER THAN 35 YEARS

PATIENTS WITH CHRONIC hepatitis B younger than 35 years are treated in accordance with the guidelines summarized in Table 1. Criteria for the treatment eligibility are: (i) serum levels of alanine aminotransferase (ALT) of 31 IU/L or more; and (ii) HBV DNA titers of 5 log copies of more in HBeAg-positive patients and 4 log copies or more in HBeAg-negative patients. In the 2008 guidelines, the indication of treatment is extended to the patients with cirrhosis due to HBV who carry HBV DNA in titers of 3 log copies/mL or more.

In Japan, most HBeAg-positive patients with 7 log copies or more of HBV DNA have been infected with HBV of genotype C by perinatal infection at birth;

Table 1 Guidelines for the treatment of patients with chronic hepatitis B younger than 35 years

Eligibility criteria	ALT	≥31 IU/L
	HBV DNA	HBeAg-positive patients: ≥5 log copies/mL HBeAg-negative patients: ≥4 log copies/mL Patients with cirrhosis: ≥3 log copies/mL

HBV DNA	≥7 log copies/mL	<7 log copies/mL
HBeAg-positive	(1) Long-term IFN for 24–48 weeks (2) Entecavir	(1) Long-term IFN for 24–48 weeks (2) Entecavir
HBeAg-negative	(1) Sequential treatment† (entecavir plus IFN) (2) Entecavir	(1) Regular follow up (2) Long-term IFN for 24 weeks
	Start with entecavir in HBeAg-negative patients who have platelet counts $<15 \times 10^3/mm^3$ and in those with advanced liver disease of stage F2 or higher.	

†Sequential treatment: patients who have lost hepatitis B virus (HBV) DNA after treatment with nucleos(t)ide analogs receive combined interferon (IFN) for 4 weeks, and then IFN monotherapy is continued for 20 weeks, and lifted thereafter. ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen.

Table 2 Guidelines for the treatment of patients with chronic hepatitis B aged 35 years or older

Eligibility criteria	ALT	≥31 IU/L
	HBV DNA	HBeAg-positive patients: ≥5 log copies/mL HBeAg-negative patients: ≥4 log copies/mL Patients with cirrhosis: ≥3 log copies/mL
HBV DNA	≥7 log copies/mL	<7 log copies/mL
HBeAg-positive	(1) Entecavir (2) Sequential treatment† (entecavir plus IFN)	(1) Entecavir (2) Long-term IFN for 24–48 weeks
HBeAg-negative	Entecavir	(1) Entecavir (2) Long-term IFN for 24–48 weeks

†Sequential treatment: patients who have lost hepatitis B virus (HBV) DNA after treatment with nucleot(s)ide analog receive combined interferon (IFN) for 4 weeks, and then IFN monotherapy is continued for 20 weeks, and lifted thereafter. ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen.

accordingly, they would be resistant to interferon (IFN) therapy. Should they receive nucleos(t)ide analogs, however, the duration would become inevitably longer, because they start the treatment when younger than 35 years old. Hence, IFN for 24–48 weeks is the first choice in their treatment. The standard treatment of 3 months is favored, which can be extended to the maximum of 6 months. Non-pegylated (standard) IFN-α is recommended to them, because self-injection at home is approved for preparations of IFN-α; it helps improve their quality of life (QOL). There are many patients who are refractory to IFN and in whom improvement of ALT levels and/or decrease in HBV DNA titers are hardly achievable. Therefore, as another option, monotherapy with entecavir can be applied for the purpose of clearing HBeAg from serum and lowering HBV DNA titers. For HBeAg-positive patients with lower HBV DNA titers (<7 log copies/mL), also, long-term IFN is endorsed as a rule.

There are HBeAg-negative patients in whom ALT levels increase to 31 IU/mL or more repeatedly. In the 2008 guidelines, sequential treatment with IFN and entecavir is introduced as a new arm of therapeutic options for such patients.[1]

For HBeAg-negative patients with less than 7 copies/mL of HBV DNA, in general, regular follow up without therapeutic intervention is deemed to suffice for the majority. For those of them in whom ALT levels flare to 31 IU/mL or more time after time, long-term IFN for 24 weeks is indicated. Because liver disease progresses in many HBeAg-negative patients, for those with platelet counts of less than $150 \times 10^3/mm^3$ or in fibrosis stage F2 or higher, treatment with entecavir is indicated.

GUIDELINES FOR THE TREATMENT OF PATIENTS WITH CHRONIC HEPATITIS B AGED 35 YEARS OR OLDER

TABLE 2 SUMS up treatment modalities for patients with chronic hepatitis B who are aged 35 years or older. HBeAg-positive patients in this age range who carry HBV DNA in titers of 7 log copies/mL or more rarely, if ever, seroconvert to the loss of HBeAg by IFN-based therapies. Hence, entecavir is the first choice in their treatment.[2,3] Because HBV mutants resistant to entecavir can be elicited by it, sequential treatment with IFN plus entecavir is amended in the 2008 guidelines.[1] In view of low viral loads in patients who possess HBV DNA in titers of less than 7 log copies/mL, entecavir is selected as the first choice, followed by long-term IFN as the second choice of treatment in these patients. HBeAg-negative patients who have high viral loads (≥7 log copies/mL), on the other hand, can normalize ALT levels by monotherapy with entecavir. Therefore, entecavir becomes their first choice, and this is the case even in patients with HBV DNA titers less than 7 copies/mL.

GUIDELINES FOR THE TREATMENT WITH NUCLEOS(T)IDE ANALOGS OF PATIENTS WITH CHRONIC HEPATITIS B WHO ARE RECEIVING LAMIVUDINE

TABLE 3 DETAILS guidelines for the treatment with nucleos(t)ide analogs of patients with chronic hepatitis B who are receiving lamivudine. Because a number of drug-resistant HBV mutants emerge increasingly with time in patients on long-term treatment with lamivudine, the fundamental rule is to switch them to ente-

Table 3 Guidelines for the treatment with nucleos(t)ide analogs in patients with chronic hepatitis who are receiving lamivudine

Lamivudine	Less than 3 years		3 years or longer
HBV DNA			
<1.8 log copies/mL persistently	May be switched to entecavir 0.5 mg daily		Continued on lamivudine 100 mg daily
≥1.8 log copies/mL	VBT (−)	May be switched to entecavir 0.5 mg daily	
	VBT (+)	Adefovir 10 mg daily add-on lamivudine	Adefovir 10 mg daily add-on lamivudine

HBV, hepatitis B virus; VBT, virological breakthrough.

cavir. For this reason, patients are stratified by the duration of lamivudine treatment, less than 3 years and 3 years or more, as well as HBV DNA titers persistently below 1.8 log copies/mL and 1.8 log copies/mL or more, and separate treatment strategies have been worked out for the patients in each category. Because by far the majority of patients with a duration of lamivudine treatment of less than 3 years and HBV DNA titers of less than 1.8 copies/mL possess drug-resistant mutants in low frequencies, they are recommended to switch to entecavir 0.5 mg daily as soon as possible. Likewise, patients who have received lamivudine for 3 years or longer, but in whom drug-resistant mutants have never developed, are recommended to switch to entecavir 0.5 mg daily. By contrast, for patients in whom drug-resistant mutants have emerged already and who have undergone virological breakthroughs,[4] adefovir 10 mg daily add-on lamivudine is started for the purpose of stabilizing liver function.[5] In regard of the patients who have received lamivudine for 3 years or longer, those without drug-resistant mutants can stay on lamivudine 100 mg daily.

SUPPLEMENTS TO GUIDELINES FOR THE TREATMENT OF CHRONIC HEPATITIS B (PART I)

FOR THE FISCAL year 2008, the following three items have been added to previous guidelines for the treatment of chronic hepatitis B (Table 4).

1 In the treatment of patients with chronic hepatitis B, IFN is the first resort for those younger than 35 years, toward the eventual goal of gaining a "drug-free state". For the patients aged 35 years or older, persistently negative HBV DNA is the aim of nucleos(t)ide analogs, with the first choice being entecavir in their primary treatment. On the other hand, for patients with HBV mutants resistant to lamivudine and/or entecavir, combined treatment with adefovir and lamivudine is the principal rule (Table 3).[6–8]
2 Therapeutic responses to antiviral treatment are much different in patients with chronic hepatitis B who are infected with HBV of distinct genotypes. It is recommended therefore to determine HBV genotypes before making a decision on the treatment choice. In particular, the patients infected with HBV of genotype A or B respond to IFN in high rates, even if they are aged 35 years or older. For these reasons, IFN becomes the first choice in their antiviral treatment.
3 The duration of IFN treatment is 24 weeks basically. In the patients in whom the efficacy of IFN has been achieved with decrease in HBV DNA titers and normalization of ALT, the treatment duration is better extended to 48 weeks.

Table 4 Supplements to guidelines for the treatment of patients with chronic hepatitis B (part I)

1 Treatment of patients with chronic hepatitis B aims at a "drug-free state" by IFN-based therapies in those younger than 35 years, and at persistently negative HBV DNA in those aged 35 years or older, with entecavir as the first choice in the primary therapy. Lamivudine plus adefovir forms the basis for the treatment of HBV mutants resistant to lamivudine or entecavir.
2 In view of antiviral response much different in patients infected with HBV of distinct genotypes, it is desired to make treatment choices based on genotypes. In particular, because genotypes A and B respond to IFN with high efficacy, even in patients aged 35 years or older, IFN is recommended as the first treatment choice in these patients.
3 The duration of IFN is for 24 weeks basically, but extension to 48 weeks is recommended in patients who respond to IFN with decrease in HBV DNA titers and normalization of ALT levels.

ALT, alanine aminotransferase; HBV, hepatitis B virus; IFN, interferon.