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A2BP1 as a Novel Susceptible Gene for Primary Biliary Cirrhosis in Japanese Patients

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Abbreviated title: Microsatellite Association Study in PBC

List of abbreviations used

PBC: primary biliary cirrhosis

SNPs: single nucleotide polymorphisms

A2BP1: ataxin 2-binding protein 1

HLA: human leukocyte antigen

STAT4: signal transducer and activator of transcription 4

CTLA4: cytotoxic T-lymphocyte antigen 4

MDR3: multidrug resistance protein 3

TNF-α: tumor necrosis factor-α

AMA: anti-mitochondrial antibody

AMA-M2:anti-mitochondrial antibody specific for the pyruvate dehydrogenase complex-E2 component

ELISA: enzyme-linked immunosorbent assay

PCR: polymerase chain reaction

Pc: corrected P-values

OR: odds ratios

CI: confidence intervals

HWP: Hardy-Weinberg proportion

CGRP: calcitonin-calcitonin gene-related peptide

Abstract

Primary biliary cirrhosis (PBC) is a complex autoimmune liver disease whose etiology 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). One hundred and twenty six patients with PBC and 95 healthy Japanese controls were enrolled. Four candidate susceptible regions and 7 candidate protective regions were statistically associated with PBC. Since 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, and 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 immunological, 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], and in monozygotic twins, the rate of concordance is 63 percent 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. Material 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 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 racial background was Japanese. All control subjects had indicated the absence of major illness on a standard questionnaire,

and were volunteers from hospital staff whose racial backgrounds were also all Japanese.

The diagnosis of PBC could be established when two of the following three criteria were met: (1) biochemical evidence of cholestasis with elevation of alkaline phosphatase activity; (2) presence of anti-mitochondrial antibody (AMA) specific for the pyruvate dehydrogenase complex-E2 component antigen; and (3) 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 the enzyme-linked immunosorbent assay (ELISA). An index of greater than seven 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 inter-marker 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. Following 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 7 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 5 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 (*Pc*). 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, a total of 126 Japanese patients with PBC and 95 healthy Japanese controls were enrolled for an association analysis 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 7 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, *Pc* = 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* = 0.02665) (Table 2). We next used the National Center for Biotechnology Information Map Viewer from the National Institute 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 the minor allele frequencies of all SNPs were

more 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% confidence interval (95% CI) 1.100-4.883, *P*=0.027) in patients compared with controls (Table 5). However, the significance of this allele was 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, *Pc*=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 4 OLT PBC cases and 122 non-OLT cases revealed no significant differences in allele frequencies (data not shown).

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 4 susceptible and 7 protective markers were significantly associated with PBC

patients. The 4 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 inter-marker 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, 4 statistically significant susceptible regions and 7 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 8 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 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 in 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 since 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 4 candidate PBC susceptibility and 7 candidate PBC resistance regions by genome-wide microsatellite analysis, which include 2 candidate susceptible genes and 8 resistant genes to PBC by statistical LD analysis. Among these, *A2BP1* gene polymorphisms might be associated with the onset of PBC. Since 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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Table 1. Demographic and clinical characteristics of 126 patients with PBC

Characteristics	
Age, years†	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)†	
AST (12-37 IU/L)	35 (12-687)
ALT (7-45 IU/L)	32 (8-885)
ALP (124-367 mg/dl)	403 (131-2,306)
γ- GTP (8-50 mg/dl)	104 (10-1,029)
Total bilirubin (0.3-1.2 mg/dl)	0.73 (0.30-6.04)
IgM (35-220 mg/dl)	232 (56-1,390)
IgG (870-1700 mg/dl)	1,530 (764-3,410)

† Median (range)

Abbreviations: PBC, primary biliary cirrhosis; AMA, anti-mitochondrial antibody specific for the pyruvate dehydrogenase complex-E2 component; ANA, anti-nuclear antibody; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γ-GTP, γ-glutamyltransferase.

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	$\chi 2$	P	Pc
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; *Pc*, corrected *P*

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

dbSNP	Alleles major/minor	Position (bp)	Patients (n=126)		Controls (n=95)	
			MAF (%)	HWE *p* value	MAF (%)	HWE *p* value
rs17139207	A/G	6026273	9.9	0.200	7.4	1.000
rs12926282	C/A	6034558	23.4	0.718	18.4	0.686
rs17139244	A/G	6045214	29.8	0.532	40.0	0.058
rs6500742	C/T	6049222	37.7	0.916	48.4	0.073
rs4146812	G/A	6055827	27.0	0.806	20.0	1.000
rs4124065	T/G	6068401	25.4	1.000	25.3	1.000
rs889699	A/G	6074528	34.9	0.494	35.8	0.489

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

Table 5. A2BP1 polymorphisms in patients with PBC and healthy subjects

	Alleles	PBC (n＝126)			Controls (n=95)			§	additive model	
dbSNP	(1/2)	1/1	1/2	2/2	1/1	1/2	2/2	*p*	*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;

§ showed the P value in genotype frequency caluculated by χ^2-test of a 2x3 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 less than 5 data samples, Fisher's exact probability test was used.

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A2BP1 as a Novel Susceptible Gene for Primary Biliary Cirrhosis in Japanese Patients

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Key words: Primary biliary cirrhosis, Microsatellite, HLA, *ataxin 2-binding protein 1* gene

Abbreviated title: Microsatellite Association Study in PBC

List of abbreviations used

PBC: primary biliary cirrhosis

SNPs: single nucleotide polymorphisms

A2BP1: ataxin 2-binding protein 1

HLA: human leukocyte antigen

STAT4: signal transducer and activator of transcription 4

CTLA4: cytotoxic T-lymphocyte antigen 4

MDR3: multidrug resistance protein 3

TNF-α: tumor necrosis factor-α

AMA: anti-mitochondrial antibody

AMA-M2:anti-mitochondrial antibody specific for the pyruvate dehydrogenase complex-E2 component

ELISA: enzyme-linked immunosorbent assay

PCR: polymerase chain reaction

Pc: corrected P-values

OR: odds ratios

CI: confidence intervals

HWP: Hardy-Weinberg proportion

CGRP: calcitonin-calcitonin gene-related peptide

Abstract

Primary biliary cirrhosis (PBC) is a complex autoimmune liver disease whose etiology 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). One hundred and twenty six patients with PBC and 95 healthy Japanese controls were enrolled. Four candidate susceptible regions and 7 candidate protective regions were statistically associated with PBC. Since 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, and 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 immunological, 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], and in monozygotic twins, the rate of concordance is 63 percent 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. Material 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 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 racial background was Japanese. All control subjects had indicated the absence of major illness on a standard questionnaire,

and were volunteers from hospital staff whose racial backgrounds were also all Japanese.

The diagnosis of PBC could be established when two of the following three criteria were met: (1) biochemical evidence of cholestasis with elevation of alkaline phosphatase activity; (2) presence of anti-mitochondrial antibody (AMA) specific for the pyruvate dehydrogenase complex-E2 component antigen; and (3) 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 the enzyme-linked immunosorbent assay (ELISA). An index of greater than seven 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 inter-marker 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. Following 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 7 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 5 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 (*Pc*). 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, a total of 126 Japanese patients with PBC and 95 healthy Japanese controls were enrolled for an association analysis 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 7 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, *Pc* = 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* = 0.02665) (Table 2). We next used the National Center for Biotechnology Information Map Viewer from the National Institute 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 the minor allele frequencies of all SNPs were

more 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% confidence interval (95% CI) 1.100-4.883, *P*=0.027) in patients compared with controls (Table 5). However, the significance of this allele was 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, *Pc*=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 4 OLT PBC cases and 122 non-OLT cases revealed no significant differences in allele frequencies (data not shown).

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 4 susceptible and 7 protective markers were significantly associated with PBC

patients. The 4 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 inter-marker 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, 4 statistically significant susceptible regions and 7 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 8 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 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 in 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 since 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 4 candidate PBC susceptibility and 7 candidate PBC resistance regions by genome-wide microsatellite analysis, which include 2 candidate susceptible genes and 8 resistant genes to PBC by statistical LD analysis. Among these, *A2BP1* gene polymorphisms might be associated with the onset of PBC. Since 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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Table 1. Demographic and clinical characteristics of 126 patients with PBC

Characteristics	
Age, years†	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)†	
AST (12-37 IU/L)	35 (12-687)
ALT (7-45 IU/L)	32 (8-885)
ALP (124-367 mg/dl)	403 (131-2,306)
γ- GTP (8-50 mg/dl)	104 (10-1,029)
Total bilirubin (0.3-1.2 mg/dl)	0.73 (0.30-6.04)
IgM (35-220 mg/dl)	232 (56-1,390)
IgG (870-1700 mg/dl)	1,530 (764-3,410)

† Median (range)

Abbreviations: PBC, primary biliary cirrhosis; AMA, anti-mitochondrial antibody specific for the pyruvate dehydrogenase complex-E2 component; ANA, anti-nuclear antibody; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γ-GTP, γ-glutamyltransferase.

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	$\chi 2$	P	Pc
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; *Pc*, corrected *P*

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

dbSNP	Alleles major/minor	Position (bp)	Patients (n=126)		Controls (n=95)	
			MAF (%)	HWE *p* value	MAF (%)	HWE *p* value
rs17139207	A/G	6026273	9.9	0.200	7.4	1.000
rs12926282	C/A	6034558	23.4	0.718	18.4	0.686
rs17139244	A/G	6045214	29.8	0.532	40.0	0.058
rs6500742	C/T	6049222	37.7	0.916	48.4	0.073
rs4146812	G/A	6055827	27.0	0.806	20.0	1.000
rs4124065	T/G	6068401	25.4	1.000	25.3	1.000
rs889699	A/G	6074528	34.9	0.494	35.8	0.489

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

Table 5. A2BP1 polymorphisms in patients with PBC and healthy subjects

	Alleles	PBC (n＝126)			Controls (n=95)			§	additive model	
dbSNP	(1/2)	1/1	1/2	2/2	1/1	1/2	2/2	*p*	*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;

§ showed the P value in genotype frequency caluculated by χ^2-test of a 2x3 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 less than 5 data samples, Fisher's exact probability test was used.