Table 3. 簡易型スコアリングシステム

自己抗体		
ANA or SMA	>1：40	+1
ANA or SMA	>1：80	+2
LKM-1 抗体	>1：40	+2
soluble liver antigen 抗体	陽性	+2
自己抗体陰性		0
IgG	正常上限の 1.1 倍以上	+2
	正常上限以上	+1
	正常範囲内	0
組織所見	典型的	+2
AIH に合致する所見	矛盾しない	+1
	なし	0
肝障害をおこすウイルスのマーカー	なし	+2
	あり	0
確診：7 点以上	疑診：6 点	

文献 4）Hepatology. 48：169-76, 2008 より和訳.

7 点での診断感受性は 81%，特異性は 99% で，感受性よりも特異性に秀でているのが特徴である．

この簡易型システムは従来型システムと比較して簡便なため，臨床現場で高頻度に用いられていると推測される．

III 両スコアリングシステムの比較検討

このように従来型，簡易型の 2 つのスコアリングシステムが存在するが，既に述べたように従来型は診断感受性に秀で，簡易型は診断特異性に秀でるという特徴がある．したがって，どのような臨床像を呈する症例にどちらのシステムを用いるべきかを明らかにしないと，診断の見落としが生じる可能性がある．特に簡易型は従来型に比し感受性が劣るとされているので注意が必要である．

そうした観点から，同一症例を従来型，簡易型両方のスコアリングシステムで点数を付け比較検討する臨床研究が世界各国から報告された．Czaja は，従来型は診断感受性に優れ，自己免疫的な所見が目立たない AIH 症例を拾い上げて診断する効力があり，簡易型は特異性に優れ，自己免疫的所見が目立つ AIH に類似した症例と AIH を鑑別する効力があると報告した[5]．Gatselis らも同様の傾向を報告し，簡易型は AIH と，免疫的異常をともなわない肝疾患が共存する症例を AIH と診断しない傾向があり，そうした“診断困難例”は簡易型でなく従来型を用いて診断すべきと考察している[6]．勝島らは簡易型では従来型に比し確診例の割合が増加するが，IgG 低値や HCV 陽性の AIH 症例は見落とされることを報告した[7]．金子らは簡易型で AIH と診断されない症例の 89% が従来型で確診・疑診とされ，それらのほとんどが IgG 低値，ANA 低力価であることを報告している[8]．

筆者らも臨床病理学的に AIH と診断された 219 例（男性 24 例，女性 195 例）の診断時データを用い，従来型，簡易型システムによりスコアリングを行い両者間で診断が不一致となる症例の臨床像を解析した[9]．その結果，従来型では確診・疑診とされるが簡易型では AIH と診断されない症例は，両型で確診・疑診とされる症例に比し，IgG 値が有意に低く，ANA 価が 40 倍以下の低力価例が有意に多かった（Table 4）．逆に簡易型で確診・疑診とされるが従来型では AIH と診断さ

Table 4. 従来型で確診または疑診，簡易型で AIH でないとされる症例の臨床像

	従来・簡易とも確診・疑診 (n=110)	従来：確診・疑診 簡易：AIH でない (n=53)
AST	281±374	216±408
ALP	344±178	309±140
IgG	2597±733	1663±483
	P<0.001	
ANA<40×	17.3%	62.6%
	P<0.001	

Table 5. 簡易型で確診または疑診，従来型で AIH でないとされる症例の臨床像

	従来・簡易とも確診・疑診 (n=110)	簡易：確診・疑診 従来：AIH でない (n=13)
AST	281±374	228±344
ALP	344±178	379±206
IgG	2597±733	2316±369
ANA<40×	17.3%	15.3%
胆管病変/AMA 陽性	32.7%	84.6%
	P<0.001	

れない症例は，両型で確診・疑診とされる症例に比し，胆管病変陽性例または AMA 陽性例が占める割合が有意に高いことが明らかとなった(Table 5)．

以上の成績を考慮すると，IgG 値や ANA 価が高い典型例の診断には簡易型が適し，簡易型作成の目的に沿った早期治療介入に有用である．しかし，これらの典型所見が明確でない非定型例は簡易型では AIH と診断されない可能性があり，従来型も含めて総合的に診断すべきと考えられる．一方で，従来から outlier とされていたこうした非定型症例を AIH に包含し治療を行うべきか否かについては，さらなる検討が必要であろう．

IV 特殊な病態を呈する症例のスコアリングシステムによる診断

1. 急性発症例，劇症発症例

近年，急性発症型，劇症発症型 AIH の存在が注目されているが[10][11]，そうした症例は自己抗体価や IgG 値が低値なことが少なくないため，簡易型を用いて診断すると見落とされる可能性がある．

Miyake らは急性発症例の 23%，組織学的に急性肝炎所見を呈する症例の 50% が簡易型では AIH と診断されないと報告し[12]，Fujiwara らは急性発症例が AIH と診断されない確率は従来型では 9%，簡易型では 60% と報告した[13]．また Yeoman らは劇症発症例が AIH と診断されない確率は従来型では 60%，簡易型では 74% と報告した[14]．以上より，急性・劇症発症型 AIH が疑われる症例の診断には，簡易型でなく従来型を用いるべきである．

また，急性発症型では従来報告されている慢性肝炎に特徴的な病理組織所見は示さず，中心静脈領域の壊死・炎症反応が特徴的であるという報告がなされており，これらの点に関するコンセンサスを含めた診断方法の確立が今後の課題である．

2. オーバーラップ症候群

AIH と PBC のオーバーラップ症候群の診断における AIH スコアリングシステムの有効性については明確なコンセンサスが得られていない．

PBC でオーバーラップの臨床像を示す症例を AIH スコアリングシステムにより診断した検討

Table 6. オーバーラップ症候群診断のための Paris Criteria

下記に示す AIH，PBC の Criteria の 3 項目のうち，それぞれ 2 項目以上を満たすこと
AIH の Criteria
ALT 値が正常上限の 5 倍以上
IgG 値が正常上限の 2 倍以上 or ASMA 陽性
中等度以上のリンパ球浸潤をともなう interface hepatitis の所見を示す
PBC の Criteria
ALP 値が正常上限の 2 倍以上 or γGTP 値が正常上限の 5 倍以上
AMA 陽性
胆管病変の所見を示す

文献 16）Hepatology. 28：296-301, 1998 より和訳.

が 2 報あり，Kuiper らは診断感受性は従来型で 60%，簡易型で 73%，診断特異性は従来型で 83%，簡易型では 78% と，簡易型の方がオーバーラップ症候群を診断しやすいとしている[15]．ただしこの論文では，オーバーラップ症候群の診断には Poupon らが提唱する Paris Criteria（Table 6）[16]が AIH スコアリングシステムより有用と結論している．一方 Neuhauser らは従来型より簡易型の方がオーバーラップ症候群の診断特異性が高く，簡易型で診断された症例は肝障害の程度も予後も悪いことを明らかにし，オーバーラップ症候群診断における簡易型の有用性を示している[17]．

前述したように従来型は AIH と PBC の鑑別を念頭において改訂されたシステムであり，一方簡易型は PBC との鑑別に重要な AMA を項目に加えていないので，従来型より簡易型の方がオーバーラップ症候群の診断に有用なのは道理ではある．しかし IAIHG が 2011 年に発表したオーバーラップ症候群に関するステートメントには「現状では AIH 診断スコアリングシステムがオーバーラップ症候群の診断に用いられているが，元来このシステムはオーバーラップ症候群の診断のために作成されたものではなく，その有用性も証明されておらず，新たなサブグループの診断のために用いられるべきではない」とコメントされている[18]．またアメリカ肝臓学会が 2010 年発表したクリニカルガイドラインでは「オーバーラップ症候群の診断には従来型が参考になる」という程度の記述に留まっている[19]．

一方，2005 年にわが国の厚生労働省・難治性肝疾患研究班で検討，提示された判別式では，オーバーラップ症候群の多くは PBC の hepatic form として診断されることが明らかにされている[20]．

3. 小児例

小児でも AIH が発症することが明らかにされているが[21]，その診断にスコアリングシステムを用いることにはいくつかの問題がある．

まず小児例は初診時 IgG 値が 2000mg/dl 未満のことが多いため，IgG の評価は年齢別基準値上限との比を用いるべきである．また小児はアルコール摂取が皆無のため，従来型では自動的に 2 点加算されてしまう点も留意すべきである．さらに ALP 値は小児期では生理的に高く年齢による幅があるため，基準値の設定が困難になる．そのため ALP の代わりに生理的変動が少ないγ GTP と ALT の比を用いることが推奨され，γGTP と ALT の比を用いた従来型スコアリングにより診断率が向上し，特に小児 AIH で高頻度に合併が見られる PSC とのオーバーラップ症例の診断に有用と報告されている[22]．一方，小児例の診断感受性は，従来型では 100% であるのに対し簡易型では 55% と低く，簡易型では AIH と PSC の鑑別ができないとの報告もある[23]．

以上より，小児例の診断には IgG 値や ALP 値を考慮した従来型を用い，簡易型による診断は避

けた方がよいと考えられる．

おわりに―どのような症例にどのスコアリングシステムを用いるか？―

簡易型は簡便で臨床的利便性があり，自己免疫的な臨床像が著明な典型例の診断には効力を発揮するが，IgG，ANA低値の非典型例，急性発症例，劇症例などはAIHと診断されない危険性がある．そうした症例では，簡易型のみでなく従来型によるスコアリングも含めた総合的な診断が行われるべきである．

しかしスコアリングシステムはあくまで診断の参考にすべきものであることを忘れてはならない．診断にあたってはわが国の診断指針を軸にし，スコアリングシステムも十分に参考にすることが大切で，スコアリングという行為や付けられた点数を過大評価するあまり，個々の症例の綿密な病態解析がおろそかになることがあってはならない．

早期の診断と適切な治療の開始がAIHの予後の規定因子として重要であり，診断に難渋する症例はスコアリングにこだわらず生検所見の評価も含め速やかに専門医のいる施設にコンサルトすることが肝要と思われる．

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

A polymorphism in the integrin αV subunit gene affects the progression of primary biliary cirrhosis in Japanese patients

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Abstract
Background Accumulating evidence indicates that multiple genetic factors are involved in the pathogenesis of primary biliary cirrhosis (PBC). The aim of this study was to investigate whether polymorphisms of the integrin αV subunit gene (*ITGAV*), a component of integrin αVβ6, which plays an important role in the process of fibrosis, are associated with susceptibility to the onset and/or progression of PBC.
Methods In the primary study, eight tag single nucleotide polymorphisms (SNPs) in *ITGAV* were analyzed by polymerase chain reaction (PCR)-restriction fragment length polymorphism, direct DNA sequencing, or high-resolution melting curve analysis in 309 Japanese patients with PBC who were registered in the National Hospital Organization Study Group for Liver Disease in Japan (PBC cohort I) and 293 gender-matched healthy Japanese volunteers (control subjects). For the replication study, 35 PBC patients who progressed to end-stage hepatic failure and underwent liver transplantation (PBC cohort II) were also analyzed.
Results Three tag SNPs (rs3911238, rs10174098, and rs1448427) in *ITGAV* were significantly associated with the severe progression of PBC, but not with susceptibility to the onset of PBC, in the primary study (PBC cohort I). Among these SNPs, rs1448427 was also significantly associated with the severe progression to end-stage hepatic failure in the replication study of PBC patients who underwent liver transplantation (PBC cohort II).
Conclusions *ITGAV* is a genetic determinant for the severe progression of PBC in Japanese patients. Genetic polymorphisms of *ITGAV* may be useful for identifying high-risk Japanese PBC patients, including those who will require liver transplantation, at the time of initial diagnosis.

Keywords PBC · Integrin αV · Hepatic fibrosis · Premature ductopenia · Progression

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Introduction

Primary biliary cirrhosis (PBC) is a chronic and slowly progressive liver disease characterized by inflammation of cholangiocytes in the interlobular bile ducts and subsequent destruction of the bile ducts, leading to hepatic cholestasis, ductopenia, fibrosis, cirrhosis, and eventually

liver failure. PBC is considered to be an autoimmune disease because autoantibodies, such as antimitochondrial antibodies, are present in more than 90% of PBC patients [1]. At present, a majority of PBC patients undergoing treatment with ursodeoxycholic acid (UDCA), which is the only therapeutic agent approved by the Food and Drug Administration, have a normal life expectancy without additional therapeutic approaches. However, one-third of PBC patients show severe progression and require additional treatments [2]. Ultimately, a few percent of PBC patients who show resistance to UDCA undergo liver transplantation or die within a decade of diagnosis [3].

PBC is a multifactorial disease that is attributed to genetic predisposition and environmental triggers. Genetic factors have been implicated in previous studies identifying familial clustering [4, 5] and a high concordance of monozygotic twin pairs compared to dizygotic twin pairs [6]. Furthermore, there are many reports describing associations of susceptibility to PBC with genetic variations of several genes, e.g., human leukocyte antigen DR and DQ [5, 7, 8], cytotoxic T lymphocyte antigen 4 [9–11], vitamin D receptor [12], interleukin-1 beta [13], interleukin-12 alpha and interleukin-12 receptor beta 2 [7], and interferon regulatory factor 5—transportin 3 [14, 15]. Additionally, a few genes have been confirmed as genetic factors affecting the progression of PBC; namely, tumor necrosis factor alpha [16], solute carrier 4, anion exchanger 2 [16], interleukin-1 beta [13], and multidrug resistance protein 3 [17]. These genes are mainly related to the innate or adaptive immune systems and the homeostasis of bile acids.

Liver fibrosis is a wound-healing process for chronic liver injuries and is associated with major alterations in both the quantity and composition of extracellular matrix (ECM) components produced by hepatic stellate cells, portal fibroblasts, and myofibroblasts, which are activated by fibrogenic cytokines including transforming growth factor beta 1 (TGF-β1) [18]. Integrin is a cell surface receptor composed of α and β chains, and forms at least 24 different heterodimers which are employed in the process of cell–cell and cell–ECM adhesion [19, 20]. Of the well-known heterodimers, integrin $\alpha V\beta 6$ is a receptor for ECM components such as fibronectin [21] and tenascin [22], and activates TGF-β1 [23]. Integrin $\alpha V\beta 6$ is expressed in the proliferative epithelial cells of the bile ducts of the liver in parallel with the progression of fibrosis in PBC patients, as well as chronic hepatitis C patients, but it is not expressed in adult epithelial cells, where there is neither inflammation nor fibrosis [24, 25]. Furthermore, polymorphisms of the integrin αV subunit gene (*ITGAV*), encoding the αV chain, are associated with chronic hepatitis B virus (HBV) infection and the progression of hepatocellular carcinoma [26]. Therefore, we investigated whether polymorphisms of *ITGAV* are associated with susceptibility to the onset and/or progression of PBC.

Methods

Subjects

In the primary association study, the study subjects comprised 309 unrelated Japanese patients with PBC, referred to as PBC cohort I, and 293 gender-matched healthy Japanese volunteers as control subjects (Table 1). The PBC patients were registered in the PBC cohort study in the National Hospital Organization Study Group for Liver Disease in Japan from August 1982 to September 2008. The time of entry was defined as the time of initial diagnosis of PBC.

The definite diagnosis of PBC was made on the basis of the following criteria: the presence of detectable antimitochondrial antibodies in serum; elevation of liver enzymes including alkaline phosphatase (greater than two times the upper limit) at the time of initial diagnosis; and compatible histological features. A liver biopsy was performed at the time of the initial diagnosis in 232 of the 309 patients. Patients receiving a maintenance dose of prednisolone >5 mg/body weight for concomitant autoimmune hepatitis or those with acute or autoimmune hepatitis (alanine aminotransferase >500 IU/l, aspartate aminotransferase >500 IU/l), persistent hepatitis virus B or C infection, alcoholic liver disease, or other chronic liver diseases were excluded from this study.

Among the 309 patients, 95 (30.7%) were complicated by other autoimmune diseases: Sjögren's syndrome ($n = 42$), autoimmune hepatitis ($n = 22$), Hashimoto's thyroiditis

Table 1 Characteristics of PBC patients and control subjects at the end of the observation period

Characteristics	Control subjects	PBC cohort I	PBC cohort II
Total number	293	309	35
Age, mean ± SD (years)	41.2 ± 12.2	64.2 ± 11.4	51.2 ± 8.1
Male/female (%)	36/257 (12.3/87.7)	43/266 (13.9/86.1)	3/32 (8.6/91.4)
Observation period, mean ± SD (months)		71.2 ± 64.3	93.9 ± 61.2
Concomitant autoimmune disease (%)		30.7	8.6

PBC primary biliary cirrhosis, *SD* standard deviation

Table 2 Characteristics of PBC patients in each clinical stage

	PBC cohort I								
	Clinical stage I	Clinical stage II	Clinical stage III	Early stage (Clinical stage I)	Late stage (Clinical stages II and III)	*P* value*	Nonjaundice stage (Clinical stages I and II)	Jaundice stage (Clinical stage III)	*P* value**
Total number	224	68	17	224	85		292	17	
Age, mean ± SD (years)	62.9 ± 11.4	69.7 ± 9.8	58.8 ± 9.8	62.9 ± 11.4	67.5 ± 10.7	<0.005	64.5 ± 11.4	58.8 ± 9.8	0.044
Male/female (%)	27/197 (12.1/87.9)	11/57 (16.2/83.8)	5/12 (29.4/70.6)	27/197 (12.1/87.9)	16/69 (18.8/81.2)	0.125	38/254 (13.0/87.0)	5/12 (29.4/70.6)	0.071
Observation period, mean ± SD (months)	63.3 ± 59.9	89.7 ± 72.0	100.2 ± 68.0	63.3 ± 59.9	91.8 ± 70.9	<0.001	69.5 ± 63.7	100.2 ± 68.0	0.055
Concomitant autoimmune disease (%)	28.6	38.2	29.4	28.6	36.5	0.179	30.8	29.4	>0.999

SD standard deviation

* Comparison between early stage and late stage in PBC cohort I

** Comparison between nonjaundice stage and jaundice stage in PBC cohort I

(*n* = 13), rheumatoid arthritis (*n* = 9), Raynaud's syndrome (*n* = 8), systemic sclerosis (*n* = 7), CREST (calcinosis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia) syndrome (*n* = 4), autoimmune thrombocytopenic purpura (*n* = 3), interstitial pneumonitis (*n* = 2), hypothyroidism (*n* = 2), polymyositis (*n* = 1), Basedow's disease (*n* = 1), sarcoidosis (*n* = 1), systemic lupus erythematosus (*n* = 1), mixed connective tissue disease (*n* = 1), and uveitis (*n* = 1).

During the observation period, patients received the following treatment: UDCA (300–900 mg/day) alone (*n* = 204), bezafibrate (200–400 mg/day) alone (*n* = 3), maintenance prednisolone (≤5 mg/day) alone (*n* = 4), UDCA + bezafibrate (*n* = 58), UDCA + maintenance prednisolone (*n* = 17), UDCA and/or bezafibrate + maintenance prednisolone (*n* = 12), or no medication (*n* = 11).

In addition to the primary association study, 35 additional Japanese PBC patients, referred to as PBC cohort II, who underwent liver transplantation at Kyushu University Hospital because of severe progression to end-stage hepatic failure, were analyzed as a replication study (Table 1).

Classification of clinical stages of PBC

In the primary association study, PBC patients in PBC cohort I were classified into the following three clinical stages based on the findings of liver biopsy and/or clinical manifestations: clinical stage I, Scheuer's stage 1 or 2 [27] in liver biopsy or unknown histological stage without any signs indicating portal hypertension or liver cirrhosis; clinical stage II, Scheuer's stage 3 or 4 in liver biopsy or any histological stage with signs indicating portal hypertension or liver cirrhosis, but without persistent jaundice (total bilirubin <2 mg/dl); and clinical stage III, any Scheuer's stage with persistent or progressive jaundice (total bilirubin >2 mg/dl). The characteristics of the three subgroups in PBC cohort I are shown in Table 2.

Clinical stage I was defined as early stage, whereas clinical stages II and III were defined as late stage. Clinical stages I and II were also defined as the nonjaundice stage, whereas clinical stage III was defined as the jaundice stage (Table 2). In addition, the progression to clinical stage III (late stage with jaundice) was defined as jaundice-type progression, which is the most severe progression, resulting in liver transplantation or death due to hepatic failure. The observation period was defined as the time from the date of the initial diagnosis until the date of death, liver transplantation, death due to non-liver-associated disease, or the end of follow-up, whichever came first.

In the secondary, replication, study, all PBC patients in PBC cohort II progressed to clinical stage III and were referred to Kyushu University Hospital where orthotopic liver transplantation was performed.

Ethics board

Written informed consent was obtained from each subject. The study protocol was approved by the Committee for Ethical Issues dealing with the Human Genome and Gene Analysis at Nagasaki University, National Hospital Organization Nagasaki Medical Center, and Kyushu University.

Preparation of genomic DNA

Genomic DNA was extracted from whole blood samples using a NucleoSpin Blood L Kit (Macherey–Nagel, Düren, Germany) according to the manufacturer's protocol. Genomic DNA was also extracted from the removed livers of PBC patients who underwent liver transplantation, using a QuickGene DNA Tissue Kit S (Fujifilm, Tokyo, Japan) according to the manufacturer's protocol.

Selection of tag single nucleotide polymorphisms in *ITGAV*

Polymorphic information on single nucleotide polymorphisms (SNPs) in *ITGAV* (GenBank accession number: NM_002210; MIM 193210) in the Japanese population was obtained using data available on the International HapMap Website (http://www.hapmap.org). Candidate tag SNPs were selected from all SNPs in the 2q31–32 chromosomal region including 2-kbp upstream of *ITGAV* with priority in minor allele frequency of more than 10% in the International HapMap data. Subsequently, linkage disequilibrium blocks and genotyped tag SNPs among the candidate tag SNPs were determined using the Haploview 4.1 software program (Broad Institute, Cambridge, MA, USA) [28]. The gene structure and positions of linkage disequilibrium blocks and genotyped tag SNPs in *ITGAV* used in this study are shown in Fig. 1.

Genotyping of tag SNPs in *ITGAV*

Eight SNPs, rs3911238 (intron 2), rs12611439 (intron 2), rs1992898 (intron 4), rs10174098 (intron 4), rs4667107 (intron 6), rs3768785 (intron 12), rs1448424 (intron 12), and rs1448427 (intron 18) were selected as genotyped tag SNPs for this study (Fig. 1). The analytic methods, sequences of primer pairs, and annealing temperature for genotyped tag SNPs in *ITGAV* are shown in Table 3.

One tag SNP, rs3911238, was analyzed by polymerase chain reaction (PCR)-restriction fragment length polymorphism. The polymorphic region was amplified by PCR with a T1 Thermocycler 96 (Biometra, Göttingen,

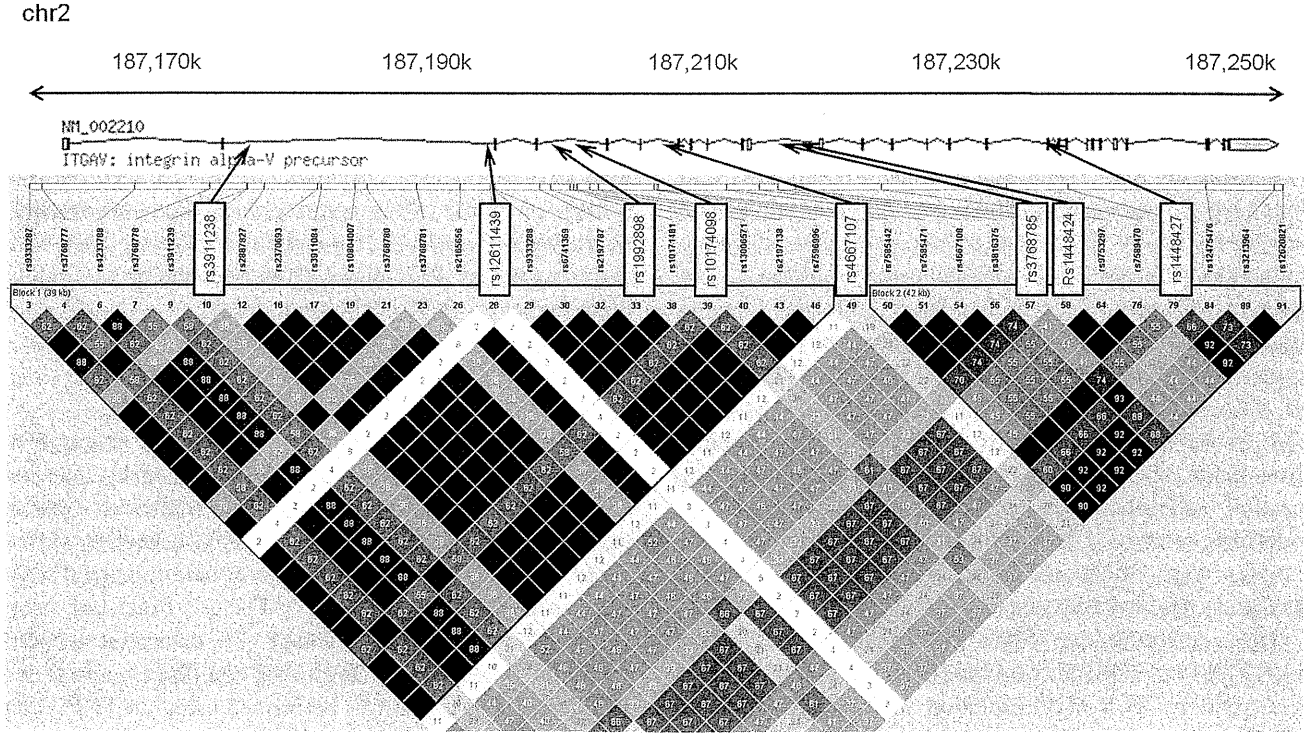

Fig. 1 Location of genotyped tag single nucleotide polymorphmisms (SNPs) in *ITGAV* and linkage disequilibrium blocks in the International HapMap data. The *horizontal wavy line* on the *top* indicates the genomic sequence of *ITGAV*. The *short vertical bars* on the sequence of *ITGAV* indicate exons. *Arrows* indicate the positions of genotyped tag SNPs used in this study, and their names are presented in *open rectangles*. Linkage disequilibrium *blocks* are aslo shown. *Each diamond* represents a pairwise linkage disequilibrium value (r^2). The *black diamonds* show an r^2 value of 1.0

Table 3 Analytic methods, sequences of primer pairs, and annealing temperature for genotyped tag SNPs in *ITGAV*

SNP	Analytic method	Primer sequence		Annealing temperature (°C)
		Forward	Reverse	
rs3911238	PCR–RFLP	TGGGCACCAGACAATGTTTA	AGTGAATGTCTCTTTCCCTCAA	55
rs12611439	PCR-HRM	TGAGACACTTCTGGGTCATCAT	TCCCCAGCTAGGTTGAGAAA	57
rs1992898	PCR-HRM	CCTAAGGGCTCTAGGCATAAAC	CCCGCTACCCTTTGTTGAAT	55
rs10174098	PCR-HRM	GGGGCATCTTGTTCAGAGAA	AAGGCCCAGAGTGGTTAGGT	55
rs4667107	PCR-sequencing	TTCTTGGTGGTCCTGGTAGC	ACAGATTGGTTTCGCACACT	55
rs3768785	PCR-HRM	ACATTGTCAAGGCAATGCTG	ATGCCACCATAAAGGGAGAT	53
rs1448424	PCR-HRM	GCCAACATCTCTTCCAGCTT	TTTTAGCCCTTTGTAAAATCATTG	55
rs1448427	PCR-HRM	CCGTACCCAGCCTCAAATAC	AGCAATGTCTTCCATGCTCA	55

SNP single nucleotide polymorphism, *ITGAV* integrin αV, *PCR–RFLP* polymerase chain reaction–restriction fragment length polymorphism, *HRM* high-resolution melting curve

Germany) using 10 ng genomic DNA in a 15-μl reaction mixture containing 1× Taq buffer (Invitrogen, Carlsbad, CA, USA), 200 μM dNTPs (Promega, Madison, WI, USA), 200 nM each of forward and reverse primers, and 0.05 U recombinant Taq DNA Polymerase (Invitrogen). The amplification protocol consisted of initial denaturation at 94°C for 3 min; followed by 40 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 20 s, and extension at 72°C for 20 s; and final extension at 72°C for 5 min. The PCR products were digested with *Mva* I (Fermentas, Burlington, Ontario, Canada), separated by electrophoresis on an 8% polyacrylamide gel (Nacalai Tesque, Kyoto, Japan), and subsequently visualized with a UV transilluminator (Alpha Innotech, San Leandro, CA, USA) after ethidium bromide staining.

Another tag SNP, rs4667107, was analyzed by the PCR-direct DNA sequencing method. The polymorphic region was amplified by PCR. The primer pairs and annealing temperature for rs4667107 are indicated in Table 3. The other constituents of the PCR mixture were the same as those described above. The PCR products were treated with ExoSAP-IT (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and cycle sequenced using a BigDye Terminator v3.1 Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). After the sequencing reaction solutions were purified using Sephadex G-50 superfine columns (Amersham Pharmacia Biotech), the samples were dried and sequenced with a 3730 DNA Analyzer (Applied Biosystems).

The remaining tag SNPs (rs12611439, rs1992898, rs10174098, rs3768785, rs1448424, and rs1448427) were genotyped by PCR-high-resolution melting curve (PCR-HRM) analysis [29]. The primer pairs and annealing temperature for each SNP are indicated in Table 3. The other constituents of the PCR mixture were the same as those described above. HRM was performed with a LightCycler 480 Instrument (Roche Diagnostics, Basel, Switzerland) in a 15-μl reaction mixture containing 10 μl of the PCR product, 2 μM fluorescent DNA intercalating dye SYTO® 9 (Invitrogen), and 4% dimethylsulfoxide (DMSO: Wako Pure Chemical Industries, Osaka, Japan). The melting program includes four steps: denaturation at 95°C for 2 min; renaturation at 40°C for 1 min; melting consisting of a continuous reading of fluorescence from 65 to 85°C at the rate of 25 acquisitions for each degree Celsius; and cooling at 40°C for 30 s. HRM raw melting curve data were normalized and temperature-shifted, and then analyzed to distinguish heterozygosity from homozygosity using the LightCycler 480 Gene-Scanning Software Version 1.5 program (Roche Diagnostics). Subsequently, we added 5 μl of the PCR products preliminarily genotyped as major allele homozygous samples into the HRM reaction mixture of homozygous samples and re-performed HRM to distinguish minor allele homozygosity from major allele homozygosity. At least 16 samples per each tag SNP were re-genotyped by PCR-direct DNA sequencing to confirm the accuracy of PCR-HRM genotyping.

Statistical analysis

Differences in age and the observation period among PBC patients and control subjects were evaluated by unpaired Student's *t*-test, using the PASW 18 statistical software package (SPSS Japan, Tokyo, Japan). Likewise, differences in gender and concomitance of autoimmune disease were compared by the χ^2 test or Fisher's exact test, using the PASW 18 software package. To determine whether each SNP was in Hardy–Weinberg equilibrium, the χ^2 test with Yates' correction was performed using the SNP Alyze 7.0 standard software package (Dynacom, Yokohama, Japan). The frequencies of alleles and genotypes among subgroups of PBC patients were compared by the χ^2 test in three different models: the allele model, the minor allele recessive model, and the minor allele dominant model,

using the SNP Alyze 7.0 standard software package. The odds ratio (OR) with 95% confidence interval (CI) was calculated using the SNP Alyze 7.0 standard software package. A P value of less than 0.05 was considered to be statistically significant.

Results

Comparison of demographics among PBC patients and control subjects

The characteristics of PBC patients in PBC cohort I were compared between early stage and late stage as well as nonjaundice stage and jaundice stage subgroups (Table 2). The mean age and observation period of PBC patients in the early stage were significantly younger and shorter, respectively, than those of patients in the late stage ($P < 0.005$ and 0.001, respectively). These results imply that a few patients in the early stage might progress to more severe stages (clinical stage II or III) in the future. In addition, the mean age of PBC patients in the jaundice stage was significantly younger than that of patients in the nonjaundice stage ($P = 0.044$). This result indicates that it is unlikely that any PBC patients in the nonjaundice stage would progress to the jaundice stage in the future.

With regard to a comparison of the characteristics between the PBC patients who underwent liver transplantation in PBC cohort II (Table 1) and the nonjaundice-stage PBC patients in PBC cohort I (Table 2), the mean age of the PBC patients who underwent liver transplantation in PBC cohort II was significantly younger than that of patients in the nonjaundice stage in PBC cohort I (51.2 vs. 64.5 years, $P < 0.001$ in Tables 1, 2). However, the mean observation period of PBC patients who underwent liver transplantation in PBC cohort II was significantly longer than that of the nonjaundice-stage PBC patients in PBC cohort I (93.9 vs. 69.5 months, $P = 0.035$ in Tables 1, 2). These results indicate that it is unlikely that any PBC patients in the nonjaundice stage would progress to requiring liver transplantation; that is, progress to the state of PBC cohort II, in the future because PBC patients who underwent liver transplantation tended to progress to more severe stages at younger ages.

Association between tag SNPs in *ITGAV* and the severe progression of PBC

The minor allele frequencies and genotype distributions of eight tag SNPs in *ITGAV* are indicated in Table 4. One tag SNP, rs1448424, was excluded from this study because the distribution of genotypes of this SNP in control subjects was not in Hardy–Weinberg equilibrium ($P = 0.045$).

The distributions of alleles and genotypes of seven tag SNPs in *ITGAV* were identified and compared between control subjects and PBC patients in PBC cohort I (Table 5). There were no significant associations with susceptibility to the onset of PBC in any genetic models.

The distributions of alleles and genotypes of tag SNPs were also compared between early- and late-stage patients, as well as between nonjaundice- and jaundice-stage patients, in PBC cohort I in the primary association study (Table 5). No significant associations were observed between *ITGAV* polymorphisms and the progression to late stage. On the other hand, three tag SNPs, rs3911238, rs10174098, and rs1448427, were significantly associated with the progression to jaundice stage in two genetic models. With regard to the minor allele recessive model, the frequencies of a minor homozygous C/C genotype at rs3911238 SNP and a minor homozygous G/G genotype at rs10174098 SNP were significantly increased in jaundice-stage patients compared with those in nonjaundice-stage patients ($P = 0.003$, OR = 5.3 and $P = 0.003$, OR = 6.7, respectively; Table 5). Conversely, with regard to the minor allele dominant model, the frequency of PBC patients possessing a minor G allele at rs1448427 SNP was significantly increased in jaundice stage as compared to that of the patients in nonjaundice stage ($P = 0.031$, OR = 2.9; Table 5).

Association of rs1448427 SNP in *ITGAV* with the severe progression of PBC in the secondary replication study

The secondary replication study in PBC cohort II was carried out by comparing the frequencies and distributions of the three tag SNPs that had shown a significant association with the jaundice-type progression of PBC in the primary association study, between nonjaundice-stage PBC patients in PBC cohort I and PBC patients who underwent liver transplantation in PBC cohort II, in order to validate this association in the primary association study (Table 6). With regard to the minor allele dominant model, the frequency of PBC patients possessing the minor G allele at rs1448427 SNP was significantly increased in PBC patients who underwent liver transplantation in PBC cohort II as compared to that in the patients in nonjaundice stage in PBC cohort I ($P = 0.033$, OR = 2.13; Table 6). However, the other two tag SNPs showed no significant association with the severe progression of PBC in the secondary replication study.

Subsequently, two tag SNPs, rs1448427 and rs3768785, which showed an association with the jaundice-type progression of PBC in PBC cohort I and are in the same linkage disequilibrium block, were utilized to infer haplotype structure and analyze haplotype frequencies, using the

Table 4 Distributions of genotypes of tag SNPs in *ITGAV* among control subjects and PBC patients in PBC cohort I

SNP	Subject	Major/major (%)	Major/minor (%)	Minor/minor (%)	MAF
rs3911238	Control	156 (53.2)	117 (39.9)	20 (6.8)	0.27
G>C	PBC cohort I	162 (52.4)	127 (41.1)	20 (6.5)	0.27
	Early stage	117 (52.2)	95 (42.4)	12 (5.4)	0.27
	Late stage	45 (52.9)	32 (37.6)	8 (9.4)	0.28
	Nonjaundice stage	157 (53.8)	119 (40.8)	16 (5.5)	0.26
	Jaundice stage	5 (29.4)	8 (47.1)	4 (23.5)	0.47
rs12611439	Control	194 (66.2)	90 (30.7)	9 (3.1)	0.19
A>C	PBC cohort I	218 (70.6)	81 (26.2)	10 (3.2)	0.16
	Early stage	162 (72.3)	56 (25.0)	6 (2.7)	0.15
	Late stage	56 (65.9)	25 (29.4)	4 (4.7)	0.19
	Nonjaundice stage	206 (70.5)	76 (26.0)	10 (3.4)	0.16
	Jaundice stage	12 (70.6)	5 (29.4)	0	0.15
rs1992898	Control	220 (75.1)	71 (24.2)	2 (0.7)	0.13
T>C	PBC cohort I	233 (75.4)	71 (23.0)	5 (1.6)	0.13
	Early stage	171 (76.3)	49 (21.9)	4 (1.8)	0.13
	Late stage	62 (72.9)	22 (25.9)	1 (1.2)	0.14
	Nonjaundice stage	222 (76.0)	66 (22.6)	4 (1.4)	0.13
	Jaundice stage	11 (64.7)	5 (29.4)	1 (5.9)	0.21
rs10174098	Control	187 (63.8)	98 (33.4)	8 (2.7)	0.19
A>G	PBC cohort I	190 (61.5)	107 (34.6)	12 (3.9)	0.21
	Early stage	137 (61.2)	80 (35.7)	7 (3.1)	0.21
	Late stage	53 (62.4)	27 (31.8)	5 (5.9)	0.22
	Nonjaundice stage	182 (62.3)	101 (34.6)	9 (3.1)	0.20
	Jaundice stage	8 (47.1)	6 (35.3)	3 (17.6)	0.35
rs4667107	Control	73 (24.9)	152 (51.9)	68 (23.2)	0.49
C>T	PBC cohort I	75 (24.3)	169 (54.7)	65 (21.0)	0.48
	Early stage	51 (22.8)	127 (56.7)	46 (20.5)	0.49
	Late stage	24 (28.2)	42 (49.4)	19 (22.4)	0.47
	Nonjaundice stage	72 (24.7)	161 (55.1)	59 (20.2)	0.48
	Jaundice stage	3 (17.6)	8 (47.1)	6 (35.3)	0.59
rs3768785	Control	154 (52.6)	123 (42.0)	16 (5.5)	0.26
G>A	PBC cohort I	172 (55.7)	124 (40.1)	13 (4.2)	0.24
	Early stage	124 (55.4)	91 (40.6)	9 (4.0)	0.24
	Late stage	48 (56.5)	33 (38.8)	4 (4.7)	0.24
	Nonjaundice stage	166 (56.8)	115 (39.4)	11 (3.8)	0.23
	Jaundice stage	6 (35.3)	9 (52.9)	2 (11.8)	0.38
rs1448424	Control	224 (76.5)	69 (23.5)	0	0.12
A>G	PBC cohort I	245 (79.3)	60 (19.4)	4 (1.3)	0.11
	Early stage	181 (80.8)	40 (17.9)	3 (1.3)	0.10
	Late stage	64 (75.3)	20 (23.5)	1 (1.2)	0.13
	Nonjaundice stage	235 (80.5)	54 (18.5)	3 (1.0)	0.10
	Jaundice stage	10 (58.8)	6 (35.3)	1 (5.9)	0.24
rs1448427	Control	178 (60.8)	108 (36.9)	7 (2.4)	0.21
A>G	PBC cohort I	202 (65.4)	97 (31.4)	10 (3.2)	0.19
	Early stage	146 (65.2)	71 (31.7)	7 (3.1)	0.19
	Late stage	56 (65.9)	26 (30.6)	3 (3.5)	0.19
	Nonjaundice stage	195 (66.8)	88 (30.1)	9 (3.1)	0.18
	Jaundice stage	7 (41.2)	9 (52.9)	1 (5.9)	0.32

MAF minor allele frequency

Table 5 Associations of tag SNPs in *ITGAV* with the progression of PBC

SNP	Model	Primary association study											
		Control subjects vs. PBC cohort I				Early stage vs. late stage				Nonjaundice stage vs. jaundice stage			
		OR	95% CI		*P* value	OR	95% CI		*P* value	OR	95% CI		*P* value
			Low	High			Low	High			Low	High	
rs3911238	Allele model	0.99	0.77	1.28	0.928	1.09	0.73	1.61	0.676	2.55	1.27	5.13	0.007
	Recessive model	1.06	0.56	2.01	0.862	1.84	0.72	4.66	0.196	5.31	1.55	18.14	0.003
	Dominant model	0.97	0.70	1.33	0.841	0.97	0.59	1.60	0.911	2.79	0.96	8.12	0.051
rs12611439	Allele model	1.16	0.86	1.56	0.339	1.35	0.85	2.13	0.204	0.88	0.33	2.32	0.791
	Recessive model	0.95	0.38	2.37	0.908	1.79	0.49	6.52	0.369	–	–	–	0.438
	Dominant model	1.22	0.87	1.72	0.252	1.35	0.79	2.31	0.268	1.00	0.34	2.92	0.997
rs1992898	Allele model	0.97	0.69	1.36	0.874	1.13	0.67	1.88	0.647	1.79	0.75	4.25	0.184
	Recessive model	0.42	0.08	2.17	0.285	0.65	0.07	5.94	0.705	4.50	0.48	42.63	0.152
	Dominant model	1.02	0.70	1.47	0.928	1.20	0.68	2.11	0.536	1.73	0.62	4.85	0.292
rs10174098	Allele model	0.90	0.68	1.19	0.453	1.05	0.68	1.61	0.832	2.13	1.03	4.43	0.039
	Recessive model	0.69	0.28	1.72	0.430	1.94	0.60	6.28	0.263	6.74	1.64	27.67	0.003
	Dominant model	0.91	0.65	1.26	0.554	0.95	0.57	1.59	0.848	1.86	0.70	4.97	0.209
rs4667107	Allele model	1.03	0.82	1.29	0.791	0.93	0.65	1.32	0.685	1.56	0.77	3.15	0.210
	Recessive model	1.13	0.77	1.67	0.521	1.11	0.61	2.04	0.726	2.15	0.77	6.06	0.138
	Dominant model	0.97	0.67	1.40	0.855	0.75	0.43	1.32	0.317	1.53	0.43	5.47	0.512
rs3768785	Allele model	1.12	0.87	1.46	0.385	0.99	0.65	1.49	0.956	2.02	0.99	4.14	0.051
	Recessive model	1.32	0.62	2.78	0.473	1.18	0.35	3.94	0.788	3.41	0.69	16.76	0.110
	Dominant model	1.13	0.82	1.56	0.445	0.96	0.58	1.58	0.860	2.42	0.87	6.71	0.082
rs1448427	Allele model	1.13	0.85	1.49	0.412	0.99	0.63	1.56	0.966	2.16	1.02	4.56	0.040
	Recessive model	0.73	0.27	1.95	0.531	1.13	0.29	4.49	0.858	1.97	0.23	16.48	0.526
	Dominant model	1.22	0.88	1.70	0.240	0.97	0.57	1.64	0.908	2.87	1.06	7.78	0.031

CI confidence interval, *OR* odds ratio

SNP Alyze 7.0 standard software package. Although significant associations between *ITGAV* haplotype and the jaundice-type progression of PBC were observed, these associations were completely dependent on the rs1448427 SNP in both the primary association and secondary replication studies (data not shown).

Discussion

This study is the first to report that *ITGAV* polymorphisms are associated with the severe (jaundice-stage) progression of PBC in Japanese patients, but not with susceptibility to the onset of PBC. The primary association study indicated that three tag SNPs (rs3911238, rs10174098, and rs1448427) in *ITGAV* were associated with the jaundice-type progression of PBC. One of these associations was reproducible in the secondary replication study for validation. In particular, in the PBC patients possessing the minor G allele of the rs1448427 SNP in *ITGAV* in the minor allele dominant model, an approximately 2.9-fold increase in susceptibility to the jaundice-type progression was shown in the primary association study and a 2.1-fold increase in susceptibility to requiring liver transplantation was shown in the secondary replication study, although such PBC patients accounted for only approximately 59% (10/17 = 58.8% in the primary association study, Table 5) and 51% (18/35 = 51.4% in the secondary replication study, Table 6) of the genetic variance observed in PBC. These findings suggest that *ITGAV* is a genetic determinant for predisposition to the jaundice-type progression of PBC in Japanese patients.

Although the reason is not clear why the two SNPs rs3911238 and rs10174098 in *ITGAV* showed no significant association with severe progression of PBC in the secondary replication study, this finding may be attributable to the difference in treatment during the pre- and post-ursodeoxycholic acid (UDCA) eras between clinical stage III patients in cohort I and those who underwent liver transplantation in cohort II, or to a strong linkage disequilibrium across the *ITGAV* region including these two SNPs (Fig. 1). Thus, it remains to be confirmed whether these associations are reproducible in a larger number of Japanese PBC patients with the jaundice-type progression

Table 6 Comparison of distributions of tag SNPs in *ITGAV* between PBC patients in nonjaundice stage in PBC cohort I and PBC patients requiring liver transplantation in PBC cohort II

SNP	Subject	Major/major (%)	Major/minor (%)	Minor/minor (%)	MAF	Model	Replication study			
							Nonjaundice stage vs. liver transplantation			
							OR	95% CI		*P* value
								Low	High	
rs3911238	Nonjaundice-stage (PBC cohort I)	157 (53.8)	119 (40.8)	16 (5.5)	0.26	Allele model	1.59	0.94	2.69	0.079
G>C	Liver transplantation (PBC cohort II)	14 (40.0)	17 (48.6)	4 (11.4)	0.36	Recessive model	2.23	0.70	7.08	0.165
						Dominant model	1.74	0.85	3.56	0.123
rs10174098	Nonjaundice-stage (PBC cohort I)	182 (62.3)	101 (34.6)	9 (3.1)	0.20	Allele model	0.98	0.53	1.81	0.941
A>G	Liver transplantation (PBC cohort II)	22 (62.9)	12 (34.3)	1 (2.9)	0.20	Recessive model	0.92	0.11	7.53	0.942
						Dominant model	0.98	0.47	2.02	0.951
rs1448427	Nonjaundice-stage (PBC cohort I)	195 (66.8)	88 (30.1)	9 (3.1)	0.18	Allele model	1.80	1.03	3.16	0.037
A>G	Liver transplantation (PBC cohort II)	17 (48.6)	16 (45.7)	2 (5.7)	0.29	Recessive model	1.91	0.39	9.20	0.414
						Dominant model	2.13	1.05	4.31	0.033

CI confidence interval, *OR* odds ratio

and those who underwent liver transplantation, as well as in other ethnic populations.

Integrin $\alpha V\beta 6$ is highly expressed in the proliferative epithelial cells of the bile duct in the livers of rodents and humans during fibrosis, including PBC [24, 25, 30]. In rodent models of biliary fibrosis, inhibition of integrin $\alpha V\beta 6$ reduced the levels of activated TGF-$\beta 1$ and fibrogenic transcripts and induced fibrolytic transcripts, resulting in the retardation of biliary fibrosis [24]. Therefore, it is possible to speculate that integrin $\alpha V\beta 6$ may play a crucial role in the progression of liver fibrosis, a key component of the pathogenesis of PBC. In this context, it would be interesting to investigate the role of *ITGAV* SNP rs1448427 in the progression of fibrosis in other liver diseases, such as hepatitis C virus (HCV) and HBV infection and alcoholic liver diseases, in patients who underwent liver transplantation.

Another possible mechanism for the contribution of integrin αV (*ITGAV*) to the severe progression of PBC is via the epimorphin signaling pathway. Epimorphin is one of the ligands of the integrin $\alpha V\beta 1$ receptor located on the cellular membrane [31] and is expressed in hepatic stellate cells and myofibroblasts during liver injury in mice [32, 33]. After binding to its receptor, epimorphin signals regulate normal bile duct formation by mediating mitosis orientation [34]. Because integrin αV, coded by *ITGAV*, is a component of the integrin $\alpha V\beta 1$ receptor, the minor G allele at rs1448427 SNP in *ITGAV* in PBC patients may affect the expression of integrin $\alpha V\beta 1$ receptor and its affinity to epimorphin, leading to diminution of the function of integrin αV, as well as the diminution of epimorphin-integrin $\alpha V\beta 1$ receptor signaling. Therefore, accumulation of the diminution of epimorphin-integrin $\alpha V\beta 1$ receptor signaling caused by *ITGAV* polymorphisms may lead to the reduction of bile duct formation and an increase in abnormal bile duct regeneration, resulting in the severe progression of PBC with premature ductopenia.

We propose the following two modes for the clinical progression of PBC: one is the typical slow progression, similar to that of hepatitis, which leads to cirrhosis over a period of 10–20 years; the other is the very rapid progression, which leads to premature ductopenia before the beginning of fibrosis, resulting in severe chronic cholestasis and jaundice in less than 5 years; this occurs in 5–10% of PBC patients [2, 35]. The latter mode of progression occurred in the PBC patients with jaundice-type progression in PBC cohort I, as well as in the PBC patients who underwent liver transplantation in PBC cohort II in our study. The *ITGAV* polymorphisms in this study were associated with the jaundice-type progression, but not with the progression to late-stage, indicating that an alteration of integrin αV due to *ITGAV* polymorphisms may affect the process of premature ductopenia and subsequent jaundice rather than affecting the progression of fibrosis in some PBC patients with jaundice-type progression.

Polymorphisms of *ITGAV* are associated with susceptibility to various diseases; e.g., rs3738919 SNP is associated

with rheumatoid arthritis [36] and rs2290083 SNP and its affiliated haplotype are associated with chronic HBV infection and HBV-infected hepatocellular carcinoma. These two SNPs were not analyzed in the present study because the minor allele frequency of the rs3738919 SNP was less than 0.10 in the Japanese population and therefore did not qualify as a genotyped tag SNP in this study, and the other SNP, rs2290083, was not included in the International HapMap data. In our study, the rs1448427 SNP was associated with the progression to jaundice and this association was independent of the status of anti-gp210 antibodies (data not shown), the strongest risk factor reported so far for jaundice-type progression in PBC [37]. Although these two studies [36, 37], as well as our own, did not investigate alterations of the expression and function of *ITGAV* due to these polymorphisms, it seems that the intronic polymorphisms of rs3738919, rs2290083, and rs1448427 SNPs in *ITGAV* may influence its expression and function by acting as enhancers or silencers.

ITGAV appears to be a genetic determinant for susceptibility to the severe progression of PBC, but not for susceptibility to the onset of PBC. Polymorphisms of *ITGAV* may be useful as new genetic biomarkers not only for predicting the progression and prognosis of PBC in Japanese patients, but also for identifying high-risk Japanese PBC patients, who may require liver transplantation in the future, at the time of the initial diagnosis.

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Conflict of interest None.

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Original Article

Primary biliary cirrhosis – Autoimmune hepatitis overlap syndrome: A rationale for corticosteroids use based on a nation-wide retrospective study in Japan

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Aims: Primary biliary cirrhosis (PBC) and autoimmune hepatitis (AIH) may simultaneously coexist in some patients, designated as PBC-AIH overlap syndrome. Previous studies suggest that combination therapy of ursodeoxycholic acid (UDCA) and corticosteroids may be effective. In the current study, we aimed to describe clinical features of these cases and to propose a rationale for combination treatment in PBC-AIH overlap.

Methods: We enrolled patients with PBC-AIH overlap from eight referral centers for liver diseases in Japan, and clinical, biochemical and immunological features were examined. Liver histology of all patients at diagnosis were analyzed altogether in detail. Eighty-nine and 44 patients with PBC and AIH alone were included and served as controls.

Results: We identified 33 patients with PBC-AIH overlap. The mean follow-up period was 6.1 years. On liver histology, the HA (hepatitis activity) score was significantly higher than the CA (cholangitis activity) score ($P < 0.001$). At the end of the follow-up period, corticosteroids were used in 23 patients (72%), and neither liver-related death nor liver transplantation had been noted. The sensitivity and specificity of the simplified AIH scoring system for prediction of patients who required corticosteroids during clinical course was 92% and 75% in the training set ($n = 17$), and 91% and 80% in the validation set ($n = 16$) of overlap. Only 3% of PBC patients were diagnosed as having indication for corticosteroid use.

Conclusion: In PBC-AIH overlap, AIH-like features are dominant in liver histology. The simplified AIH scoring system could predict patients who needed corticosteroids with a higher specificity.

Key words: corticosteroids, the revised International Autoimmune Hepatitis Group scoring system, the simplified scoring system

Abbreviations: AIH, autoimmune hepatitis; ALP, alkaline phosphatase; AMA, anti-mitochondrial antibodies; CA, cholangitis activity; GGT, gamma-glutamyltranspeptidase; HA, hepatitis activity; PBC, primary biliary cirrhosis; SMA, anti-smooth muscle antibody; UDCA, ursodeoxycholic acid.

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INTRODUCTION

PRIMARY BILIARY CIRRHOSIS (PBC) and autoimmune hepatitis (AIH) are the two main autoimmune liver disorders. PBC is a chronic and progressive cholestatic liver disease, characterized by elevated cholestatic liver enzymes, presence of serum antimitochondrial antibodies (AMA), and pathologically chronic nonsuppurative destructive cholangitis.[1,2] On the other hand, AIH is defined as a chronic and progressive hepatitis, characterized by elevated aminotransferases, hypergammaglobulinemia, detectable serum autoantibodies, and histological findings such as interface hepatitis and portal plasma cell infiltration.[3,4] In general, differential diagnosis of PBC and AIH is not problematic, and is easily performed by biochemical findings, autoantibody profiles and liver histology. However, both clinical features of PBC and AIH may simultaneously or successively exist in some patients, and this relatively rare variant form of autoimmune liver disorders has been designated as PBC-AIH overlap syndrome.[5]

Although the first appearance of PBC-AIH overlap in the literature was back in the 1970s,[6,7] there has been no general agreement on the etiological concept and diagnostic criteria that could distinguish PBC-AIH overlap from various atypical forms of autoimmune liver disease.[8] By definition, simultaneous or successive presence of both PBC and AIH features are required for diagnosis of PBC-AIH overlap. In most cases the diagnosis of PBC features is straightforward, presumably because it usually depends on detection of serum AMA, an effective diagnostic tool for PBC with very high sensitivity and specificity.[2] However, controversies exist in the confirmation of AIH features, which lacks diagnostic markers with acceptable sensitivity and specificity.[9] The revised scoring system proposed by the International Autoimmune Hepatitis Group (IAIHG)[10,11] has served as an international standard for diagnosis of AIH, and several clinical studies describing PBC-AIH overlap used the revised IAIHG scoring system as inclusion criteria.[12,13] However, the revised IAIHG scoring system was not suitable for the purpose of diagnosis of PBC-AIH overlap,[14] because AMA positivity and biliary changes, both of which are essential in PBC, are regarded as negative score, −4 and −3, respectively.[10] In 2008, the IAIHG proposed a new simplified scoring system for AIH to assist early diagnosis,[15] with no negative scores for AMA positivity and biliary changes. A recent study demonstrated the simplified scoring system could be used for diagnosis of PBC-AIH overlap as well.[16] On the other hand, Chazouilleres *et al.* proposed another diagnostic criterion for PBC-AIH overlap, later called the "Paris criteria".[17] The Paris criteria was initially proposed in the clinical study for PBC-AIH overlap, and several following studies have adopted Paris criteria as inclusion criteria of patients.[18–23]

In spite of the diagnostic criteria, it seems that the clinical studies describing treatment and prognosis of PBC-AIH overlap generally agree that prognosis of patients with PBC-AIH overlap could be worse than those with PBC, and combination therapy of ursodeoxycholic acid (UDCA) and immunosuppressive agents such as corticosteroids for overlap patients may be justified,[12,16,18,22] yet there seems to be no agreement on which cases should be treated with immunosuppressive drugs in addition to UDCA. In the current study, we aimed to describe clinical features of PBC-AIH overlap cases retrospectively enrolled in a nation-wide manner, compared to those with PBC and AIH alone, and to propose a rationale for combination treatment with UDCA and corticosteroids in PBC-AIH overlap based on this cohort.

METHODS

Study population and data collection

IN THIS NATION-WIDE retrospective study, we collected currently followed-up patients with PBC-AIH overlap syndrome from eight referral centers for liver diseases in Japan. The diagnosis of PBC-AIH overlap was established by simultaneous presence of clinical, biochemical, serological and histopathological features of both PBC and AIH at presentation. We excluded patients with sequential development of PBC and AIH features in the current study due to two reasons; first, in the current study we mainly paid attention to liver histological findings of PBC-AIH overlap, and it was obvious that liver specimens at presentation from simultaneous forms included enough histological information, both PBC and AIH features. Second, most recent studies regarding PBC-AIH overlaps enrolled only simultaneous forms of PBC-AIH.[18,21,22] Patients were diagnosed as having PBC features if they met at least two of three criteria: (i) chronic elevation of cholestatic liver enzymes, alkaline phosphatase (ALP) and gamma-glutamyltranspeptidase (GGT), for at least 6 months; (ii) presence of serum AMA detected by either indirect immunofluorescence or enzyme linked immunosorbent assay (ELISA) using commercially available kits; and (iii) typical histological findings of

biopsied liver specimens.[1,24] The diagnosis of AIH features was based on the revised scoring system according to the IAIHG,[10,11] in which the allocation of AMA positivity was modified as described below. The patients with other etiologies for chronic liver diseases, including viral hepatitis, excess drinking of alcohol, drug-induced liver diseases, fatty liver, were excluded in this study, even if clinical parameters of the patient met the criteria of PBC and/or AIH. The diagnosis of PBC-AIH overlap syndrome was initially made at each referral center, and finally re-evaluated and confirmed altogether with recalculating the AIH scores with histopathological findings. Liver biopsied specimens of each patient at diagnosis were collected and interpreted altogether by two blinded pathologists independently (Y.N and K.H.). Six categories of histopathological findings, i.e. interface hepatitis, rosette formation, plasma cell infiltration, bile duct injury, bile duct loss and atypical ductular reaction, were mainly analyzed and scored as four grades, from 0 (no finding) to 3 (very strong findings) in each case. Also, grading of necroinflammatory activities (CA; cholangitis activity and HA; hepatitis activity) was performed as previously reported; CA and HA 0 (no activity), 1 (mild activity), 2 (moderate activity) and 3 (marked activity).[25]

In addition, we included patients with PBC and AIH alone in this study. Overall, 89 patients with PBC and 44 patients with AIH, both being currently followed up at the out-patient department of Teikyo University Hospital, were served as controls. The diagnosis of PBC was established using the similar criteria described above: chronic elevation of cholestatic liver enzymes, presence of serum AMA, and typical histological findings.[1,24] The diagnosis of AIH was made using the revised scoring system according to the IAIHG,[10,11] without modification in the allocation of AMA positivity.

Application of the revised IAIHG scoring system for the diagnosis of AIH features

We demonstrated the variables required for the calculation of the revised IAIHG scores in Table 1. The current study was designed as a multi-center collaborative study, and upper normal limits (UNL) of liver enzymes varied with the liver centers. Therefore, we obtained UNL of aspartate aminotransferase (AST), alanine aminotransferase, (ALT), ALP and GGT as well as values of enzymes from each center, and used UNL of AST/ALP for calculation of ALP : AST ratio in the revised IAIHG system, as suggested in the original report.[10] AMA positivity was graded as −4 in the revised IAIHG scoring system, and this allocation for AMA positivity for exclusion of patients with PBC features is rational to the diagnosis of "pure" AIH. However, negative score for AMA positivity seems to be inadequate for diagnosis of PBC-AIH overlap, since patients with PBC-AIH overlap are usually AMA seropositive. In this regard, we did not apply this negative allocation for AMA positivity to diag-

Table 1 The variables required for the revised International Autoimmune Hepatitis Group (IAIHG) scoring system and the number of patients allocated in each item

Clinical parameters	Score	*n* (%)
Female gender	+2	31 (94)
ALP : AST ratio		
<1.5	+2	31 (94)
1.5–3.0	0	2 (6)
>3.0	−2	0
Serum globulin or IgG above normal		
>2.0	+3	8 (24)
1.5–2.0	+2	8 (24)
1.0–1.5	+1	11 (33)
<1.0	0	5 (15)
Missing		1 (3)
ANA, SMA, LKM-1		
>1:80	+3	19 (58)
1:80	+2	6 (18)
1:40	+1	2 (6)
<1:40	0	6 (18)
AMA positivity		
Positive	−4	29 (88)
Negative	0	4 (12)
Hepatitis viral markers		
Positive	−3	0
Negative	+3	33 (100)
Illicit drug use history		
Positive	−4	0
Negative	+1	33 (100)
Average alcoholic intake		
<25 g/day	+2	33 (100)
>60 g/day	−2	0
Histological findings		
Interface hepatitis	+3	32 (97)
Predominant lymphoplasmacytic infiltrate	+1	33 (100)
Rosetting of liver cells	+1	8 (24)
None of the above	−5	0
Biliary changes	−3	27 (82)
Other changes	−3	25 (76)
Other autoimmune disease	+2	9 (27)
Response to therapy		
Complete	+2	33 (100)
Relapse	+3	0

nose AIH features in this study. The –3 allocation for biliary changes was adopted in this study. Both "definite" AIH (>15 at pre-treatment and >17 at post-treatment) and "probable" AIH (10–15 at pre-treatment and 12–17 at post-treatment) are included in this study as having AIH features.

Application of the simplified IAIHG scoring system for the diagnosis of AIH features

On the other hand, the simplified IAIHG scoring system included only four items; autoantibodies (ANA, SMA, LKM, SLA), serum immunoglobulin G (IgG) level, liver histology and absence of viral hepatitis. In liver histology, "typical" AIH is defined as presence of all three findings; interface hepatitis, emperipolesis and hepatic rosette formation, and is regarded as 2 points. "Compatible" AIH is a picture of chronic hepatitis with lymphocytic infiltration without all the features considered typical.[15] However, it seems that emperipolesis is not familiar to clinicians and even pathologists in Japan. Therefore, we adopted the grading of necroinflammatory activities, especially the HA scores, instead of these histological findings. We defined allocations using the HA scores as follows: 0 point for HA 0 and 1, 1 point for HA 2 and 2 points for HA 3. The diagnosis of "definite" AIH and "probable" AIH is established if the score is ≥7 and ≥6 points, respectively,[15] and both were defined as having AIH features in the current study.

Statistical analysis

Continuous variables are presented as means ± standard deviations (SD) if they were normally distributed, or medians if not. Comparison was performed using Student's *t*-test for normal distributed variables, or Mann–Whitney *U*-test for non-normal distributed variables. Dichotomous variables were compared using χ^2 test. All tests were two-tailed and conducted at a 5% level of significance.

RESULTS

Characteristics of the identified patients with PBC-AIH overlap syndrome

WE IDENTIFIED 33 patients with PBC-AIH overlap who fulfilled both PBC and AIH criteria. In eight referral centers, the number of patients with PBC and AIH were 1081 and 597 overall, respectively. Thus, the frequency of PBC-AIH overlap was 3.1% (33/1 081) of PBC and 5.6% (33/597) of AIH. In Table 2, we demonstrated the baseline characteristics of these patients, with those of patients with PBC or AIH alone. Here, we demonstrated minimum values of liver enzymes of the enrolled patients, and indeed some patients diagnosed as having PBC-AIH overlap had normal cholestatic enzyme or normal AST/ALT. However it should be kept in mind that a single patient having PBC features never had normal values in both ALP and GGT; instead all patients with PBC features had either elevated ALP (two patients out of 33) or elevated GGT (2/33), or both elevated (29/33) at presentation. Similarly, all patients with AIH features had either elevated AST (1/33) or elevated ALT (2/33), or both elevated (30/33). In PBC-AIH overlap cases, the mean follow-up period was 6.1 years. Ten patients out of 33 (30%) developed any symptom at presentation: jaundice in five patients, ascites in four, pruritus in four and varices in one. The concomitant autoimmune diseases of the patient were found in nine patients (27%), including Sjögren syndrome in six patients and chronic thyroiditis in three. By comparison with patients with PBC alone, patients with PBC-AIH overlap developed at younger age, and had more symptoms at presentation, higher values of AST, ALT, IgG and IgM, and higher positive rate of ANA/SMA. By comparison with patients with AIH, patients with PBC-AIH overlap had higher values of ALP, GGT and IgM, and higher positive rate of AMA.

Revised IAIHG scoring system

The number of patients who fulfilled each item of the revised IAIHG scoring system was shown in Table 1, and the interpretation of the revised IAIHG aggregate scores was shown in Table 3. We did not apply negative allocation for AMA positivity in the revised IAIHG scoring system for diagnosis of AIH features in this study. As a result, although six patients were diagnosed as "Not AIH" according to the revised IAIHG scoring system due to negative scoring for AMA positivity, all 33 patients were defined as either "probable AIH" or "definite AIH" after dismissal of this negative allocation for AMA positivity (Table 3).

Histopathological findings

We collectively analyzed histopathological findings at diagnosis in each case, and the results are summarized in Table 4 and Figure 1. Among three AIH-like pathological features (interface hepatitis, rosette formation and plasma cell infiltration), the grades of interface hepatitis were 2.0 ± 0.8 (mean ± SD), while those of rosette formation and plasma cell infiltration were