Dendritic Cells in Autoimmune Liver Diseases

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Abstract: Dendritic cells (DC) are professional antigen presenting cells that maintain immune tolerance to self-antigens by controlling the pathogenicity of autoreactive T cells, and a lack of immune tolerance against self-antigens results in autoimmune diseases. Therefore, DCs play an essential role in the induction and/or maintenance of autoimmunity. In the present review, we focus on the role of DCs in the pathogenesis of autoimmune liver diseases. In addition, recent developments in DC-based immunotherapy using regulatory (tolerogenic) DCs in autoimmune diseases will be discussed.

Keywords: Autoimmune liver disease, dendritic cells, tolerance.

INTRODUCTION

Autoimmune responses can arise because the repertoire of both T- and B-cell receptors, which allow the recognition of pathogens, may contain receptors recognizing self-compartments. Ideally, autoreactive lymphocytes are destroyed in the thymus during negative selection and the induction of autoimmunity should thus be controlled. However, a great number of self-reactive lymphocytes escape the thymic negative selection process and form a peripheral pool of potentially autoimmune disease-mediated lymphocytes. On the other hand, self-tissues are routinely destroyed in our body and thus self-antigens (Ag) are available *in situ*. If self-reactive lymphocytes are not completely destroyed in the thymus, autoantigens can activate these lymphocytes, and the features of autoimmunity are begun. To block the activities of these autoreactive lymphocytes and minimize clinically apparent autoimmune diseases, a population of tolerogenic immunocytes (regulatory T cells: Tregs) is present in our body. When these lymphocytes also fail to block the progression of an autoimmune process, then the pathological consequences of autoimmunity manifest themselves. Therefore, it is important to clarify the mechanisms leading to the initial activation of self-reactive lymphocytes that induce and sustain the autoimmune response. The underlying cause of the inability of tissue-derived dendritic cells (DC) to induce immune tolerance to self-Ag remains to be fully elucidated. In addition, another population of DCs, called regulatory (tolerogenic) DCs, might counteract the autoimmune process.

DCs are one of the main players that maintain tolerance at the tissue level, and may have a dominant role in the initiation and perpetuation of autoimmunity and autoimmune diseases [1, 2]. In the present paper, we mainly discuss the role of DCs in the pathogenesis of autoimmune liver diseases.

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DENDRITIC CELLS IN AUTOIMMUNITY

A major source of self-antigens in the steady state is apoptotic cells that arise from normal steady-state cell turnover. Immature DCs are widely distributed in almost all tissues of the body. These cells efficiently internalize apoptotic cells and can present apoptotic cell-derived peptides in MHC molecules to Ag-specific T cells. However, this process does not induce DC maturation, and these cells do not stimulate T cell immunity [3, 4]. Recently, Ohnmacht *et al.* [1] demonstrated that constitutively DC-depleted mice develop spontaneous severe autoimmune diseases, thus indicating that DCs help protect against autoimmunity under normal conditions.

In addition to immature DCs, another population of DCs, regulatory DCs, has been characterized [5, 6]. These cells may also be committed immunocytes that contribute to induction of the tolerogenic state. The precise mechanisms by which induce specific T cell-tolerance to self-Ags are unlikely to be mutually elusive; current evidence suggests that multiple mechanisms, such as the induction of T cell anergy, T cell deletion, and Tregs, may be involved [5-7].

These facts indicate that impaired or improper DC functions may be related to the development of autoimmune diseases. During the development and progression of autoimmune diseases, several factors including the pro-inflammatory milieu and the nature of self-Ags appear to be involved. Various factors determine the induction of immune tolerance and immune responses by DCs. DCs are predicted to be involved induction and progression of autoimmune diseases, because DCs are regulators of immune responses and tolerance, and are capable of modulating different factors related to the induction and progression of autoimmune diseases. In addition, DC is one of the key players of innate immunity and produces various types of cytokines. It is possible that under certain conditions and in genetically susceptible individuals, pro-inflammatory cytokines may be produced by DCs when encountering self-Ags, distorting the immune balance and providing conditions that are favorable for the induction of autoreactive lymphocytes. This event may be induced by either viral infections or drugs, which triggers autoimmunity. Among

the DC subset, myeloid DCs (mDC) represent a promising population to regulate Ag-specific immune regulation. These cells have already been used for development of Ag-specific T cells in clinical trials. In addition, plasmacutoid DCs (pDC) are potent producers of type I interferon (IFN), which is also implicated in various autoimmune disease [8, 9]. Therefore, the production of high levels of proinflammatory cytokines by DCs may disrupt the inflammatory balance of different tissues, and may thus cause the formation of autoreactive T cells which can induce the destruction of these tissues.

The role of DC in the pathogenesis of human autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis has been investigated extensively (reviewed in [10, 11]).

DENDRITIC CELLS IN THE PATHOGENESIS OF AUTOIMMUNE LIVER DISEASES

The exact roles of DCs in the pathogenesis of autoimmune diseases are not well characterized, but accumulating evidence suggest that DC activity may contribute to the development of autoimmune diseases in animal models and in patients with autoimmune disease.

The liver is known to induce tolerance rather than immunity toward Ags, and local hepatic immune regulation is governed by the unique bacterial degradation product-rich hepatic microenvironment, and by liver cells that possess with unique functions [12-14]. Antigen presenting cells, including DCs [14-18], appear to be involved in this process. When the self-Ag tolerogenic property is perturbed, DCs can induce autoreactive T cell immunity, thereby causing tissue destruction in the liver. The nature of DCs in autoimmune liver diseases is summarized in Table 1.

Table 1.   Dendritic Cells in Autoimmune Liver Diseases

		Reference
AIH	Decreased expression of HLA-DR on mDC	[21]
	Decreased expression of HLA-DR and CD123 on pDC	[21]
	Increased expression of mDC	[23]
PBC	Located predominantly in portal area	[26, 27]
	Decreased expression of HLA-DR and CD123 on pDC	[21]
	Decreased allostimulatory capacity	[28]
	Increased production of nitric oxide	[28, 29]
PSC	Induction of α4β7 and CCR9 on T cells by gut-derived DC	[33]

AUTOIMMUNE HEPATITIS (AIH)

AIH is defined as chronic liver disease of unknown etiology, and it is associated with aberrant autoreactivity and a genetic predisposition to this condition [19]. The target antigens on the hepatocyte membrane are unknown, but it is likely that liver membrane-specific activated T cells are important in the development or the progression of these diseases. Recently, the numeral and functional Treg impairments have been identified in patients with AIH, particularly during the active phase [20].

Few studies have examined the role of DCs in AIH pathogenesis. Our group analyzed the phenotypic characteristics of DCs in peripheral blood, and demonstrated that the expression of HLA-DR on mDCs was decreased in AIH patients compared to control subjects. In addition, HLA-DR and CD123 expressions on pDCs were significantly lower in AIH patients than that in controls [21]. Because pDCs are involved in the induction of immunogenic tolerance and T helper (Th) 2 polarization [8, 9, 22], defective surface antigen expression on pDCs may underlie the immune response switch to the Th1 type in AIH patients.

Chen *et al.* [23] reported the expression of CD274 (B7-H1) on mDCs in peripheral blood to significantly increase in patients with AIH compared to the control subjects. However, the number of enrolled patients was small, and no functional assay was performed in either study. Further studies will be necessary to confirm these results.

In a mouse study, DCs were used to establish an animal model of AIH. Tamaki *et al.* [24] described an animal model of AIH using DCs loaded with the well-differentiated Hepa1-6 hepatocellular carcinoma cell line. DC/Hepa1-6-vaccined mice induced cytotoxic activity of spleen and liver T cells to the syngeneic hepatocytes. However, the induction of a significant inflammatory response in the liver required additional IL-12 treatment. These data indicate that in addition to an Ag-DCs encounter, a pro-inflammatory milieu is essential for the induction of autoimmune diseases.

PRIMARY BILIARY CIRRHOSIS (PBC)

PBC is a chronic cholestatic liver disease characterized by the destruction of small and medium-size intrahepatic bile ducts. Although several studies have examined the autoimmune mechanisms for biliary damage in PBC, the underlying cause of the disease remains largely unknown. Autoreactive $CD4^+$ and $CD8^+$ T cells are believed to be involved in the pathogenesis of PBC [25].

DCs have been frequently observed in the portal area in the early stages of PBC, but are less frequent in advanced disease, where they may often be periductually located [26]. Mature DCs expressing CD83 have also been found in the liver tissue of PBC patients [27], suggesting a role for DCs in different states in PBC.

Yamamoto *et al.* [28] demonstrated that the capacity of monocyte-derived DCs (Mo-DC) to stimulate allogeneic T cells was significantly lower compared to control subjects. Increased production of nitric oxide by DC from PBC patients was responsible to this finding. In addition, the levels of NO produced by DCs were decreased in all PBC patients treated with the hypolipidemic drug bezafibrate, which has been reported to improve liver function tests in PBC patients [29]. In addition, the expression levels of HLA-DR and CD123 on pDCs were significantly lower in the PBC patients than that in the control subjects [21].

Patients with PBC are characterized by the presence of antibodies to the pyruvate dehydrogenase complex (PDC) in the sera and PDC-specific T cells in the liver. However, most of the patients did not show a peripheral blood T cell response to PDC when a conventional lymphoproliferative assay was performed using PDC. Kita *et al.* [30]

demonstrated the presence of PDC–E2 specific cytotoxic T cells in the peripheral blood of PBC patients using PDC-E2-pulsed DCs. We have also shown that DCs can be used to detect autoantigen-specific T cells from patients with PBC. PDC-specific T cells were detected using PDC-pulsed Mo-DCs from most of the patients with PBC, even those who did not have detectable levels of anti-PDC antibodies in the sera [31]. These studies provide definitive evidence regarding the existence of autoantigen-specific lymphocytes in PBC patients.

PRIMARY SCLEROSING CHOLANGITIS (PSC)

PSC is a fibrosclerotic disease of the bile ducts, with diffuse structuring of the intrahepatic and extrahepatic biliary tree. The etiology and pathogenesis of PSC still remains poorly understood, but autoimmune mechanisms are believed to contribute to this disease state. The biliary epithelium appears to be a target for immune-mediated injury.

There is a strong association between PSC and inflammatory bowel diseases. Aberrant expression of CCL25 and mucosal addressin cell-adhesion molecule-1 (MAdCAM-1) in the liver of patients with PSC is associated with hepatic infiltration by CC chemokine receptor 9 (CCR9) $^+$ $\alpha 4\beta 7^+$ T cells [32]. Intestinal DCs imprint the gut-homing receptors CCR9 and $\alpha 4\beta 7$ on lymphocytes, which facilitates their tissue-specific homing to the gut [33]. Conversely, liver DCs do not have capacity to imprint these adherent molecules, indicating that CCR9$^+$$\alpha 4\beta 7^+$ T cells in the liver are primed in the gut. These data suggest that therapies targeted to intestinal DCs and T cells might have applications for patients with PSC.

IgG4-RELATED SCLEROSING CHOLANGITIS (IgG4-SC)

IgG4-SC is a recently described biliary disease of unknown etiology that presents with biochemical and cholangiographic features similar to PSC and is often associated with autoimmune pancreatitis (AIP) and other fibrotic conditions. The serum level of IgG4 is elevated and IgG4-positive plasma cells are infiltrated in the bile ducts and liver tissue. In addition, Treg cells are increased in the bile ducts and liver tissue of patients with IgG4-SC [34, 35]. Therefore, the immunopathogenesis of IgG4-SC appears to distinct from that of PBC and PSC.

The nature of DCs in IgG4-SC has not yet been clarified. In patients with AIP, the number of mDCs and pDCs in peripheral blood were not significantly different from those in the control subjects [36].

DC-BASED THERAPY FOR AUTOIMMUNITY

To elucidate the role of DCs in regulating autoimmunity and tolerance, DCs have been developed as an immunotherapeutic tool for the treatment of patients with autoimmune diseases. Over the last decade, many different strategies have been proposed to create DCs in the laboratory that can induce T cell tolerance, and are termed regulatory (or tolerogenic) DC [5, 6, 37]. The prophylactic and therapeutic potential of regulatory DCs has been demonstrated predominantly in animal models.

In general, regulatory DCs are characterized by 1) the low expression of co-stimulatory molecules; 2) low levels of pro-inflammatory cytokines; and 3) high production of immunosuppressive cytokines. Various strategies have been developed to create DCs with stable tolerogenic functions (Table 2) [7, 37, 38].

Table 2. Strategy of the Creation of Regulatory DC for Therapy Against Autoimmunity

Genetic modification	Transduction of immunoregulatory molecules
	Silence of immunostimulatory molecules
Modification by immunosuppressive agents	Treatment of DC *in vitro* with anti-inflammatory cytokines and/or immunosuppresive drugs
Modification of culture condition	Semi-maturation (alternatively-activation)

An important challenge for regulatory DC therapy for the treatment of autoimmune disease is the re-balancing of dysregulated immune responses that have already been ongoing for some time. The patients with autoimmune disease may already have developed this condition many years before the first clinical manifestations of the disease. In animal models, various studies found that regulatory DCs can inhibit the established clinical manifestations of autoimmune disease. Despite the many limitations of animal model for the study of human autoimmune disease, it is becoming clear that regulatory DCs can have beneficial effects of pathogenic autoimmune response under highly inflammatory conditions.

We have previously reported that regulatory DCs, which were produced by culturing mouse bone marrow-derived DCs with IL-10, lipopolysaccharide, and parietal cell antigen, can improve autoimmune gastritis in a mouse model [39]. These cells produced increased levels of IL-10 and, which also increasing the induction of Treg cells *in vivo*. In a murine model of PBC [40], these regulatory DC (PDC was used as auto-Ag) also exhibited a therapeutic effect (unpublished observations). This finding indicates that DC-based immunotherapy using Ag-loaded alternatively-activated DC night be promising for the treatment of autoimmune liver diseases.

These promising results in pre-clinical models have prompted a number of groups to consider the application of regulatory DC therapy to human autoimmune disease. Several groups started phase I clinical trials with regulatory DCs to rheumatoid arthritis and type-I diabetes [37]. However, there remain several important considerations related to the clinical application of this therapy for autoimmune diseases. A significant consideration is the choice and source of auto-Ags. Most of the auto-Ags in human autoimmune diseases, including autoimmune liver disease, are not well characterized. In addition, the choice of DC with stable tolerogenicity, even *in vivo*, is critically important. Furthermore, the optimal route, timing, dose, and frequency of administration have not been clarified. Most importantly, biomarkers of tolerance induction have not been

established. However, it remains very difficult to solve most of these issues using *in vitro* or animal studies. Therefore, it is important to collect data in current clinical trials and to establish the future design of additional studies.

CONCLUSION

Studies of the immune pathogenesis of various autoimmune diseases indicate that DCs may play a dominant role in the initiation and/or maintenance of autoimmunity and autoimmune diseases, although there is currently only preliminary evidence for this in autoimmune liver diseases thus far. The identification of auto-Ag in theses pathological conditions is important to reaffirm the role of DCs in autoimmune diseases. The therapeutic potential of regulatory DCs has been demonstrated in pre-clinical animal models, and the current goal is to translate these findings into clinical applications. It is essential to determine a strategy for DC-based immunotherapy for treating human autoimmune liver diseases in patients.

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CONFLICT OF INTEREST

Declared none.

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③ わが国における非B非C型肝癌の実態
(6) AIHにおける肝癌発生

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Key words：自己免疫性肝炎, 肝細胞癌, 肝硬変

┌─ 要 旨 ─

　自己免疫性肝炎(AIH)に肝細胞癌が合併することはきわめてまれと考えられていたが, 長期観察例の増加に伴い, 肝細胞癌合併例の報告が増加している. 最近の全国集計からみても, AIH全体での肝細胞癌合併率は低いが, 線維化進展例, とくに肝硬変症例はハイリスク群であり, 画像検査を含めた定期的なフォローアップが必要である. 肝細胞癌の発症予防には十分な免疫抑制療法により再燃を抑制することも重要である. AIHの診療にあたっては, 肝細胞癌の合併についても注意を払う必要がある.

はじめに

　自己免疫性肝炎(autoimmune hepatitis；AIH)は, 中年以降の女性に好発し, 通常は慢性, 進行性に肝障害をきたす疾患である[1),2)]. 肝細胞障害の成立の成因は不明であるが, 他の自己免疫疾患の合併, 自己抗体の出現や高γグロブリン血症などから, 免疫寛容システムの破綻による自己免疫機序の関与が想定されている.

　従来, AIHに肝細胞癌を合併することは例外

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的とされていたが, AIHの疾患概念の普及と適切な診断・治療により長期観察例の増加したことに伴って, 近年その報告例は増加している.

　本稿では, AIHにおける肝細胞癌合併について, 最近行われた調査結果を中心に概説する.

I. 本邦の肝細胞癌合併AIHの特徴

この項のポイント
● 肝細胞癌合併例でもAIHの肝障害度が予後に関与すると考えられる.
● AIHの適切な診断と治療が必要である.

　Watanebeら[3)]は, 医学中央雑誌を用いた検索で1990年以降にAIHに合併した肝細胞癌症例38例を解析している. 男性7例, 女性31例であり, 全国調査などに比し相対的に男性が多い. 肝細胞癌発見後の予後は約1年(14±12カ月)と不良であった. 全国原発性肝癌追跡調査報告のデータ[4)]と比較すると, 腫瘍マーカーや腫瘍数, 最大腫瘍径については通常の肝細胞癌と差はない. 治療については, 肝動脈塞栓術(TACE)が選択される割合が多く, 手術や局所療法(ラジオ波焼灼療法；RFA, 経皮的エタノール注入療法；PEIなど)の割合が低い. 死因では, 癌死の報告はなく, 半数が肝不全で死亡していることが, 一般の肝細胞癌と大きく異なってい

る．すなわち，腫瘍側の因子ではなく，肝障害度の因子により十分な治療ができず，予後不良となっていることが推測される．早期にAIHと診断して，適切な治療を行うことが予後改善のために重要である．

Ⅱ．本邦におけるAIHの現状と肝細胞癌の合併：全国アンケート調査

この項のポイント
● AIHの病像は以前と比べ変化してきているため，診断に注意が必要である．
● AIH新規症例での肝細胞癌の合併頻度は約1％である．

　厚生労働省「難治性の肝・胆道疾患に関する調査研究」班(坪内博仁班長)では，2006〜2008年の新規症例を対象にAIHの全国アンケート調査を行った[5]．本調査では全国153施設から回答を得て，1,056例のデータを解析した．その結果，おもな変化としては以下のようなものがあった．①診断時年齢は60歳代に一峰性のピークがあり，平均年齢は59.9歳と高齢化していた．診断時60歳以上の割合は57.7％と半

数以上を占めた．②男女比は男性：女性が1：6で以前の調査より男女差が縮まっていた．③抗核抗体陰性・低力価の症例，血清IgG低値の症例が増加した．④診断時に肝硬変まで至っている症例は6.4％と減少し，急性肝炎の症例は10.9％と増加していた．以上から，従来からいわれているAIHの病像と変化してきていることが考えられる．

　本調査においては，肝細胞癌の合併は14例，全体の1.3％にみられ，肝硬変症例に限ると8.9％にみられていた[5),6)]．平均年齢は69歳と，肝細胞癌非合併例より高かった．また，男女比は1：3.7と肝細胞癌非合併例より男性の比率が多かった．HCV抗体陽性例を除くと，全例が背景肝は肝硬変であった．

Ⅲ．AIH肝硬変における肝細胞癌の合併：肝硬変の成因別実態調査

この項のポイント
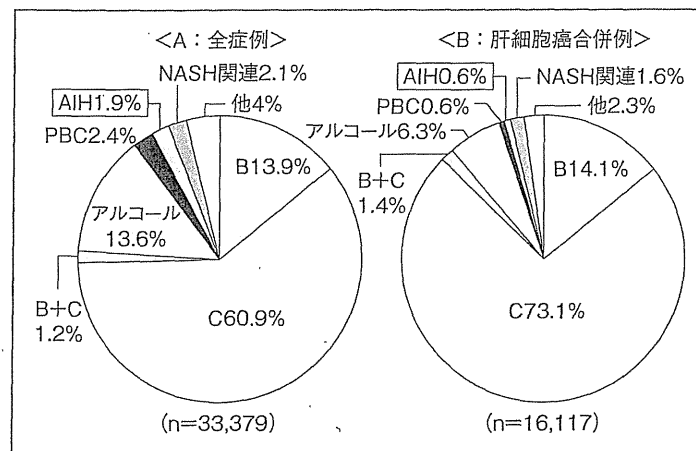● AIHにおいても肝硬変に至ると肝細胞癌の合併頻度が高い．

　2008年の第44回日本肝臓学会総会におい

図　肝硬変の成因別実態調査(1999〜2008年)
NASH：非アルコール性脂肪性肝炎
〔文献8)より一部改変〕

て，主題ポスター「肝硬変の成因別実態調査」が行われた[7),8)]．本調査では1999〜2008年の肝硬変症例を対象として，全国58施設より集積した33,379例について解析した（図）．肝硬変の成因のうち，AIHは肝硬変全体の1.9%（631例），非B非C型肝硬変の7.9%を占めていた．女性に限ると，AIHは肝硬変全体の4.3%，非B非C型肝硬変の18.7%を占めていた．非B非C型肝硬変に占めるAIHの割合は，2011年の第15回日本肝臓学会大会で行われた「非B非C型肝硬変の実態調査」とほぼ同等であった．

次に，肝細胞癌の有無別に肝硬変の成因を解析したところ，肝細胞癌合併があった肝硬変16,117例のうち，AIHは97例（0.6%）ときわめて少なかった（図）．しかし，AIH肝硬変631例のみに着目すると，肝細胞癌の合併はそのうち15.4%にみられている．したがって，肝硬変はAIHにおいても肝細胞癌合併のリスクであると考えられる．

IV．AIHに合併した肝細胞癌の現状：第47回日本肝癌研究会ワークショップ

この項のポイント
- 専門施設におけるAIHでの肝細胞癌の合併頻度は約5%である．
- 肝細胞癌を合併したAIHの背景肝では肝硬変が約8割を占める．

2011年に行われた第47回日本肝癌研究会では，ワークショップ「自己免疫性肝炎（肝硬変）からの肝細胞癌」（担当：東京慈恵会医科大学 銭谷幹男教授，福島県立医科大学 大平弘正教授）が行われた．その際に，全国の専門施設に対してアンケート調査が行われている．

AIH症例に占める肝細胞癌合併は4,869例中250例（5.1%）にみられた．二次調査を行っ

た142例の肝細胞癌合併例の解析では，男女比は男性：女性が1：5.1，平均年齢は69歳であった．肝硬変の合併は78.2%にみられていた．ステロイド剤の使用は55.8%と低く，免疫抑制剤は7.2%で使用されていた．AIH診断後の肝細胞癌合併までの期間は7.8年（0〜29年）であり，生存率は54.8%であった．

診断時の臨床検査所見では，血小板数は13.3万（2.6〜60.5万）と低下しており，肝障害度はA 62%，B 30%，C 8%であった．腫瘍数は単発がもっとも多く，61%を占めている．腫瘍最大径は4.6 cm（0.9〜24.7 cm）だった．病期stageとしては，I 22%，II 45%，III 25%，IV 8%となっていた．治療法としては，手術，局所療法，塞栓療法がそれぞれ1/3程度ずつ行われていた．肝細胞癌発見後の予後は3.2年（0〜17年）であった．これらのデータについては，全国原発性肝癌追跡調査報告[4)]と比べても大きな差はない．前述のWatanabeらのレビュー[3)]に比して手術と局所療法の割合が多くなっている．

二つの結果を直接比較することは難しいが，専門施設においてはAIHにおける肝細胞癌合併に対する認識ができてきており，早期発見とRFAをはじめとした肝細胞癌の治療法の改善に伴って予後が改善してきている可能性がある．

V．AIHにおける肝細胞癌合併の頻度とリスク因子

この項のポイント
- 肝硬変は肝細胞癌合併のリスクである．
- 十分な免疫抑制療法により肝炎を抑制することも重要である．

2000年以降のAIHにおける肝細胞癌の合併頻度に関する報告を（表1）にまとめた[9)〜14)]．肝線維化進展例，とくに肝硬変がAIHにおける肝細胞癌合併の危険因子であることは，おおむ

表1　自己免疫性肝炎における肝細胞癌の合併頻度

文献	国	報告年	平均観察期間	全症例における頻度	(AIH 診断時)肝硬変における頻度
9)	USA	2000	8 年	1/212(0.5%)	1/88(1.1%)
10)	Japan	2006	8 年	3/69(4.3%)	3/29[*1](10.3%)
11)	USA	2008	11.2 年	9/227(4.0%)	4/53(7.5%) [*2]
12)	UK	2008	11 年	15/243(6.2%)	11/118(9.3%) [*3]
13)	Germany	2009	4.8 年	0/278	0/22[*4]
14)	USA	2011	6.3 年	6/322(1.9%)	6/50(12.0%)

[*1]：F3/F4 症例
[*2]：他の 5 例は全例経過中に肝硬変へ進展した症例
[*3]：他の 4 例は全例経過中に肝硬変へ進展した症例
[*4]：経過中に肝硬変へ進展した症例 68 例を含めても肝細胞癌合併なし.
　　　ただし，同時期に肝細胞癌合併 AIH 3 例紹介あり(全例肝硬変).

表2　自己免疫性肝炎における肝細胞癌合併のおもな危険因子

	文　献
・男性	11)
・輸血歴あり	11)
・血小板減少	11)，14)
・(診断時)黄疸なし	12)
・低アルブミン血症	14)
・腹水	11)
・門脈圧亢進症(食道静脈瘤など)	11)，14)
・3 年以上の治療期間	11)
・治療効果が不十分(経過中 ALT 高値)	10)
・ステロイド治療中の肝機能検査の増悪	11)
・肝硬変で長期経過	9)，10)，11)，12)，14)，15)

ね一致している．それ以外の因子についても報告されているが，一定の見解に至っていないものも多い(表2).

　AIH では標準治療としてステロイドあるいはステロイド＋アザチオプリンといった免疫抑制療法が行われる．ステロイド長期投与による免疫抑制が発癌と関与する可能性も指摘されていたが，肝細胞癌合併例では経過中の血清ALT 値が高くコントロールが不十分である[10),11)]ことから，十分な免疫抑制療法により肝炎を制御して，再燃を極力抑制することが重要と考えられている．また，肝炎ウイルスの関与については以前より指摘されている．本邦ではHBV，HCV 感染者が多く，とくに HBV 既往感染者は多い．したがって，肝炎ウイルスの関与を完全に否定するためには，血中・肝組織中のHBV-DNA，HCV-RNA の検索が必要となる．ステロイド投与に伴う肥満や耐糖能異常が肝細胞癌合併の危険因子との報告もあり，今後検討が必要である．

おわりに

　AIH における肝細胞癌合併について概説した．AIH においても線維化進展例については，肝細胞癌を合併する頻度が高いので，スクリーニング検査を定期的に行う必要がある．

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Summary

Hepatocellular Carcinoma in Autoimmune Hepatitis

Masanori Abe* and Morikazu Onji**

　Hepatocellular carcinoma (HCC) has traditionally been considered a rare complication in patients with autoimmune hepatitis (AIH). The number of reported cases has however increased recently. The prevalence of HCC in patients with AIH was low in a recent nationwide survey. However, advanced fibrosis stage, particularly liver cirrhosis, is a major risk factor for the development of HCC. A focused surveillance strategy, based on imaging studies, is recommended for these patients. In addition, sustaining remission by implementing adequate immunosuppressive therapy may be important for prevention of the development of HCC in patients with AIH. Careful attention should be paid to the potential development of HCC in patients with AIH.

Key words： autoimmune hepatitis, hepatocellular carcinoma, liver cirrhosis

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原発性胆汁性肝硬変
Primary biliary cirrhosis

阿部雅則，恩地森一　Masanori Abe, Morikazu Onji

疫学

わが国における年間推定発生患者数は約500人であり，推定患者総数は約5万〜6万人（2008年度）である．好発年齢は50〜60歳代であり，男女比は1：7で女性に多い．近年は診断時年齢が高齢化する傾向にある．

症状

原発性胆汁性肝硬変（primary biliary cirrhosis；PBC）は臨床上，症候性PBCと無症候性PBCに分類される．症候性PBCは特定疾患治療研究事業の対象となっている．多くの症例（70〜80％）は無症候の時期に診断される．進行すると種々の症候が生じる．皮膚瘙痒感は多くのPBC患者で最初に現れる本症の特徴的な症状である．さらに進行すると黄疸や肝不全症状（腹水，肝性脳症など）を呈する．他の肝疾患と異なり，食道・胃静脈瘤などの門脈圧亢進症状は肝硬変に至っていない比較的早期にもみられる．また，従来ではまれとされていた肝細胞がんの合併例も増加してきている．

診断

1．病歴

皮膚瘙痒感は多くのPBC患者で最初にみられる症状であるが，現在では無症状で健康診断あるいは他疾患での加療中に肝機能検査異常を指摘されて医療機関を受診することが多い．ほかの自己免疫疾患（シェーグレン症候群，関節リウマチ，慢性甲状腺炎など）に肝機能検査異常がみられた場合には，本症も鑑別に挙げる必要がある．

2．検査

肝機能検査において胆道系酵素（ALP, γ-GTP, LAP）の上昇が肝逸脱酵素（AST, ALT）の上昇に比して顕著である場合に，本症の可能性を考える．本症が疑われた場合にはまず抗ミトコンドリア抗体（antimitochondrial antibody；AMA）あるいはM2抗体を測定する．これらに加え，IgM上昇，コレステロール値の上昇があればPBCを強く疑う．他に自己抗体として，抗核抗体，抗セントロメア抗体が陽性のことがある．

確定診断のために腹腔鏡および肝生検を行う．PBCに特徴的な組織所見は慢性非化膿性破壊性胆管炎（chronic non-suppurative destructive cholangitis；CNSDC）であり，それを連続切片で確認できれば診断は確定する（表1）．厚生労働省「難治性の肝・胆道疾患に関する調査研究」班では，組織学的病期分類を提唱している．

3．鑑別診断

肝炎ウイルスの関与やアルコール性肝障害，非アルコール性脂肪性肝疾患，薬物性肝障害，閉塞性黄疸を除外する．慢性胆汁うっ滞性肝疾患として，原発性硬化性胆管炎や慢性薬物性肝内胆汁うっ滞を鑑別する．血液検査以外にCT，MRCP，ERCPなどの画像検査による鑑別が必要となる．また，自己免疫性肝炎でも約10％はAMA陽性であり，病理組織学的検索が鑑別に有用である．

表1 ● 原発性胆汁性肝硬変の診断基準（平成22年度）

概念
原発性胆汁性肝硬変は，病因・病態に自己免疫学的機序が想定される慢性進行性の胆汁うっ滞性肝疾患である．中高年女性に好発し，皮膚瘙痒感で初発することが多い．黄疸は出現後，消退することなく漸増することが多く，門脈圧亢進症状が高頻度に出現する．PBCは臨床上，症候性（symptomatic）PBC（sPBC）と無症候性（asymptomatic）PBC（aPBC）に分類され，皮膚瘙痒感，黄疸，食道胃静脈瘤，腹水，肝性脳症など肝障害に基づく自他覚症状を有する場合は，sPBCと呼ぶ．これらの症状を欠く場合はaPBCと呼び，無症候のまま数年以上を経過する場合がある．sPBCのうち2mg/dL以上の高ビリルビン値を呈するものをs2PBCと呼び，それ未満をs1PBCと呼ぶ．

1．血液・生化学検査所見
症候性，無症候性を問わず，血清胆道系酵素（ALP，γ-GTP）の上昇を認め，抗ミトコンドリア抗体が約90%の症例で陽性である．また，IgMの上昇を認めることが多い．

2．組織学的所見
肝組織では，肝内小型胆管（小葉間胆管ないし隔壁胆管）に慢性非化膿性破壊性胆管炎を認める．病期の進行に伴い胆管消失，線維化を生じ，胆汁性肝硬変へと進展し，肝細胞がんを伴うこともある．

3．合併症
慢性胆汁うっ滞に伴い，骨粗鬆症，高脂血症が高率に出現し，高脂血症が持続する場合に皮膚黄色腫を伴うことがある．シェーグレン症候群，関節リウマチ，慢性甲状腺炎などの自己免疫性疾患を合併することがある．

4．鑑別診断
自己免疫性肝炎，原発性硬化性胆管炎，慢性薬物性肝内胆汁うっ滞，成人肝内胆管減少症など．

診断
次のいずれかの1つに該当するものをPBCと診断する． 1）組織学的にCNSDCを認め，検査所見がPBCとして矛盾しないもの． 2）AMAが陽性で，組織学的にはCNSDCの所見を認めないが，PBCに矛盾しない（compatible）組織像を示すもの． 3）組織学的検索の機会はないが，AMAが陽性で，しかも臨床像および経過からPBCと考えられるもの．

[平成22年度 厚生労働省難治性克服研究対策事業（難治性の肝・胆道疾患に関する調査研究班），原発性胆汁性肝硬変（PBC）のガイドライン．2011より引用]

治療

1．薬物治療

a．ウルソデオキシコール酸

例） ウルソ®（100mg）：6錠，分3，毎食後．

　PBCの治療としてウルソデオキシコール酸（ursodeoxycholic acid；UDCA）の投与が行われる．UDCAは臨床検査の改善のみならず予後改善効果も有することが証明されている．1日600mgの投与が標準とされ，効果不十分の場合には900mgまで増量できる．
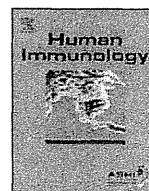
b．ベザフィブレート

例） ベザトール®（200mg）：2錠，分2，朝・夕．

　UDCAで効果不十分の場合には，ベザフィブレートの併用が試みられている（ただし，PBCに対するベザフィブレート投与は保険収載されていないので，注意が必要である）．ベザフィブレートとUDCAとは作用機序が異なることから，併用投与が望ましい．

c．症候・合併症への対策

　皮膚瘙痒感に対しては抗ヒスタミン薬，コレスチラミン，コレスチミドなどが用いられる．また，脂溶性ビタミンの吸収障害に加え，本疾患が中高年以降の女性に多いことから骨粗鬆症の合併にも注意する必要があり，骨粗鬆症が疑われる症例ではビスホスホネート製剤などで治療を開始する．

2．肝移植

　血清総ビリルビンが5mg/dL以上の症例では予後はきわめて悪いため，肝移植も考慮に入れて専門医に相談すべきである．移植適応の決定にはMayo Clinicの式，日本肝移植研究会モデルの式などが重要である．また，末期肝不全の重症度の評価にはMELD（model for end-stage liver disease）スコアが用いられる．これらを参考にしながら，脳死肝移植の登録や生体肝移植の準備を行う必要がある．肝移植後の予後は5年生存率70%以上と比較的良好である．

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Association analysis of toll-like receptor 4 polymorphisms in Japanese primary biliary cirrhosis

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ABSTRACT

Primary biliary cirrhosis (PBC) is characterized by portal inflammation and immune-mediated destruction of intrahepatic bile ducts that often result in liver failure. Toll-like receptor (TLR) 4 recognizes lipopolysaccharides of Gram-negative bacteria. Infectious agents have been suspected to play a crucial role in PBC pathogenesis since TLR4 expression was found in bile duct epithelial cells and periportal hepatocytes in liver tissues of PBC. To assess the potential contribution of TLR4 SNPs to the development of this disease, we genotyped five SNPs in TLR4 in 261 PBC patients and 359 controls using a TaqMan assay. No significant positive associations with either PBC susceptibility or progression were uncovered. These results indicate that TLR4 polymorphisms do not play a prominent role in the development of PBC in Japanese patients.
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1. Introduction

Primary biliary cirrhosis (PBC) is an autoimmune liver disease characterized by portal inflammation and immune-mediated destruction of intrahepatic bile ducts that often result in cirrhosis and liver failure [1]. The cause of PBC remains poorly understood [2], although population and family studies suggest that genetic factors contribute to disease susceptibility and severity [3]. Significant associations of genetic factors, including HLA alleles [4–6], cytotoxic T-lymphocyte antigen 4 [7–10], and other loci [11] have been reported for PBC. Only HLA has consistently been associated with PBC among these susceptibility genes. Specifically, the DRB1*08 family of alleles has been the most frequently described determinant for this disease [4–6].

Toll-like receptors (TLRs) are a class of evolutionarily conserved pathogen recognition receptors that play an important role in innate identification of foreign material [12]. Activation of TLRs in-

duces both innate and adaptive immune reactions against invading pathogens. TLR4 is a receptor for bacterial lipopolysaccharide (LPS) which selectively binds the lipid A portion of LPS. It was also found to be expressed in bile duct epithelial cells and periportal hepatocytes in PBC patient liver tissues [13,14]. Since several bacterial products were detected in sera or liver tissues of PBC patients [15–17], infectious agents might play a crucial role in disease pathogenesis [18]. TLR4 single nucleotide polymorphisms (SNPs) have been reported to be associated with genetic susceptibility to autoimmune diseases [19–22], but these genes have not been examined with respect to PBC. As such, we hypothesized that TLR4 SNPs may be associated with PBC in the Japanese population and examined eight SNPs for associations with susceptibility and progression in Japanese patients.

2. Subjects and methods

2.1. Study subjects

Between January 2005 and December 2011, a total of 261 patients with PBC (234 women, median age: 58 years, range: 27–86 years) and 359 healthy subjects (319 women) participated in this study. All control subjects had indicated the absence of major illness on a standard questionnaire. Racial backgrounds were all

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Japanese. The diagnosis of PBC in all patients was based on criteria from the American Association for the Study of Liver Diseases [23]. Serum anti-mitochondrial antibody-M2 was determined by ELISA, where a >7.0 index was considered to be positive, as previously reported [24]. All patients were negative for hepatitis B surface antigen and antibodies to hepatitis B core antigen, hepatitis C virus, and human immunodeficiency virus. Patients were grouped into two stages of PBC based on their most recent follow-up: early stage patients were histologically classified as Scheuer stage I or II [25] or of unknown histological stage without liver cirrhosis, and late stage patients were histologically Scheuer stage III or IV or clinically diagnosed with liver cirrhosis or hepatic failure [10]. Liver cirrhosis was diagnosed by histological examination and/or characteristic clinical signs of advanced liver disease [26]. Patients with late stage disease or cirrhosis were 53 (20%) and 44 (17%), respectively. All subjects and controls provided written informed consent for testing of DNA samples. This study was approved by the institutional ethics committee.

2.2. Genotypoing of TLR4 SNPs

Genomic DNA was isolated from whole blood extracts for all patients and controls using QuickGene-800 (FUJIFILM, Tokyo, Japan) and adjusted to 10–15 ng/μl. TLR4 is composed of four exons and has four transcript isoforms. We evaluated five SNPs (rs10759930, rs2149356, rs11536889, rs7037117, and rs7045953) which were localized within the exons and introns of the TLR4 gene. SNPs were selected from among previous reports [27,28] and had minor allele frequencies of >5%. SNP spans were approximately 1–5 kb and included 5 kb of the predicted 5'-untranslated region and 6 kb of the predicted 3'-untranslated region of the TLR4 gene. Genotyping of all SNPs was performed with a TaqMan 5' exonuclease assay using primers supplied by ABI (Applied Biosystems, Foster City, CA, USA). The probe fluorescence signals were detected with a TaqMan Assay for Real-Time PCR (7500 Real Time PCR System, Applied Biosystems) according to the manufacturer's instructions.

HLA typing was carried out using a Luminex multi-analysis profiling system with a LAB type® SSO OneLambda typing kit One (Lamda, Ganoga Park, CA), which is based on polymerase chain reaction sequence-specific oligonucleotide probes. HLA genotypes were determined by sequence-based typing [6].

2.3. Statistical analysis

The Hardy–Weinberg equilibrium test was performed for each SNP between control and patient groups. Pairwise linkage disequilibrium pattern, haplotype block structure, and haplotype frequency analysis were assessed for all SNPs by the block definition by Gabriel et al. [29], and were based on a 95% confidence interval (CI) of D' with Haploview version 4.2 software [30]. We plotted r^2 values. The significance of allele distribution between patients with PBC and healthy subjects was evaluated using the χ^2 test for 2 × 2 comparisons. A P value of <0.05 was considered statistically significant. Statistical analysis was performed using SPSS software (version 18.0J; SPSS, Chicago, IL).

3. Results

A total of five SNPs in the TLR4 gene were genotyped in 261 patients with PBC and 359 healthy subjects. The observed genotype frequencies for patients and controls were all in Hardy-Weinberg equilibrium, and the minor allele frequencies of all SNPs were >5%. All five SNPs were located in one haplotype block, and the magnitude of linkage disequilibrium between each SNP was high (Fig. 1). Analysis of allelic frequencies revealed no significant differences between PBC and controls for TLR4 SNPs (Table 1).

The haplotype frequency of the five SNPs was estimated with the expectation-maximization algorithm. Six unique SNP haplotypes were identified, and five of them had frequencies of >5% (Table 2). Association analysis using haplotypes calculated by expectation-maximization algorithms showed that none of them were associated with either susceptibility or resistance to PBC.

Since we previously reported that the HLA DRB1*08:03-DQ*06:01 haplotype was associated with PBC in Japan, we further investigated the genetic association between this haplotype and the TLR4 SNPs. Analysis of allelic frequencies revealed no significant differences between the presence and absence of the HLA DRB1*08:03-DQ*06:01 haplotype and these SNPs (data not shown).

Next, we examined associations between the five TLR4 SNPs and disease progression. There were neither significant allelic associations nor significant haplotype associations found in comparisons of early and late stage groups with regard to liver cirrhosis or non-cirrhosis (data not shown).
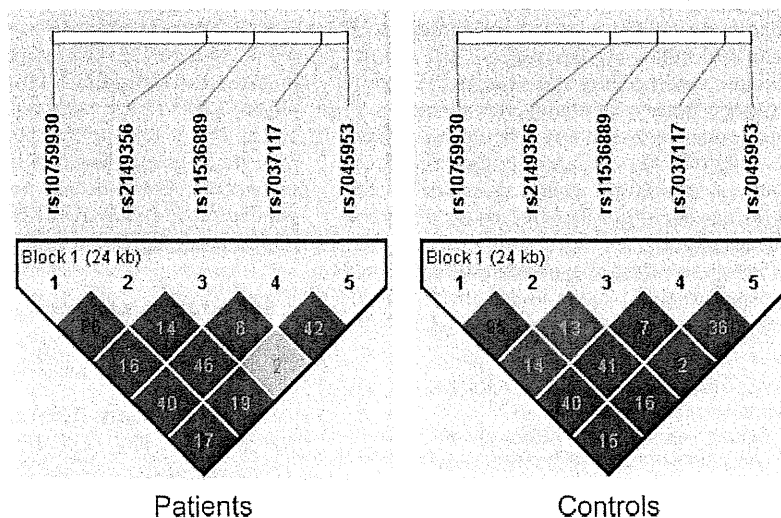

Fig. 1. Linkage disequilibrium plot of five SNPs of the TLR4 gene in 261 patients with primary biliary cirrhosis and 359 healthy controls. Values of r^2 corresponding to each SNP pair are expressed as a percentage and shown within the respective squares. Higher D' values are indicated by a brighter red color. The five SNPs constitute a haplotype block spanning 24 kb of the TLR4 gene.

Table 1
Allele frequencies of SNPs in the TLR4 gene of PBC patients and healthy subjects.

SNP no.	dbSNP	Position (bp)	Minor allele	MAF in PBC	MAF in controls	P value	OR	95%CI
1	rs10759930	119,501,442	C	0.366	0.350	0.55	1.07	0.85-1.36
2	rs2149356	119,514,020	T	0.337	0.345	0.76	0.96	0.76-1.22
3	rs11536889	119,517,952	C	0.226	0.253	0.27	0.86	0.66-1.12
4	rs7037117	119,523,484	G	0.190	0.187	0.89	1.02	0.76-1.36
5	rs7045953	119,525,616	G	0.090	0.078	0.45	1.17	0.78-1.75

CI, confidence interval; MAF, minor allele frequency; OR, odds ratio; PBC, primary biliary cirrhosis; TLR4; toll-like receptor 4; SNP, single nucleotide polymorphism, P values were calculated with a χ^2-test 2 × 2 contingency table (df = 1).

Table 2
TLR4 haplotypes in PBC patients and healthy subjects.

Haplotype	SNPs					Haplotype frequencies		P value
	1	2	3	4	5	PBC	Controls	
1	T	G	G	A	A	0.409	0.402	0.78
2	T	G	C	A	A	0.225	0.243	0.46
3	C	T	G	A	A	0.144	0.151	0.73
4	C	T	G	G	A	0.098	0.106	0.65
5	C	T	G	G	G	0.090	0.078	0.44

PBC, primary biliary cirrhosis; TLR4, toll-like receptor 4.

4. Discussion

In the present study, we investigated the possibility of an association between TLR4 SNPs and PBC in Japan. We found no associations for any of the SNPs analyzed. Several infectious organisms have been proposed as potential causes of PBC [15–17], and TLR4, a specific receptor for LPS, was found in bile duct epithelial cells and periportal hepatocytes in liver tissues of PBC patients [13,14]. Ballot et al. [31] reported that 64% of PBC sera was positive for IgM antibodies against lipid A, an immunogenic and toxic component of LPS. This finding was specific for the disease and correlated with more florid histological lesions. Moreover, Mao et al. reported that PBC patients were hyper-responsive to LPS stimulation, and suggested that aberrant signaling through TLR4 may precipitate disease onset [32]. Therefore, it has been hypothesized that TLR4 and its ligands would be implicated in the development of PBC, but the results of our SNP analysis indicated otherwise. Until now, no reports have been published regarding an association between PBC and TLR4 SNPs in other ethnicities. Furthermore, genome-wide association studies have shown no significant associations between TLR4 SNPs and PBC in Caucasians, so our negative association of TLR4 SNPs with Japanese PBC may be valid.

Of the two co-segregating missense mutations in the gene encoding TLR4 rs4986790 (Asp299Gly) and rs4986791 (Thr399Ile), only rs4986790 interrupts TLR4 signaling. Most studies that reported disease associations with TLR4 SNPs have shown significantly higher frequencies of SNPs related to Asp299Gly and Thr399Ile [33], but none have detected these nonsynonymous mutations in Asian populations. Moreover, they were monomorphic in our Japanese healthy controls, which was consistent with other reports, including HapMap data [28].

The HLA DRB1*08:03-DQB1*06:01 haplotype has been associated with susceptibility to PBC in a Japanese population [6]. Therefore, we investigated whether the HLA DRB1*08:03-DQB1*06:01 haplotype and TLR4 SNPs or haplotypes were independently associated with PBC, but found no confounding associations. Although our prior study showed that the HLA DRB1*09:01-DQ*03:03 haplotype was associated with disease progression [6], we observed no significant associations between TLR4 SNPs or haplotypes with late stage PBC or cirrhosis in this study.

In conclusion, it appears that TLR4 SNPs and haplotypes are not associated with susceptibility to PBC in Japan. Genetic variations associated with PBC vulnerability remain open for further investigation, indicating the need for a genome-wide association study of PBC in Japan.

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Genome-wide Association Study Identifies *TNFSF15* and *POU2AF1* as Susceptibility Loci for Primary Biliary Cirrhosis in the Japanese Population

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For the identification of susceptibility loci for primary biliary cirrhosis (PBC), a genome-wide association study (GWAS) was performed in 963 Japanese individuals (487 PBC cases and 476 healthy controls) and in a subsequent replication study that included 1,402 other Japanese individuals (787 cases and 615 controls). In addition to the most significant susceptibility region, human leukocyte antigen (HLA), we identified two significant susceptibility loci, *TNFSF15* (rs4979462) and *POU2AF1* (rs4938534) (combined odds ratio [OR] = 1.56, p = 2.84 × 10^{-14} for rs4979462, and combined OR = 1.39, p = 2.38 × 10^{-8} for rs4938534). Among 21 non-HLA susceptibility loci for PBC identified in GWASs of individuals of European descent, three loci (*IL7R*, *IKZF3*, and *CD80*) showed significant associations (combined p = 3.66 × 10^{-8}, 3.66 × 10^{-9}, and 3.04 × 10^{-9}, respectively) and *STAT4* and *NFKB1* loci showed suggestive association with PBC

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(combined p $= 1.11 \times 10^{-6}$ and 1.42×10^{-7}, respectively) in the Japanese population. These observations indicated the existence of ethnic differences in genetic susceptibility loci to PBC and the importance of TNF signaling and B cell differentiation for the development of PBC in individuals of European descent and Japanese individuals.

Primary biliary cirrhosis (PBC, MIM 109720) is a chronic and progressive cholestatic liver disease, presumably caused by autoimmune reactions against biliary epithelial cells, leading to liver cirrhosis and hepatic failure.[1] The incidence and prevalence of PBC range from 0.33 to 5.8 and from 2 to 40 per 100,000 inhabitants, respectively, in different geographical areas.[2] This may indicate the contribution of environmental or genetic factors in the development of PBC, whereas the clinical profiles of PBC are thought to be similar between different ethnicities and/or different geographical areas, including European-descent and eastern Asian populations. The high concordance rate in monozygotic twins compared to dizygotic twins[3] and familial clustering of individuals with PBC indicate the involvement of strong genetic factors in the development of PBC; however, the pathogenesis of PBC is still poorly understood. Previous genome-wide association studies (GWASs) and subsequent meta-analyses have identified HLA and 21 non-HLA susceptibility loci (IL12A [MIM 161560], IL12RB2 [MIM 601642], STAT4 [MIM 600558], IRF5 [MIM 607218], IKZF3 [MIM 606221], MMEL1 [MIM 120520], SPIB [MIM 606802], DENND1B [MIM 613292], CD80 [MIM 112203], IL7R [MIM 146661], CXCR5 [MIM 601613], TNFRSF1A [MIM 191190], CLEC16A [MIM 611303], NFKB [MIM 164012], RAD51L1 [MIM 602948], MAP3K7IP1 [MIM 602615], PLCL2 [MIM 614276], RPS6KA4 [MIM 603606], TNFAIP2 [MIM 603300], 7p14, and 16q24) to PBC in individuals of European descent,[4-7] indicating the important role of several autoimmune pathways (i.e., IL12A signaling, TNF/TLR-NF-κB signaling, and B cell differentiation) in the development of PBC. However, GWASs for PBC have never been reported for ethnicities other than European descent, limiting our knowledge of the genetic architecture of PBC. Here, we conducted a GWAS for PBC in the Japanese population to identify host genetic factors related to PBC, which would not only expand our knowledge of pathogenic pathways in PBC but also lead to the development of rationale for therapies in the future.

Samples from 2,395 individuals (1,295 cases with PBC and 1,100 healthy volunteers working at the National Hospital Organization (NHO) in Japan as a medical staff who declared having no apparent diseases, including chronic liver diseases and autoimmune diseases [healthy controls]) were collected by members of the Japan PBC-GWAS Consortium, which consists of 31 hospitals participating in the NHO Study Group for Liver Disease in Japan (NHOSLJ) and 24 university hospitals participating in the gp210 Working Group in Intractable Liver Disease Research Project Team of the Ministry of Health and Welfare in Japan. Most of the case and control samples were collected from the mainland and the neighboring islands of Japan (Honshu, Kyushu, and Shikoku). Previous studies have shown that there is little genetic heterogeneity in resident populations in these areas.[8] In fact, the genetic inflation factor was close to 1.00, and only a small portion of the samples were identified as outliers in the principal component analysis. The cases were diagnosed with PBC if they met at least two of the following internationally accepted criteria:[9] biochemical evidence of cholestasis based mainly on alkaline phosphatase elevation, presence of serum anti-mitochondrial antibodies, histological evidence of non-suppurative destructive cholangitis, and destruction of interlobular bile ducts. The demographic details of PBC cases are summarized in Table S1, available online. Of the 487 PBC cases in the GWAS, 57 were male and 430 were female, ages ranged from 33 to 90 years, the median age was 66 years, 320 cases had early-stage PBC (a stage without any signs indicating portal hypertension or liver cirrhosis), 110 had late-stage PBC without jaundice (a stage with signs of portal hypertension or liver cirrhosis but without persistent jaundice), and 57 were at the late stage with jaundice (persistent presence of jaundice [total bilirubin >2 mg/dl]). Of the 476 healthy controls in the GWAS, 170 were male and 306 were female, ages ranged from 25 to 87 years, and the median age was 40. Of the 808 PBC cases in the replication set, 120 were male and 688 were female, ages ranged from 24 to 85 years, the median age was 61 years, 646 had early-stage PBC, 121 had late-stage PBC without jaundice, and 39 were at the late stage with jaundice. Of the 624 healthy controls in the replication set, 271 were male and 353 were female, ages ranged from 24 to 74 years, and the median age was 33 years. Concomitant autoimmune diseases are also shown in Table S1. As for inflammatory bowel diseases such as Crohn disease (CD, MIM 266600) and ulcerative colitis (UC, MIM 266600), only one out of 1,274 PBC cases had UC, but none had CD. DNA was extracted from whole peripheral blood with the QIAamp DNA Blood Midi Kit (QIAGEN, Tokyo).

For the GWAS, we genotyped 1,015 samples (515 Japanese PBC cases and 500 Japanese healthy controls) using the Affymetrix Axiom Genome-Wide ASI 1 Array, according to the manufacturer's instructions. After excluding three PBC samples with a Dish QC of less than 0.82, we recalled the remaining 1,012 samples (512 cases and 500 controls) using the Genotyping Console v4.1 software. Here, Dish QC represents the recommended sample quality control (QC) metric for the Axiom arrays.[10] Of the 600,000 SNPs embedded in the array, samples with an overall call rate of less than 97% were also excluded. As a result, 508 cases and 484 controls were subjected to further analysis. All samples used for GWAS passed a heterozygosity check, and no duplicated and related samples were identified in identity by descent testing. Moreover, principal component analysis found 29 outliers to be excluded via the Smirnov-Grubbs test
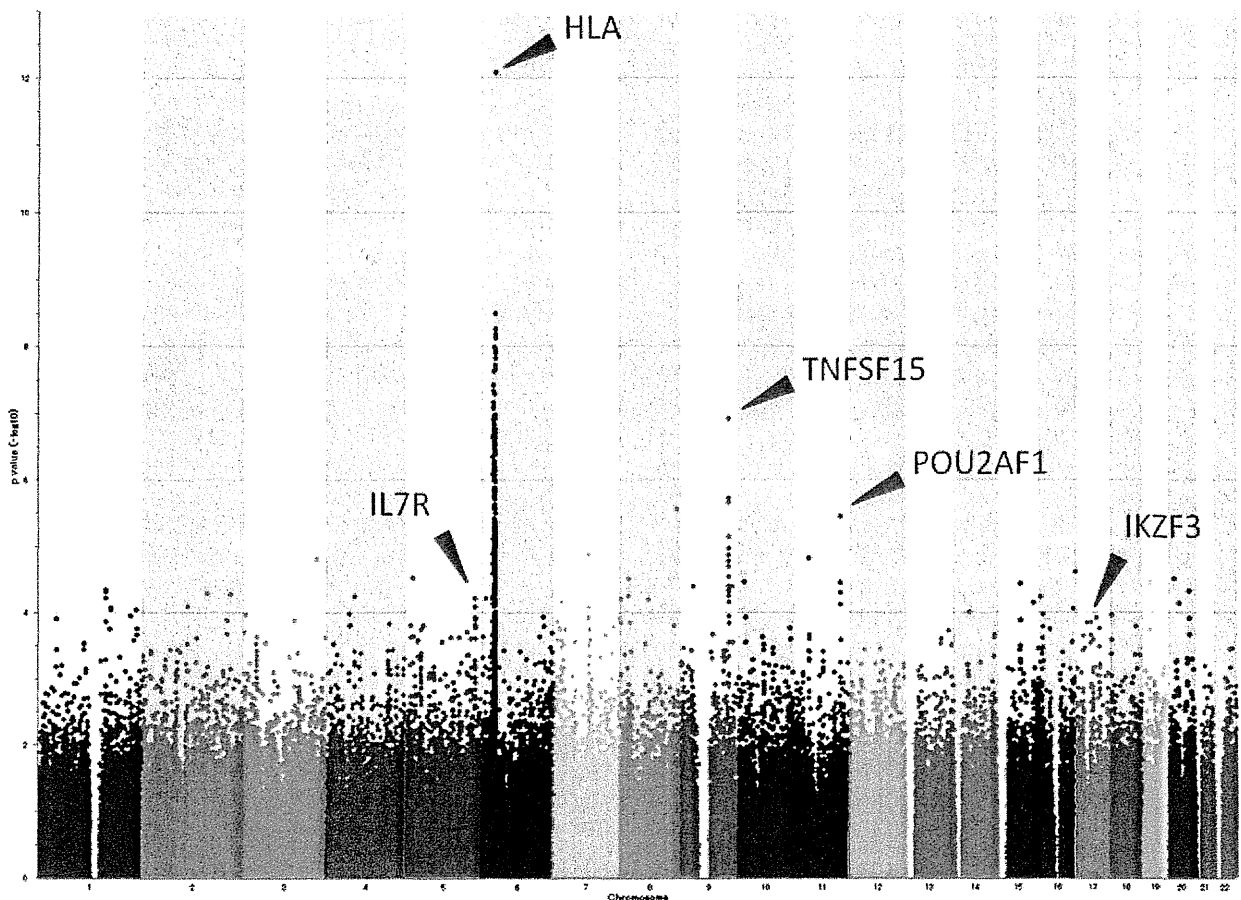
Figure 1. GWAS Results
From 963 samples (487 Japanese PBC cases and 476 Japanese healthy controls), p values were calculated with a chi-square test for allele frequencies among 420,928 SNPs.

and finally showed that all PBC cases (n = 487) and healthy controls (n = 476) formed a single cluster together with the HapMap JPT (Japanese in Tokyo from the CEPH collection), but not with CHB (Han Chinese in Beijing) samples (Figure S1, Table S2). These results indicate that the effect of population stratification was negligible. The average overall call rates of the remaining 487 PBC cases and 476 healthy controls were 99.38% (97.15–99.80) and 99.27% (97.01–99.81), respectively.[11] We then applied the following thresholds for SNP quality control during the data cleaning: SNP call rate $\geq 95\%$, minor allele frequency $\geq 5\%$ in both PBC cases and healthy controls, and Hardy-Weinberg Equilibrium (HWE) p value ≥ 0.001 in healthy controls.[12] Of the SNPs on autosomal chromosomes and in the pseudoautosomal regions on the X chromosome, 420,928 and 317 passed the quality control filters and were used for the association analysis, respectively (Table S3). A quantile-quantile plot of the distribution of test statistics for the comparison of genotype frequencies in PBC cases and healthy controls showed that the inflation factor lambda was 1.039 for all the tested SNPs, including those in the HLA region, and was 1.026 when SNPs in the HLA region were excluded (Figures S2A

and S2B). Table S4 shows the 298 SNPs with p < 0.0001 in the GWAS. All cluster plots for the SNPs with a p < 0.0001 from a chi-square test of the allele frequency model were checked by visual inspection, and SNPs with ambiguous genotype calls were excluded. For the GWAS and replication study, a chi-square test was applied to a two-by-two contingency table in an allele frequency model.

Figure 1 shows a genome-wide view of the single-point association data, which are based on allele frequencies. We found that the *HLA-DQB1* locus (MIM 604305) had the strongest association with susceptibility to PBC (rs9275175, odds ratio [OR] = 1.94; 95% confidence interval [CI] = 1.62–2.33, p = 8.30×10^{-13}) (Figure 1 and Table S4); this finding was consistent with findings from previous studies.[4-7] In addition to the HLA class II region, loci *TNFSF15* and *POU2AF1* showed evidence indicative of association with PBC (rs4979462, OR = 1.63; 95% CI = 1.36–1.95, p = 1.21×10^{-7} for *TNFSF15*; rs4938534, OR = 1.53; 95% CI = 1.28–1.83, p = 3.51×10^{-6} for *POU2AF1*).

In a subsequent replication analysis, 27 SNPs with p < 0.0001 in the initial GWAS were also studied, in addition to SNPs at the *TNFSF15* and *POU2AF1* loci. Tagging SNPs were selected from the regions surrounding *TNFSF15* and
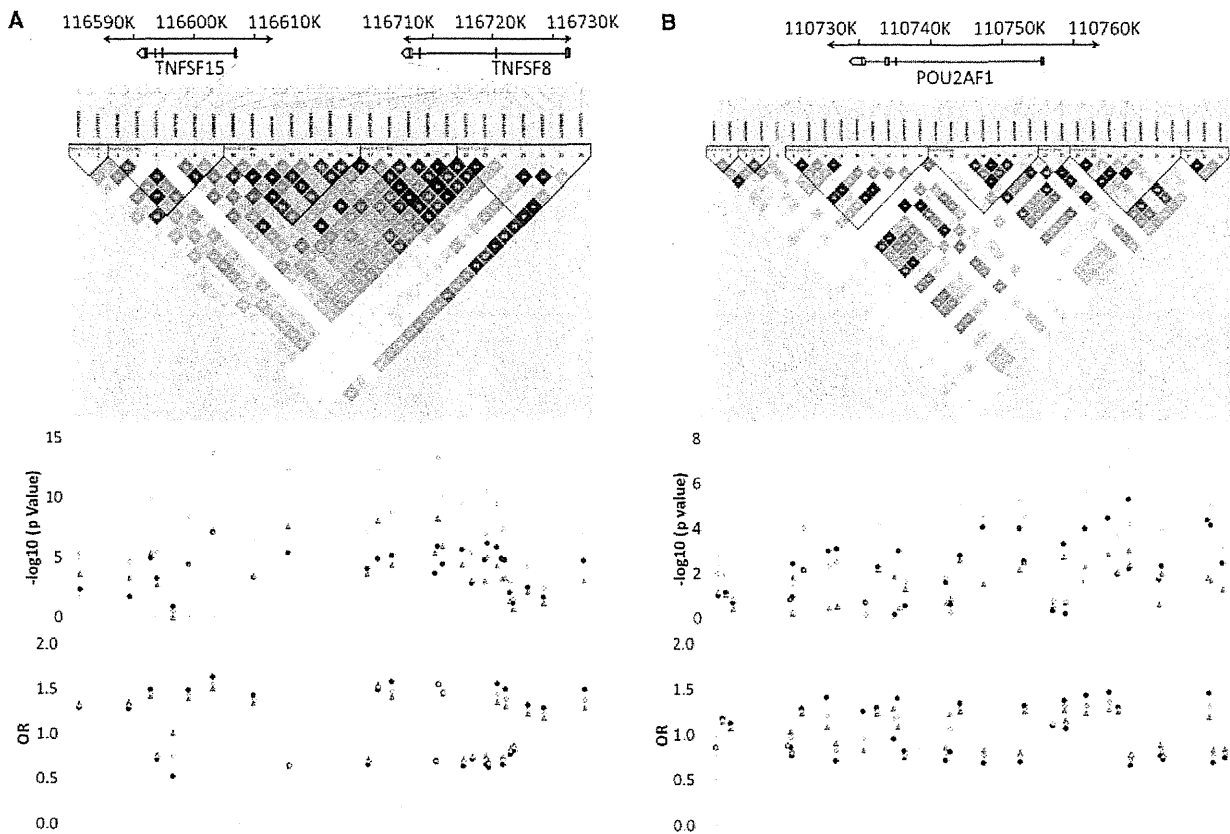
Figure 2. LD Structure, p Values, and OR Plots in the Association Analysis

LD maps (A) around *TNFSF15* (chr9: nucleotide position: 116561403–116733452; build 36.3) and (B) around *POU2AF1* (chr11: nucleotide position: 110684600–110802128; build 36.3). The middle panels show estimates of pairwise r^2 for (A) 28 SNPs and (B) 33 SNPs in the high-density mapping with a total of 2,365 samples used. The bottom panels show p values and OR-based chi-square tests for the allelic model for the left panels of 963 samples in the GWAS (●), the right panels of 1,402 samples in the replication study (▲), and the combined analysis (◇).

POU2AF1 (28 and 33, respectively) for high-density association mapping (Table S5, Figures 2A and 2B). For this follow-up replication analysis, an independent set of 1,402 samples (787 Japanese PBC cases and 615 Japanese healthy controls) and the original set of 963 samples (487 PBC cases and 476 healthy controls) were genotyped with the DigiTag2[13] and custom TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA, USA) on the LightCycler 480 Real-Time PCR System (Roche, Mannheim, Germany). The strongest associations identified in the initial GWAS were replicated in the independent set of 1,402 samples (OR = 1.52, p = 5.79 × 10^{-8} for rs4979462; OR = 1.29, p = 9.32 × 10^{-4} for rs4938534, Table 1). The combined p values were 2.84 × 10^{-14} (OR = 1.56; 95% CI = 1.39–1.76) for rs4979462 and 2.38 × 10^{-8} (OR = 1.39; 95% CI = 1.24–1.56) for rs4938534 (Table 1), both of which reached the genome-wide significance level of p < 5 × 10^{-8}. In contrast, the other 27 weakly associated SNPs identified in the initial GWAS (p values <0.0001) were not found to have significant associations with PBC (Table S5). Moreover, no strongly associated SNPs were observed when comparing PBC cases between the early and late stages (Table S5).

A haplotype analysis of the *TNFSF15* and *POU2AF1* regions was conducted with the use of the genotype data from all 2,365 samples (1,274 PBC cases and 1,091 healthy controls). Linkage disequilibrium (LD) blocks were analyzed with Gabriel's algorithm,[14] and five blocks were observed in the *TNFSF15* region and seven blocks in the *POU2AF1* region (Figures 2A and 2B). There were no differences in the LD blocks between PBC cases and healthy controls. The risk haplotypes in each region showed a lower level of association than did the individual SNPs (p = 8.26 × 10^{-14} for *TNFSF15* and p = 1.00 × 10^{-4} for *POU2AF1*) (Tables S6 and S7).

Next, we focused on data from our initial GWAS in 21 loci that are reportedly associated with susceptibility to PBC in populations of European descent.[4–7] We found that three such loci (*IL7R, IKZF3,* and *STAT4*) had p values of less than 0.001 and eight other such loci (*RAD51L1, CXCR5, PLCL2, IL12RB2, NFKB1, CD80, DENND1B,* and 7p14) showed evidence of marginal associations (p < 0.05) in the initial GWAS in 487 Japanese PBC cases and 476 Japanese healthy controls (data not shown). We genotyped three SNPs (rs6890503 for *IL7R*, rs9303277 for *IKZF3*, and rs7574865 for *STAT4*) in an independent set