Title
Autoantibodies by line immunoassay in patients with primary biliary cirrhosis

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Citation

Issue Date
2012

URL
http://ir.fmu.ac.jp/dspace/handle/123456789/338

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DOI
10.5387/fms.58.107

Text Version
publisher
AUTOANTIBODIES BY LINE IMMUNOASSAY IN PATIENTS WITH PRIMARY BILIARY CIRRHOSIS

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(Received July 20, 2012, accepted September 13, 2012)

Abstract : Objectives : We attempted to measure multiple autoantibodies simultaneously using line immunoassay (LIA) in patients with primary biliary cirrhosis (PBC) with or without antimitochondrial antibody (AMA) and patients with PBC-autoimmune hepatitis (AIH) overlap, and we examined the clinical significance of measuring these autoantibodies.

Methods : The study population consisted of 80 patients with PBC (including 12 AMA-negative patients), 16 patients with PBC-AIH overlap and 40 patients with AIH as controls. Nine antibodies (AMA-M2, M2-3E, Sp100, PML, gp210, Ro-52, LKM-1, LC-1 and SLA/LP) were detected by LIA, and AMA-M2 and anti-centromere antibody (ACA) were detected by ELISA. We examined the relationship between these autoantibodies and clinical findings.

Results : The positive prevalence of each autoantibody and ACA in the PBC group, as determined by LIA, was as follows : 13.8% for anti-Sp100, 8.7% for anti-PML, 40% for anti-gp210 and 27.5% for anti-Ro-52 antibodies and 32.5% for ACA. In the PBC-AIH overlap group, the prevalence of anti-gp210 antibody (68.7%) and that of anti-Ro-52 antibody (81.2%) were significantly higher than those in the PBC and AIH groups. Only a few patients were positive for 2 or more autoantibodies. Nine patients were determined to be negative for all autoantibodies by LIA, of whom 7 were positive for ACA. Patients positive for anti-gp210 antibody included more patients classified as stage 4 on histology than did the negative group. Those positive for ACA included more patients with varices than did the negative group.

Conclusion : LIA can measure multiple autoantibodies simultaneously and thus is considered useful in diagnosing PBC and PBC-AIH overlap. In addition, ACA is a useful marker for identifying AMA-negative PBC.

Key words : primary biliary cirrhosis, antimitochondrial antibody, line immunoassay, autoimmune hepatitis

INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic cholestatic disease that is characterized by the destruction and fibrosis of liver cells and may progress from cirrhosis to hepatic failure. The pathogenesis of the condition involves autoimmune mechanisms, as evidenced by the presence of various types of immune abnormalities in patients with PBC[1]. One such abnormality is autoantibody production; more than 60 types of autoantibodies have been detected from PBC patients[2]. Among these autoantibodies, antimitochondrial antibody (AMA) has been detected with particularly high levels of sensitivity and specificity and thus has been used as a disease marker for PBC[3]. Antinuclear antibody has also been detected from PBC patients at a relatively high frequency; detection of anti-gp210 antibody or...
anti-centromere antibody (ACA) has been shown to correlate with the prognosis of PBC\(^6\). However, since not all PBC patients are positive for AMA, histological examination is required for diagnosing AMA-negative PBC. Therefore, detection of autoantibodies other than AMA would help the diagnosis of PBC\(^{5,6}\). There is also a subset of PBC patients who have autoimmune hepatitis (AIH), referred to as PBC-AIH overlap cases\(^7,8\), and no detailed analysis of autoantibodies in these overlap cases has been conducted. Most of the preceding studies on autoantibodies in PBC focused on only a single type of autoantibody, and the positivity pattern of different autoantibodies in a single serum sample, as well as its clinical significance, has not been elucidated.

A line immunoassay (LIA) kit that can simultaneously measure 9 different autoantibodies known to be involved in autoimmune diseases has recently become available and has been used in clinical practice. Of the 9 autoantibodies, the antibodies recognizing the following 6 antigens known to be involved in PBC can be detected from a single serum sample under the same conditions: AMA-M2 (pyruvate dehydrogenase complex, PDC), M2-3E (a fusion protein of the E2 subunits of alpha-2-oxoacid dehydrogenases of the inner mitochondrial membrane), Sp100, PML (promyelocytic leukemia protein), gp210 and Ro-52. The remaining 3 antigens, LKM-1 (liver-kidney microsomes-1), LC-1 (cytosolic liver antigen type 1) and SLA/LP (soluble liver antigen/liver-pancreas antigen), are recognized by autoantibodies detected from AIH patients.

Study of autoantibodies in Japanese patients with PBC by LIA has only been attempted in small populations, and no detailed analysis of these autoantibodies in a large Japanese population has been conducted. The objective of this study was to determine the prevalence and positivity pattern of these autoantibodies, as well as their clinical significance, through autoantibody measurement by LIA to determine the prevalence of AMA-M2 and ACA by ELISA in patients with PBC with or without AMA and those with PBC-AIH overlap.

**MATERIALS AND METHODS**

This study included 80 patients with PBC (including 12 AMA-negative patients), 16 patients with PBC-AIH overlap and 40 patients with AIH as controls, all of whom were diagnosed at Fukushima Medical University Hospital or its affiliated hospitals between 1989 and 2011. Patients were diagnosed as having PBC features if they met at least two of the following three criteria: (1) chronic elevation of cholestatic liver enzymes, alkaline phosphatase (ALP) and gamma-glutamyltranspeptidase (GTP), for at least 6 months, (2) presence of serum AMA detected by either indirect immunofluorescence or ELISA using commercial available kits, and (3) typical histological findings of biopsied liver specimens\(^9\). The diagnosis of AIH features was based on the revised scoring system according to the International Autoimmune Hepatitis Group (IAIHG)\(^10\).

PBC-AIH overlap was diagnosed on the basis of Paris criteria\(^7\) proposed by Chazouilleres et al. More specifically, PBC-AIH overlap was defined as meeting at least 2 of 3 criteria for PBC, that is, 1) serum ALP level ≥ 2 × upper limit of normal (ULN) or GTP level ≥ 5 × ULN, 2) positive for AMA, and 3) presence of a florid duct lesion on histology, and at least 2 of the 3 criteria for AIH, that is, 1) serum ALT level ≥ 5 × ULN, 2) serum immunoglobulin level ≥ 2 × ULN or positive for anti-smooth muscle antibody (ASMA), and 3) presence of moderate to severe interface hepatitis on histology. Serum samples were collected from these patients between March 2010 and July 2011 (i.e., on-treatment serum samples) and stored at −20°C until used for autoantibody detection. Clinical staging was also performed according to Sheuer’s classification\(^11\) for 61 patients with PBC for whom histological examination was performed.

Autoantibody detection by LIA was performed using the Euroimmune Test System (Euroimmun, Lübeck, Germany) for PBC-related autoantibodies, including AMA-M2, M2-3E, anti-Sp100, anti-PML, anti-gp210 and anti-Ro-52 antibodies. The assay was performed according to the protocol provided with the kit. Briefly, a 1:100 dilution of serum was reacted with a strip on which antigens were immobilized for 30 minutes at room temperature, followed by reaction with alkaline phosphatase-labeled goat anti-human IgG antibody for 30 minutes for color development. The positivity for each antibody was then determined using EUROLine Scan software. Figure 1 shows representative strips after color development by LIA. Positivity was graded using EUROLine Scan software into 0, (+), +, ++ or ++++, with + or higher grades defined as positive. AMA-M2 and ACA were measured by ELISA using the MESACUP-2 Test Mitochondria M2 kit and the CENP-B kit (MBL, Nagoya, Japan), respectively.

Based on the above test results, we examined whether there was any difference between the prevalence of AMA-M2 determined by ELISA and that
of AMA-M2 and M2-3E determined by LIA. We then compared the prevalence and positivity pattern of each autoantibody in the PBC and PBC-AIH overlap groups. In addition, for the PBC group, age at diagnosis, gender, laboratory test values (albumin [Alb], total bilirubin [T.Bil], AST, ALT, ALP, GTP, platelets [PLT], prothrombin time [PT], total cholesterol [T.Chol], IgG and IgM levels), histological staging of liver tissue, presence/absence of esophageal/gastric varices, presence/absence of jaundice, and response to treatment (considered as positive when 40% or more of improvement in ALP level was observed within 1 year of treatment with oral ursodeoxycholic acid (UDCA), according to the Barcelona criteria) were compared between positive and negative groups for each antibody.

For statistical analysis, data were expressed as means ± standard deviation (SD). The chi-square test was used for comparison between two groups, and the Mann-Whitney U test was used for comparison of continuous data. Two-sided P values were determined, with P < 0.05 considered as statistically significant.

The present study was conducted with approval by the ethics committee at Fukushima Medical University, and all patients provided consent before participating in the study.

### RESULTS

1) **Difference between prevalence of AMA-M2 determined by ELISA and that of AMA-M2 and M2-3E determined by LIA**

Among the PBC patients included in the present study, 68 patients were determined to be positive and 12 patients were determined to be negative for AMA-M2 by ELISA (Table 1). Of those determined to be positive for AMA-M2 by ELISA, 16 patients were determined to be negative for AMA-M2 and 6 were determined to be negative for M2-3E by LIA. In contrast, all patients determined to be negative for AMA-M2 by ELISA were also determined to be negative for AMA-M2 and M2-3E by LIA. Table 2 shows the 6 patients determined to be positive for AMA-M2 by ELISA but determined to be negative for M2-3E by LIA. These patients also showed relatively low AMA-M2 antibody titer as determined by ELISA. Five of them were also negative for AMA-M2 by LIA, while the remaining one patient was positive (+ +) for AMA-M2, despite being negative for M2-3E, by LIA.

2) **Positivity prevalence of each autoantibody in the PBC, PBC-AIH overlap, and AIH groups**

Table 3 summarizes the positivity prevalence of each autoantibody as determined by LIA and that of

![Fig. 1. Representative strips (16 cases) after color development by line immunoassay. The LIA test strips have been coated with 9 antigens. A strong color reaction is visible on the positive antibody similar to control band.](image-url)
ACA as determined by ELISA. The positivity prevalence of each antibody in the PBC group was as follows: 13.8% for anti-Sp100, 8.7% for anti-PML, 40% for anti-gp210 and 27.5% for anti-Ro-52 antibodies and 32.5% for ACA. In the PBC-AIH overlap group, the positivity prevalence of anti-gp210 antibody (68.7%) and that of anti-Ro-52 antibody (81.2%) were significantly higher than those in the PBC group. In the AIH group, 17.5% of the patients were positive for anti-Ro-52 antibody, while no patients were found to be positive for anti-Sp100, anti-PML or anti-gp210 antibody.

3) Positivity pattern of each autoantibody in the PBC group

As the results described in 2) above suggest a relatively low specificity of anti-Ro-52 antibody in identifying PBC, the antibody was excluded from analysis of antibody positivity pattern. Figure 2a shows the positivity patterns of AMA-M2, M2-3E, anti-Sp100, anti-PML, and anti-gp210 antibodies as determined by LIA. Among those patients negative for AMA-M2 and M2-3E, 2 patients were positive for anti-Sp100 antibody and 5 patients were positive for anti-gp210 antibody. Nine patients were determined to be negative for these autoantibodies by LIA, of whom 7 were posi-
The positivity patterns of autoantibodies other than AMA are shown in Figure 2b. Only one patient was determined to be positive for all autoantibodies and only a few patients were positive for 2 or more autoantibodies, with only 3 patients determined to be positive for both anti-gp210 antibody and ACA.

4) Relationship between autoantibodies and laboratory test results in patients with PBC

The relationship between autoantibodies and laboratory test results is summarized in Table 5. Patients positive for anti-PML antibody showed significantly higher levels of IgG than those negative for the antibody, while those positive for anti-gp210
antibody showed significantly lower serum levels of Alb and included more patients classified as stage 4 on histology and more patients with jaundice or esophageal varices than did the negative group. Those positive for ACA showed significantly lower levels of ALT and gamma-GTP and included more patients with varices than did the negative group. Those positive for anti-Ro-52 antibody showed significantly higher levels of ALP and included more patients with jaundice than did the negative group. No significant differences in laboratory test results were found between AMA-positive and AMA-negative groups as determined by LIA or between those positive for any of the autoantibodies tested other than AMA and those negative for all of the autoantibodies tested.

**DISCUSSION**

AMA has been demonstrated to be a useful marker for PBC, and indirect immunofluorescence assay and ELISA are widely used for the measurement of AMA-M2. The major corresponding antigen of AMA is the E2 component of pyruvate dehydrogenase complex (PDC-E2), a component of 2-oxoacid dehydrogenase complex (2-OADC) localized in the inner mitochondrial membrane. Other known corresponding antigens of AMA include branched-chain 2-oxoacid dehydrogenase complex (BCOADC)-E2 and 2-oxoglutarate dehydrogenase complex (OGDC)-E2. AMA includes IgA, IgG and IgM-class antibodies. Kuroda et al. reported that some patients negative for IgG-class anti-PDC-E2 antibody are positive for IgA- or IgM-class anti-

| Table 4. The prevalence of AMA and autoantibodies in patients with PBC. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | Anti-Sp100  | Anti-PML  | Anti-gp210 | Anti-Ro-52 | ACA  |
| AMA-M2 (positive) N=52 | 8 (15.4%) | 5 (9.6%) | 20 (38.5%) | 12 (23.1%) | 14 (26.9%) |
| AMA-M2 (negative) and M2-3E (positive) N=12 | 2 (16.7%) | 1 (8.3%) | 7 (58.3%) | 6 (50.0%) | 5 (41.7%) |
| AMA-M2 (negative) and M2-3E (negative) N=16 | 1 (6.3%) | 1 (6.3%) | 5 (31.3%) | 4 (25.0%) | 7 (43.8%) |
| ELISA | 0 (0%) | 1 (8.3%) | 4 (33.3%) | 4 (33.3%) | 6 (50.0%) |

| Table 5. Relationship between autoantibodies and laboratory test results in patients with PBC. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | Anti-Sp100  | Anti-PML  | Anti-gp210 | Anti-Ro-52 | ACA  |
| Age 54.5 58.6 | 59.2 57.2 | 54.3 59.1 | 53.3 58.8 | 61.2 56.1 |
| Sex (M/F) 2.9 | 13.56 | 0.7 15.58 | 3/29 12.36 | 3/19 12.46 | 1/25* 14/40 |
| Alb (g/dl) 4.1±0.3 3.9±0.7 | 4.0±0.3 3.9±0.7 | 4.0±0.7* 4.1±0.5 | 3.7±0.9 4.0±0.5 | 4.0±0.5 3.9±0.7 |
| TBI (mg/dl) 1.2±0.9 1.9±1.1 | 0.7±0.3 1.9±1.0 | 2.6±5.1 1.2±1.6 | 2.5±5.4 1.5±3.0 | 1.0±1.3* 2.2±4.6 |
| ALT (IU/L) 55±31 62±52 | 51±35 62±51 | 67±45 57±53 | 65±64 69±43 | 44±30 70±55 |
| ALP (IU/L) 578±305 741±654 | 518±147 738±648 | 796±685 666±578 | 947±1,013* 631±35 | 570±273 791±728 |
| GTP (IU/L) 188±130 275±283 | 130±74 275±277 | 277±295 252±252 | 342±362 232±219 | 145±131* 320±299 |
| IgG (g/dl) 1.766±578 1.768±594 | 2.316±648* 1.710±555 | 1.929±686 1.668±499 | 1.767±537 1.768±612 | 1.729±501 1.755±635 |
| IgM (g/dl) 426±355 400±274 | 368±141 407±296 | 465±327 363±249 | 364±259 419±295 | 382±246 415±304 |
| Stage | 4/4/0/1 | 32/8/4/4 | 2/2/1/0 | 34/10/7/5 | 10/5/3/5* | 26/7/5/0 | 9/3/2/2 | 27/9/6/3 | 10/6/4/0 | 25/6/4/5 |
| UDCA response (+/-) | 9/2 | 46/14 | 5/0 | 50/16 | 21/8 | 34/8 | 12/8 | 43/8 | 17/4 | 37/12 |
| Varices (+/-) | 2.9 | 12/50 | 1.3 | 13/56 | 9/20* | 5/39 | 5/14 | 9/45 | 7/13* | 7/45 |
| Icterus (+/-) | 0/11 | 9/60 | 9/7 | 9/64 | 7/19* | 2/52 | 5/14* | 4/57 | 1/25 | 8/46 |

*p<0.05, compared negative for each antibody
body. The current ELISA-based method for measuring AMA-M2 uses 3 recombinant proteins (PDC-E2, BCOADC-E2 and OGDC-E2) as antigens and can detect the 3 immunoglobulin classes, providing high levels of diagnostic sensitivity (=90%) and specificity (=95%).

Since LIA uses native PDC as the only corresponding antigen of AMA-M2 and can only detect IgG class antibodies, it is easy to imagine that LIA is less sensitive than ELISA in detecting AMA. In contrast, M2-3E uses a fusion protein formed by binding 3 specific enzyme components, PDC-E2, BCOADC-E2 and OGDC-E2, as a corresponding antigen. However, the fact that 6 patients were determined to be positive for AMA-M2 by ELISA but found to be negative for M2-3E by LIA indicates that the current ELISA-based method is more sensitive than LIA. The detection sensitivity and specificity of LIA were 79.2% and 100%, respectively, which were lower than those of ELISA. Although the detection sensitivity for other antibodies is also expected to differ between ELISA and LIA, anti-Sp100, anti-PML, anti-gp210, and anti-Ro-52 antibodies were not detected by ELISA in the present study. This issue should be addressed in future studies. One patient was found to be positive (+++) for AMA-M2 by LIA but found to be negative for M2-3E, which may be explained by the presence of other corresponding antigens outside the fusion protein.

This study is the first attempt to determine the prevalence of different autoantibodies in a relatively large population of Japanese patients with PBC under the same conditions. Stinton et al. determined the prevalences of different autoantibodies among 109 patients with PBC (including 6 patients negative for AMA) using LIA (82% for AMA-M2, 73% for M2-3E, 27% for anti-Sp100 antibody, 17% for anti-PML antibody, 27% for anti-gp210 antibody, 32% for anti-Ro-52 antibody and 11% for ACA), which were different from those observed in the present study (65% for AMA-M2, 77.5% for M2-3E, 13.8% for anti-Sp100 antibody, 8.7% for anti-PML antibody, 40.0% for anti-gp210 antibody, 27.5% for anti-Ro-52 antibody and 32.5% for ACA). The reported prevalences of these autoantibodies vary among studies: 75-96% for AMA-M2, 57% for M2-3E, 20-30% for anti-Sp100 antibody, 19% for anti-PML antibody, 17-25% for anti-gp210 antibody, 25-28% for anti-Ro-52 antibody, and 10-30% for ACA. This variability may reflect differences in target populations, such as racial difference, timing of sample collection and other variables among institutions. Further studies in larger populations are thus needed.

PBC-AIH overlap has been considered a rare condition that shows characteristics of both PBC and AIH, but it is increasingly regarded as a form of PBC accompanied by hepatitis-like changes as observed in AIH. The 16 patients with PBC-AIH overlap included in the present study showed AIH-specific findings, such as being positive for antinuclear antibody, high IgG level and histological evidence of interface hepatitis, while showing a similar pattern of autoantibody detection by LIA to that observed in PBC. It is particularly interesting that the prevalence of anti-gp210 and anti-Ro-52 antibodies in the overlap group was higher than that in the PBC and AIH groups. Gp210 is a glycoprotein that consists of 3 domains and is an essential component of nuclear pores. The antibody recognizing this antigen is detected mainly in PBC patients and has been shown to correlate with the stage and prognosis of PBC. A recent multicenter study conducted by Nakamura et al. using a C-terminal peptide of gp210 on an ELISA system revealed the presence of anti-gp210 antibody to be a risk factor for progression of liver failure. Although it is not clear how anti-gp210 antibody is involved in the pathogenesis of PBC, the fact that gp210 protein is ectopically expressed in small bile duct cells of PBC patients suggests the involvement of mechanisms similar to those involved in the breakdown of immune tolerance to PDC-E2 antigen.

The anti-Ro-52 antibody is a type of anti-SS-A/Ro antibody that has been detected in many autoimmune diseases, including Sjogren's syndrome. Granito et al. reported that the population of PBC patients positive for anti-Ro-52 antibody includes significantly more patients with advanced histological stage and high serum bilirubin levels than dose the negative population. In the present study, both the subset of patients positive for anti-gp210 antibody and that of patients positive for anti-Ro-52 antibody included significantly more patients with jaundice than did the subsets of patients negative for these antibodies, being consistent with results of previous studies. However, changes in the expression of each autoantibody over time and the long-term outcome of individual patients were not examined in the present study and should be addressed in future studies.

ACA is a disease marker antibody for localized scleroderma and is detected in approximately 30% of patients with scleroderma. The antibody is also detected in a subset of patients with PBC. Corresponding antigens of ACA include 3 major pro-
tein antigens, CENP-A (17 kD), CENP-B (80 kD) and CENP-C (140 kD), and several other centromere antigens. The most important among these antigens is CENP-B; ELISA using an epitope located at the C-terminus of CENP-B as a recombinant antigen has been used for detecting ACA. ACA-positive PBC has been shown to progress to portal hypertension at a high frequency. In the present study, the prevalence of esophageal/gastric varices was significantly higher among ACA-positive patients than among ACA-negative patients. The IgG fraction extracted from sera of ACA-positive patients has been shown to induce apoptosis in vascular endothelial cells. A possible correlation between presence of ACA and vascular endothelial cellular damage in ACA-positive PBC patients should be examined in future studies. Furthermore, the fact that many of the other autoantibody-negative PBC patients were positive for ACA suggests that liver histological examination should be performed for ACA-positive patients with impaired liver function in order to detect possible PBC.

The anti-Sp100 antibody has been shown to be relatively specific in identifying PBC and has also been detected in patients with SLE and scleroderma. The antibody has also been detected in patients with AMA-negative PBC and thus is considered a useful marker for serological diagnosis of PBC. The anti-PML antibody has been detected in PBC patients positive for anti-Sp100 antibody, and patients positive for these antibodies have been shown to show faster progression of PBC than those negative for these antibodies. In the present study, however, the prevalence of each of these antibodies among patients negative for AMA-M2 and M2-E3 was only 6.3% and was seldom correlated with laboratory test results. The roles of these antibodies should be investigated in larger Japanese populations.

The present study is the first study to show positivity patterns of different autoantibodies among Japanese patients with PBC. Anti-gp210 antibody, ACA, and anti-Sp100/anti-PML antibodies were rarely detected simultaneously, and there was a correlation between the positivity patterns of individual autoantibodies, such as anti-gp210 antibody, ACA and anti-Ro-52 antibody, and clinical findings, suggesting the significance of detecting autoantibodies other than AMA. However, one of the limitations of the present study is that we did not examine changes in the expression pattern of each antibody before and after treatment and its relationship with treatment effect or treatment outcomes over time. Considering that a correlation has been demonstrated between post-treatment change in anti-gp210 antibody titer and treatment outcomes, long-term follow-up of changes in autoantibody titers may facilitate prognosis prediction. It is therefore necessary to continue to increase the number of cases and follow them for a longer period of time. The fact that some of the patients found to be negative for AMA, a marker to be examined at the initial visit for potential PBC, were found to be positive for other autoantibodies also suggests the usefulness of assays that can detect multiple autoantibodies under the same conditions, such as LIA, for use in routine clinical practice. On the basis of the present results, for autoantibody measurement in Japanese patients with PBC, we propose that AMA should be measured by the conventional ELISA-based method and that a LIA system on which gp210, ACA, Ro-52, Sp100 and PML are immobilized should be developed.

CONCLUSION

In the present study, we assessed the clinical usefulness of measuring autoantibodies in patients with PBC and those with PBC-AIH overlap using ELISA for AMA-M2 and LIA for anti-Sp100, anti-PML, anti-gp210 and anti-Ro-52 antibodies. LIA can measure multiple autoantibodies simultaneously and thus is considered useful in diagnosing PBC in the Japanese population. The development of an assay system that incorporates PBC-related autoantibodies other than AMA-M2 and ACA is needed.

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