Construction and expression of a humanized M2 autoantigen trimer and its application in the diagnosis of primary biliary cirrhosis

Xiao-Hua Jiang, Ren-Qian Zhong, Sheng-Qian Yu, Yin Hu, Weng-Weng Li, Xian-Tao Kong

Xiao-Hua Jiang, Department of Laboratory Medicine, 85 Hospital the Chinese PLA, Shanghai 200052, China
Ren-Qian Zhong, Weng-Weng Li, Xian-Tao Kong, Clinical Immunology Center of the Chinese PLA, Changzheng Hospital, Second Military Medical University, Shanghai 200003, China
Sheng-Qian Yu, Department of Nephrology, Changzheng Hospital, Second Military Medical University, Shanghai 200003, China
Yin Hu, Department of Basic Science, Shanghai University of Engineering Science, Shanghai 200335, China
Correspondence to: Dr Xiao-Hua Jiang, Department of Laboratory Medicine, 85 Hospital of the Chinese PLA, Huashan Road, Shanghai 200052, China. jhulu@citiz.net
Telephone: +86-21-62528805 Fax: +86-21-33110236
Received: 2002-07-18 Accepted: 2002-08-07

Abstract
AIM: To construct and express a humanized M2 autoantigen trimer designated as BPO and to apply it in the diagnosis of primary biliary cirrhosis (PBC).

METHODS: cDNA fragments encoding M2-reactive epitopes of pyruvate dehydrogenase complex E2 (PDC-E2), branched 2-oxo-acid dehydrogenase complex E2 (BCOADC-E2) and 2-oxo-glutarate dehydrogenase complex E2 (OGDC-E2) were amplified with PCR using total RNA extracted from human peripheral mononuclear blood cells. The fragments were cloned into the plasmid vector pQE-30 and then transferred into E. coli M15 (pREP4) for expression, which was induced by isopropylthio-β-D-galactoside. The expressed recombinant BPO protein was demonstrated by SDS-PAGE, Western-blotting and Immunoabsorption test, its antigenic reactivity and specificity were identified with seven M2-positive sera confirmed at Euroimmun Research Center (Germany). Using the purified BPO, M2 antibodies in sera from patients with PBC and other liver related diseases were detected with ELISA.

RESULTS: The expressed BPO was observed with both antigenic reactivity and specificity of M2 autoantigens. The determination of M2 antibodies by BPO with ELISA was more sensitive than using the Euroimmun’s kit with the coefficients of variation less than 10 % in both interassay and intraassay. With the newly established method, M2 antibodies were found in 100 % (20/20) of patients with PBC. Six cases of liver disease with unknown etiology and 1 patient with drug induced liver injury had detectable levels of serum M2 antibodies. There were also 2 patients with autoimmune cholangitis and 1 with autoimmune hepatitis showing M2 antibody positive.

CONCLUSION: Compared with the routine immunofluorescence assay and commercially available assay kit using porcine heart mitochondrial protein as the antigen, the detection system established in the present study shows higher sensitivity and specificity and may be used as a powerful tool for the diagnosis of PBC.

INTRODUCTION
Primary biliary cirrhosis (PBC) is a chronically progressive cholestatic liver disease with autoimmune basis. According to some reports, the incidence of this disease has been consistently increased in recent years[1-4]. One of the remarkable features of PBC is the appearance of high titer antimitochondrial antibodies (AMA) in the patient’s sera. Generally, these antibodies are categorized into nine subgroups termed M1-M9 according to the antigens they recognize, in which only M2 antibodies are considered specific for PBC patients that are detectable years or decades before the clinical and histological diagnosis[5,11].

The major autoantigens recognized by M2 antibodies are the members of 2-oxo-acid dehydrogenase complex including pyruvate dehydrogenase complex E2 (PDC-E2), branched chain 2-oxo-acid dehydrogenase complex E2 (BCOADC-E2) and 2-oxo-glutarate dehydrogenase complex E2 (OGDC-E2), whose immunodominant epitopes have been mapped within lipoyl domains. Antibodies to these corresponding autoantigens have been reported in PBC patients with a positive rate of 95 %, 53-55 % and 39-88 % respectively[6,12]. However, when all of these antibodies are determined simultaneously, the patients with PBC can be diagnosed as high as 92-100 %[13-16]. These facts suggest such a possibility that if there is a constructed antigen containing the specific immunodominant epitopes and the antibodies above can be detected synchronously, the diagnosis of PBC patients would be more specific, sensitive and convenient.

Therefore, we designed and constructed a M2 autoantigen trimer (BPO) expression vector, which could coexpress the immunodominant lipoyl domains of PDC-E2, BCOADC-E2 and OGDC-E2 from human origin, in an attempt to establish a more accurate and sensitive method with BPO to detect M2 antibodies in patients with PBC. Besides, because it has never been reported that M2 antibodies were found in other liver related diseases other than PBC[17,20], a survey to detect M2 antibodies under these circumstances with our constructed M2 autoantigen trimer was also included in the present study.

MATERIALS AND METHODS
Patients
Eight groups of adult patients with both sexes who were treated in Shanghai Changzheng Hospital were enrolled in the present study. Group 1 consisted of 20 patients with PBC diagnosed on the criteria: the presence of AMA and at least one of the followings: (1) Elevation of serum alkaline phosphatase (ALP) and/or gamma glutamyl transpeptidase (γ-GT), (2) Liver biopsy with PBC characteristics[21]. Group 2 consisted of 5 patients with autoimmune hepatitis (AIH)[22]. Two patients diagnosed

• CLINICAL RESEARCH •
as autoimmune cholangitis (AIC) were included in group 3, and group 4 was composed of 18 patients diagnosed as liver disease with unknown etiology (LDUE) that was defined as lack of obvious causes including drug use, alcohol abuse, exposure to hepatotoxic medication or chemicals and virus infection. Group 5 consisted of 8 patients with drug induced liver injury (DILI). Group 6 enrolled 201 patients with other liver diseases (Post-viral hepatitis and liver cirrhosis, n=153; Obstructive jaundice, n=25; Acute hepatitis A, n=15; Hepatic abscess, n=3; Wilson’s disease, n=1; Cardiac cirrhosis, n=4). Thirty-three patients with various autoimmune diseases (AID) (Rheumatoid arthritis, n=12; Systemic lupus erythematosus, n=12; Polymyositis, n=4; Vasculitis, n=3; Hashimoto’s thyroiditis, n=2) were included in group 7 and 1 225 healthy volunteers taking a health checkup aged less than 28 served as the control. In the experiment, fasting serum from each patient was prepared with routine procedures and stored at -20 °C until further analysis.

**Materials**

Reverse transcriptase and PCR amplification system were purchased from Roche Company (U.S.A). Restriction endonucleases and T4 DNA ligase were from New England Biolabs (U.S.A). Plasmid vector pQE-30 and E.coli M15 (prep 4) were from Qiagen Company (Germany). Indirect immunofluorescence (IIF) test kit for AMA and Western-blotting kit for M2 antibodies were all from Euroimmun Company (Germany).

**Results**

### Identification of expressed M2 autoantigen trimer

The segment analysis by restriction endonuclease digestion confirmed that inserted cDNA sequences in the constructed plasmids were completely consistent with that of the published data (Figure 2). The molecular mass of BPO protein was examined by SDS-PAGE in 15 % polyacrylamide gel, in which a specific 42 KD protein band was clearly visualized (Figure 3).

**Figure 1** Construction protocol of recombinant plasmids.

**Expression and identification of M2 autoantigen trimer**

Recombinant plasmids were constructed as illustrated in Figure 1. Briefly, total RNA was extracted from human peripheral mononuclear blood cells. The objective cDNAs were synthesized by reverse transcriptase and used as the template to amplify the immunodominant epitopes of BCOADC-E2, PDC-E2 and OGDC-E2 with polymerase chain reaction. The PCR products were digested with relevant restriction endonuclease and purified cDNA fragments were inserted into the expression vector pQE-30 to form recombinant plasmids pQE-30/BCOADC-E2, pQE-30/PDC-E2, pQE-30/OGDC-E2 and pQE-30/BPO respectively. The pQE-30/BPO was then transferred into E. Coli M15 (pREP4) and induced by isopropylthio-β-D-galactoside to express BPO protein, which was finally confirmed with SDS-PAGE, Western-blotting and Immunoabsorption test.

The antigenic reactivity and specificity of the recombinant BPO trimer were identified with seven M2-positive sera confirmed at Euroimmun Research Center (Germany) by immunoblotting using beef heart mitochondrial preparations.

### Detection of M2 antibodies with BPO

The obtained recombinant BPO protein fused with the 6×His affinity tag in the N-terminus was purified by Ni-NTA affinity chromatography under denaturing conditions. After renatured by removing denaturants slowly with dialysis, the BPO protein was used as the specific antigen to detect M2 antibodies with the routine procedures of ELISA. The coefficients of variation for this assay method, the mean OD±SD for the control sera, as well as the critical OD value for the positive determination were respectively calculated or defined based on the experimental results. The measurements of M2 antibodies and AMA with Euroimmun’s kits as a comparison of the present assay method were also simultaneously performed in the study.

**Figure 2** Segment analysis of recombinant plasmids by restriction endonuclease digestion. 1. Markers; 2. pQE-30 (BamH1); 3. pQE-30/BCOADC-E2 (BamH1+Sphl); 4. pQE-30/PDC-E2 (Sph1+Sac1); 5. pQE-30/OGDC-E2 (Sac1+Sal1); 6. pQE-30/BPO (BamH1+Sal1).

**Figure 3** Expression products of recombinant plasmids detected by SDS-PAGE stained with Coomassie Brilliant Blue R-
The expressed BPO protein could react with all of the seven M₂-positive sera confirmed at Euroimmun Research Center (Germany) by immunoblotting using beef heart mitochondrial preparations, which identified the antigenic reactivity of the recombinant BPO trimer (Figure 4). When mixed beforehand with the lysates of E.coli expressing BPO overnight, the sera became M₂-negative by Western blotting, which confirmed the BPO specificity determined by the immunodominant epitopes of PDC-E₂, BCOADC-E₂ and OGDC-E₂.

**Table 1**

| Group      | n | AMA positive | M₂-positive | Euroimmun’ s kit | ELISA | AASLD’s guideline (+) |
|------------|---|--------------|-------------|-----------------|-------|-----------------------|
| PBC        | 20| 20           | 16          | 20              | 20    | -                     |
| AIH        | 5 | 0            | 0           | 1               | 0     | -                     |
| AIC        | 2 | 0            | 1           | 2               | 0     | -                     |
| LDUE       | 18| 7            | 6           | 6               | 6     | -                     |
| DILI       | 8 | 1            | 1           | 1               | 1     | -                     |
| AID        | 33| 3            | 0           | 0               | 0     | -                     |
| Control    | 1225| ND        | ND          | ND              | ND    | -                     |

**Table 2**

| Group | n | AMA positive | M₂-positive | Euroimmun’ s kit | ELISA | AASLD’s guideline (+) |
|-------|---|--------------|-------------|-----------------|-------|-----------------------|
| PBC   | 20| 20           | 16          | 20              | 20    | -                     |
| AIH   | 5 | 0            | 0           | 1               | 0     | -                     |
| AIC   | 2 | 0            | 1           | 2               | 0     | -                     |
| LDUE  | 18| 7            | 6           | 6               | 6     | -                     |
| DILI  | 8 | 1            | 1           | 1               | 1     | -                     |
| Other liver diseases | 201| ND          | ND          | ND              | ND    | -                     |
| AID   | 33| 3            | 0           | 0               | 0     | -                     |
| Control | 1225| ND        | ND          | ND              | ND    | -                     |

**DISCUSSION**

In the guideline by AASLD in 2000 and the standards by other researchers, AMA has long been used as an important marker for the primary biliary cirrhosis; however, only M₂ antibodies are considered as specific for the PBC diagnosis. Other AMA sub-types have been found in drug-induced disorders, cardiomyopathies, systemic lupus erythmatosus, rheumatoid arthritis, tuberculosis, syphilis and hepatitis C, indicating the nonspecific nature of AMA in the diagnosis of PBC. Besides, there were about 5-17% of the patients with biochemical and histological features compatible with PBC not having detectable AMA with the IIF method. To get better diagnostic results, approaches to detect M₂ antibodies by ELISA or Western-blotting using recombinant antigen of PDC-E₂, BCOADC-E₂ and OGDC-E₂ have been reported in several literatures.

In 2001, Miyakawa and his coworkers developed a new ELISA for the detection of M₂ antibody using porcine heart mitochondrial protein as the antigen. The sensitivity of this method was only 78%, despite the specificity was 100%. In the present study, we employed BPO as the antigen to determine M₂ antibodies with ELISA, which was more sensitive than the Euroimmun’s kit. The reason for this was partially because the antigen used in our approach was derived from human sources instead of that from porcine used in Euroimmun’s kit. The antigen heterogeneity might affect the assay results. Furthermore, the three major autoantigens, BCOADC-E₂, PDC-E₂ and OGDC-E₂, with no cross-reactivity between, were constructed together as a trimer by molecular biological techniques, which could provide more positive chance for the detection of M₂ antibodies. Therefore, the use of this recombinant molecule offered a rapid, simple and sensitive ELISA for the immunodiagnosis of PBC.

According to the investigation by James and his associates, the incidence of PBC has been increased in recent years. In northern England, the prevalence of PBC from 201.9 per 10⁵ adults and 541.4 per 10⁶ women over 40 in 1987 rose to 334.6 and 939.8 respectively in 1994. Owing to the lack of sensitive diagnostic methods, there have no reliable data related to the epidemiology of PBC in China so far and more seriously.
clinical doctors have not yet paid appropriate attention to this disease. We checked 10 patients with liver cirrhosis hospitalized in January, February and April in 2000 whose serum immunological variables showed no signs of viral infection, and the reason for liver cirrhosis seemed unclear. However, 7 of the 10 patients were found M2 antibody positive by the detailed studies at the Euroimmun Research Center (Germany). In the past six months since we detected M2 antibody by BPO with ELISA for the PBC diagnosis, over 120 patients’ sera have been examined, in which 69 demonstrated M2 antibody positive and 30 cases with comparatively complete clinical data listed in this paper. Our recent research and the related domestic reports in 2001 indicate that PBC is probably not so rare in China as it has been thought[4,7].

REFERENCES

1 James OF, Bhopal R, Howell D, Gray J, Burt AD, Metcalf JV. Primary biliary cirrhosis once rare, now common in the United Kingdom. Hepatology 1999; 30: 390-394
2 Metcalf J, James O. The geopidemiology of primary biliary cirrhosis. Semin Liver Dis 1997; 17: 13-22
3 Metcalf JV, Bhopal RS, Gray J, Howell D, James OF. Incidence and prevalence of primary biliary cirrhosis in the city of Newcastle upon Tyne, England. Int J Epidemiol 1997; 26: 830-836
4 Medina J, Jones EA, Garcia Monzon C, Moreno Otero R. Immunopathogenesis of cholestatic autoimmune liver diseases. Eur J Clin Invest 2001; 31: 64-71
5 Heathcote EJ. Evidence-based therapy of primary biliary cirrhosis. Eur J Gastroenterol Hepatol 1999; 11: 607-615
6 Joplin RE, Neuberger J. Immunopathology of primary biliary cirrhosis. Eur J Gastroenterol Hepatol 1999; 11: 587-593
7 Metcalf JV, Mitchison HC, Palmer J, Jones DE, Bassendine MF, James OF. Natural history of early primary biliary cirrhosis. Lancet 1996; 348: 1399-1402
8 Kisand KE, Metskula K, Kisand KV, Kivist T, Gershwin ME, Uibo R. The follow-up of asymptomatic persons with antibodies to pyruvate dehydrogenase in adult population samples. J Gastroenterol 2001; 36: 248-254
9 Koizumi H, Onozuka Y, Shibata M, Sano K, Ooshima Y, Morizane T, Ueno Y. Positive rate of anti-mitochondrial antibody in Japanese corporate workers. Rinsho Byori 2000; 48: 966-970
10 Turchany JM, Uibo R, Kivist T, Van DeWater J, Prindiville T, Coppell RL, Gershwin ME. A study of antimitochondrial antibodies in a random population in Estonia. Am J Gastroenterol 1997; 92: 124-126
11 Nakano T, Inoue K, Hirohara J, Arita S, Higuchi K, Omata M, Toda G. Long-term prognosis of primary biliary cirrhosis (PBC) in Japan and analysis of the factors of stage progression in asymptomatic PBC (a-PBC). Hepatology 2002; 31: 250-260
12 Migliaccio C, Van DeWater J, Ansari AA, Kaplan MM, Coppell RL, Lam KS, Thompson RK, Stevenson F, Gershwin ME. Heterogeneous response of antimitochondrial autoantibodies and bile duct apical staining monodonal antibodies to pyruvate dehydrogenase complex E2 in the mouse versus the human. Hepatology 2001; 33: 792-803
13 Kitami N, Komada T, Ishii H, Shimizu H, Adachi H, Yamaguchi Y, Kitamura T, Oide H, Miyazaki A, Iwai M. Immunological study of anti-M in antimitochondrial antibody-negative primary biliary cirrhosis. Intern Med 1995; 34: 496-501
14 Jones DE. Autoantibodies in primary biliary cirrhosis. J Clin Pathol 2000; 53: 813-821
15 Miyakawa H, Tanaka A, Kikuchi K, Matsubashia M, Kitazawa E, Kawaguchi N, Fujikawa H, Gershwin ME. Detection of antimitochondrial autoantibodies in immunofluorescent AMA-negative patients with primary biliary cirrhosis using recombinant autoantibodies. Hepatology 2001; 33: 243-248
16 Kitami N, Ishii H, Shimizu H, Adachi H, Komada T, Mikami H, Yokoi Y, Sato N. Immunoreactivity to M2 proteins in antimitochondrial antibody-negative patients with primary biliary cirrhosis. J Gastroenterol Hepatol 1994; 9: 7-12
17 Jensen WA, Josi JA, Murphy P, De Giorgio J, Brown B, Rowley MJ, Mackay IR. Automated enzymatic mitochondrial antibody assay for the diagnosis of primary biliary cirrhosis. Clin Chem Lab M 2000; 38: 753-758
18 Leung PS, van de Water J, Coppell RL, Nakayama Y, Munoz S, Gershwin ME. Molecular aspects and the pathological basis of primary biliary cirrhosis. J Autoimmun 1996; 9: 119-128
19 Strassburg CP, Manns MP. Autoimmune tests in primary biliary cirrhosis. Baillieres Best Pract Res Clin Gastroenterol 2000; 14: 585-599
20 Quaranta S, Shulman H, Ahmed A, Shoenfeld Y, Peter M, MacDonald GB, Van de Water J, Coppell R, DeWald C, Woman-HJ, Rizzeto M, Tsuneyama K, Nakayama Y, Ansari A, Locatelli F, Paganin S, Rosina F, Manns M, Gershwin ME. Autoantibodies in human chronic graft-versus-host disease after hematopoietic cell transplantation. Clin Immunol 1999; 91: 106-116
21 Parikh Patel A, Gold EB, Worman H, Krivy KE, Gershwin ME. Risk factors for primary biliary cirrhosis in a cohort of patients from the United States. Hepatology 2001; 33: 16-21
22 Ma X, Qiu DK. Relationship between autoimmune hepatitis and HLA-DR4 and DRβ1 allelic sequences in the third hypervariable region in Chinese. World J Gastroenterol 2001; 7: 718-721
23 Heathcote EJ. Management of primary biliary cirrhosis. The American association for the study of liver diseases practice guidelines. Hepatology 2000; 31: 1005-1013
24 Strassburg CP, Jaceckl E, Manns MP. Anti-mitochondrial antibodies and other immunological tests in primary biliary cirrhosis. Eur J Gastroenterol Hepatol 1999; 11: 595-603
25 Michielli P, Wandel S, Kast A, Scheuer PJ, Yeaman S, Bassendine MF, Palmer JM, Heathcote EJ. Antimitochondrial antibody negative primary biliary cirrhosis: a distinct syndrome of autoimmune cholangitis. Gut 1994; 35: 260-265
26 Lacerra MA, Ludwig J, Dickson ER, Jorgensen RA, Lindor KD. Antimitochondrial antibody-negative primary biliary cirrhosis. Am J Gastroenterol 1995; 90: 247-249
27 Heathcote J. Autoimmune cholangitis. Gut 1997; 40: 440-442
28 Ikuno N, Sealey J, Davies JM, Whittingham SF, Omagari K, Mackay IR, Rowley MJ. A comparative study of antibody expression in primary biliary cirrhosis and autoimmune cholangitis using phage display. Hepatology 2001; 34: 478-486
29 Kinoshita H, Omagari K, Whittingham S, Kato Y, Ishibashi H, Sugi K, Yano M, Kohno S, Nakayama Y, Penner E, Wiesierska Gadek J, Reynoso Paz S, Gershwin ME, Anderson J, Jois JA, Mackay IR. Autoimmune cholangitis and primary biliary cirrhosis--an autoimmune enigma. Liver 1999; 19: 122-128
30 Invernizzi P, Crosignani A, Baltezzar PM, Covini G, De Vallee G, Larghi A, Zuin M, Potthoff T. Comparison of the clinical features and clinical course of anti-mitochondrial antibody-positive and negative primary biliary cirrhosis. Hepatology 1997; 25: 1090-1095
31 Kaserer K, Exner M, Mosbeger I, Penner E, Wirba F. Characterization of the inflammatory infiltrate in autoimmune cholangitis. A morphological and immunohistochemical study. Virchows Arch 1998; 432: 217-222
32 Mayo MJ, Lipsky PE, Miller SN, Sladny P, Combes B. Similar T-cell oligoclonality in anti-mitochondrial autoantibody-positive and negative primary biliary cirrhosis. Di G Dis Sci 2001; 46: 345-351
33 Fujikoa S, Yamanoto K, Okamoto R, Miyake Y, Ujiike K, Shimada N, Terada R, Miyake Y, Nakajima H, Piao C, Iwasaki Y, Taninami M, Tsuiji T. Laparoscopic features of primary biliary cirrhosis in AMA-positive and AMA-negative patients. Endoscopy 2002; 34: 318-321
34 Nakajima M, Shimizu H, Miyazaki A, Watanabe S, Kitami N, Sato N. Detection of IgA, IgM, and IgG subclasses of anti-M antibody by immunoblotting in autoimmune cholangitis: is autoimmune cholangitis-an autoimmune cholangitis? Zhonghua Neike Zazhi 2001; 101: 107
35 Miyakawa H, Kikuchi K, Jing Hou H, Kawaguchi N, Yamakawa H, Ito Y, Maekubo H. High sensitivity of a novel ELISA for anti-M2 in primary biliary cirrhosis. J Gastroenterol 2001; 35: 33-38
36 Miyakawa H, Kawaguchi N, Kikuchi K, Fujikawa H, Kitazawa E, Matsubashia M. Detection of antigen specificity for antimitochondrial antibodies detected by Western blotting using native mitochondrial proteins in primary biliary cirrhosis. Hepatology 2001; 33: 101-107
37 Zhang F, Jiai J, Wang B, Qian L, Yin S, Wang Y, Cui Y, You H, Ma H, Wang H, Zhang C. Clinical characteristics of primary biliary cirrhosis: a report of 45 cases. Zhonghua Nake Za Zhi 2002; 41: 163-167

Edited by Zhu L