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• BRIEF REPORTS •

Characterization of M2 antibodies in asymptomatic Chinese population

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Abstract

AIM: To investigate the presence of M2 antibodies specific for primary biliary cirrhosis (PBC) in asymptomatic Chinese and identify patients with early PBC.

METHODS: Enzyme-linked immunosorbent assay (ELISA) tests for M2 antibodies to recombinant protein were performed in 5 011 subjects (age range, 26-85 years; mean age: 45.81±15.02 years) who took an annual physical examination. M2-positive subjects were further analyzed for immunoglobulin (Ig) classes and subclasses of M2 antibodies. Clinical, biochemical and immunological data were obtained for M2-positive subjects. In addition, ultrasonography (US) or endoscopic retrograde cholangio-pancreatography (ERCP) was performed to exclude any disorders other than PBC.

RESULTS: M2 antibodies were detected in 8 (0.16 %) of the 5 011 subjects studied. Of the 8 subjects, 7 were female and 1 was male (age range: 40-74 years). An unexplained increase of serum alkaline phosphatase (ALP) and gamma glutamyl transpeptidase (γ-GT) values, often to striking levels, was detected in 4 M2-positive subjects, 3 of them accorded with the diagnostic criteria recommended by the American Association for the Study of Liver Diseases, even though they had no symptoms of PBC (such as fatigue, pruritus or jaundice). Liver biopsy was performed in two M2-positive subjects and the histology was compatible with PBC in both cases.

CONCLUSION: Our data, while not assessing the true prevalence of asymptomatic PBC in the general population, suggest that asymptomatic PBC is much more common in China than has been supposed.

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INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic progressive cholestatic liver disease with autoimmune basis. Patients are typically females aged 35-65[1, 2]. Many patients with PBC have no specific symptoms, rather, they have unexplained liver function abnormalities. Progression occurs over years or decades. The end stage is an established biliary cirrhosis[1].

A hallmark feature of PBC is the presence of high titer antimitochondrial antibodies (AMA) in patients sera. AMA are divided into nine subgroups termed M1-M9 according to the autoantigens they recognize. It has been established that only those antibodies known as M2 are specific for PBC. M2 antibodies are detectable years or decades before the presence of clinical and histological features of PBC[3-7].

The major autoantigens recognized by M2 antibodies are members of the 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). The immunodominant epitopes of PDC-E2, BCOADC-E2 and OGDC-E2 have been mapped within the lipoyl domains. Antibodies to these corresponding autoantigens have been reported to appear in PBC patients with a positive rate of 95 %, 53-55 % and 39-88 % respectively[8-10]. Using the above three immunodominant lipoyl domains, 92-100 % of patients with PBC can be verified[9-12]. In contrast, there is no report that M2 are found in diseases other than PBC[13-18]. We have designed a triple hybrid clone, designated as BPO, that coexpresses the three immunodominant lipoyl domains of PDC-E2, BCOADC-E2 and OGDC-E2 from human sources. We established and then used clinically specific immunological methods with purified BPO to detect the M2 antibodies[19,20].

According to some research, there is a wide spread impression that the number of patients with PBC is on the rise, many of whom, thanks to the application of the immunological diagnostic methods, are diagnosed at a stage of asymptomatic PBC in recent years[21-23]. However, the M2-positive rate in Chinese general population has not yet been determined. In the present research, we investigated the prevalence of M2 antibodies which are specific for PBC in asymptomatic Chinese population and identified patients with early diseases.

MATERIALS AND METHODS

Establishment of the immunological method

Recombinant fusion protein fragments of human BCOADC-E2, PDC-E2, OGDC-E2 and the triple-expression hybrid clone (BPO) were expressed in *Escherichia coli*, and then purified with Ni-NTA affinity chromatography under denaturing conditions. Enzyme-linked immunosorbent assay (ELISA) method for detecting M2 antibodies was established using purified recombinant proteins. The cut-off O.D. value for positive ELISA was defined as 0.303[19,20].

Subjects

Sera were collected from subjects aged over 26 years who took an annual physical examination in 85 Hospital the Chinese PLA in Shanghai, China from January 1, 2000 to August 30, 2001. A total of 5 011 adults including 3 108 females and 1 903 males,

aged between 26-85, with a median age of 45.81±15.02 years were enrolled in this investigation. ELISA method was used for screening M2 antibodies. M2-positive sera were verified using the kit provided by the Euroimmun Research Center (Germany) which used purified porcine heart mitochondrial protein as antigen. Clinical, biochemical and immunological data were obtained for M2-positive persons. In addition, ultrasonography (US) or endoscopic retrograde cholangiopancreatography (ERCP) was performed to exclude any disorders other than PBC.

In 2000, the American Association for the Study of Liver Diseases (AASLD) recommended a guideline to aid the practicing physicians in diagnosing PBC: the diagnosis of PBC can be made with confidence in a patient with high-titer AMA (>1:40) and elevation of serum ALP and γ-GT in the absence of an alternative explanation (normal bile ducts on ultrasound)[24].

RESULTS

Immunology

Of the 5 011 subjects tested, 8 (0.16 %) had detectable titers of M2 antibodies using the ELISA containing the purified BPO. The reactivities were confirmed using the Euroimmun' s kit. Of the 8 M2-positive subjects, 7 were females and 1 was male (age range: 40-75 years). 6 M2-positive subjects were also AMA positive tested by indirect immunofluorescence (IIF) at a titer of >1:100. Antinuclear autoantibodies (ANA) were present in subjects 2, 5 and 8. Anti-smooth-muscle antibodies (SMA) were found in subject 5. Elevated IgM was found in 5 of 8, 4 had elevated IgG, and 2 had elevated IgA (Table 1). Of the 8 reactive sera, 2 recognized PDC-E2, 2 recognized BCOADC-E2, 2 recognized both PDC-E2 and BCOADC-E2, and 2 recognized BCOADC-E2 and OGDC-E2. Each positive serum was analyzed for the identification of the reactive classes and subclasses of Ig: 2/8 had detectable IgM antibodies against BCOADC-E2 and 3/8 against PDC-E2, 2/8 contained IgA antibodies to BCOADC-E2 and 1/8 against PDC-E2, 4/8 contained IgG antibodies to BCOADC-E2, 3/8 against PDC-E2 and 2/8 against OGDC-E2. The active IgG subclass of M2 antibodies were mostly in the IgG1 and IgG2 subclasses (Table 2).

Table 2 Class and subclass of reactive M2 antibodies

BCOADC-E2	PDC-E2	OGDC-E2
IgM 2/8[a]	3/8	0/8
IgA 2/8	1/8	0/8
IgG13/8	0/8	2/8
IgG21/8	2/8	0/8
IgG30/8	1/8	0/8
IgG40/8	0/8	0/8

[a]Positive samples/total samples.

Biochemistry

An unexplained increase of serum ALP and γ-GT values, often to striking levels, was detected in 4 subjects (subjects 4, 5, 6, 8). These subjects were previously unaware of the rise in serum ALP and γ-GT. The types of clinical conditions of these subjects (such as cardiac, respiratory diseases) did not explain their high serum ALP and γ-GT levels. Other aetiological studies for viral hepatitis, haemochromatosis, α_1 antitrypsin deficiency and Wilson' s disease were also excluded. Subjects 4, 5 and 6 accorded with the AASLD' s guideline, even though they had no symptoms of PBC (such as fatigue, pruritus or jaundice).

Histology

With written consent, a percutaneous liver biopsy was obtained in two patients (Patients 5 and 6). Biopsies were routinely examined by two different pathologists. Both biopsies showed the presence of lymphoid aggregates within the portal areas and were thought to be consistent with the diagnosis of PBC.

Table 1 Clinical and laboratory data of M2-positive subjects

	M2-positive subjects No.							
	1	2	3	4	5	6	7	8
Age (y)	52	42	40	62	47	67	53	74
Sex	F	F	F	F	F	F	F	M
Laboratory parameters								
AMA titer	1:160	1:640	Negative	1:1 000	1:640	1:100	1:640	Negative
M2 (O.D.)	0.544	0.578	0.583	0.602	0.655	0.673	0.987	2.157
M2 reactivity	P	B,P	B	B	B,O	B,O	P	B,P,
Other autoantibodies	Negative	ANA	Negative	Negative	ANA,SMA	Negative	Negative	ANA
IgG (g/l)	12.12	13.73	22.95	10.57	19.30	11.65	16.35	15.66
IgM (g/l)	2.37	4.32	4.17	4.78	3.98	2.80	2.73	9.52
IgA (g/l)	3.5	1.7	3.3	2.6	3.7	14.2	1.4	9.3
ALP (U/l)	86	110	81	465	302	125	97	796
γ-GT (U/l)	43	48	20	237	211	150	42	494
T-Bil (umol/l)	12	8	13	25	8	15	14	62
ALT (U/l)	24	36	24	55	56	61	33	65
AST (U/l)	28	35	30	22	42	35	37	47
Ultrasonography	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
ERCP	ND	ND	ND	ND	Normal	Normal	ND	Normal
Liver histology	ND	ND	ND	ND	Compatible	Compatible	ND	ND
Accorded with the AASLD's guideline	No	No	No	Yes	Yes	Yes	No	No

Upper limits of normal for IgG<15.5 g/L, IgM<2.8 g/L, IgA<4.0 g/L, ALP<112 U/L, γ-GT <54 U/L, T-Bil<21 umol/L, ALT<40 U/L, AST<40 U/L; T-Bil: total serum cholesterol; ND: not done.

Clinical data

Of these 8 subjects, three were considered to be healthy (Subjects 1, 4 and 5), subject 2 had thyroid disease, subject 3 had rheumatoid arthritis who had taken rifampicin for one year, subject 6 had hypertension and non-insulin-dependent diabetes, subject 7 had pain and effusion in her knees and ankles, and subject 9 had lung cancer. None of the 8 M2-positive persons had symptoms of liver diseases.

DISCUSSION

In this study, we took advantage of the recombinant protein: BPO to investigate the prevalence of M2 antibodies in asymptomatic Chinese population and identified patients with early diseases. We revealed 8 M2-positive cases in 5 011 subjects who took an annual physical examination. All these 8 M2-positive persons had no symptoms attributed to liver diseases. Three (Subjects 4, 5 and 6) of the 8 fulfilled the AASLD' s guideline. Liver biopsy was performed in subjects 5 and 6 and the histology was compatible with PBC in both cases.

One M2-positive subject (subject 8) had an unexplained increase of serum ALP, γ-GT levels asympatomatically, but AMA was negative using IIF method. According to the guideline recommended by AASLD in 2000 and the standards recommended by other researchers, AMA have long been used as an important marker for the diagnosis of PBC[21,23], but AMA is inferior to M2 in sensitivity and specificity. Among AMA, only M2 antibodies are specific for PBC. Other sub-types of AMA have been found in diverse conditions, including drug-induced disorders, cardiomyopathies, systemic lupus erythematosus, rheumatoid arthritis, tuberculosis, syphilis and hepatitis C. Using IIF, AMA cannot be sub-classified, indicating the nonspecific nature of AMA in diagnosis of PBC[25]. About 5-17 % of patients with liver biochemcal and histological features compatible with the diagnosis of PBC had no detectable AMA using IIF method. Autoimmune cholangitis (AIC) has been proposed as a better term for these patients[26-28]. In recent years, some researches have indicated that no difference was found between AMA-positive PBC and AIC, with respect to age, liver function tests, serum IgA, IgG and IgM, T-cell oligoclonality, laparoscopic features or pathologic stage(detection)[29-34], while the detection of M2 antibodies could be achieved by ELISA or Western-blot using recombinant antigen of PDC-E2, BCOADC-E2 and OGDC-E2[11,14,15,35]. Therefore, this subject should also be diagnosed as asymptomatic PBC.

In this research, we also found that 4 M2-positive subjects with normal liver biochemistry were women over 40 years old. Many studies indicated that the presence of AMA (M2 antibodies more accurately), the hallmark of PBC, preceded the clinical manifestations of the disease for many years. In 1986, Mitchison *et al*. reported 29 patients with AMA detected at a titer of at least 1:40 by IIF when screened for an autoimmune disease. None had any symptoms of a liver disease and all had normal liver biochemistry. An 18-year follow-up of this cohort revealed that 83 % developed persistent abnormal liver biochemistry and 76 % developed symptoms of PBC[3]. In 2001, Kisand *et al*. performed ELISA tests for antibodies to PDC-E2 in 1 961 persons, then identified 14 (0.71 %) asymptomatic persons with antibodies to PDC-E2. Eight of the 14 were followed-up. Three of the 8 persons with high levels of anti-PDC-E2 developed abnormal liver biochemical test results by the ninth year of follow-up[4]. Also in 2001, Koizumi *et al*. tested sera from 1 145 corporate workers who took an annual physical examination and evaluated the liver of AMA-positive subjects. AMA were detected in 5 of 1 145 (0.44 %) workers. AMA-positive people were all females aged over 40. All of the AMA-positive sera were also positive for M2 antibodies. Liver biopsy was performed in two M2-positive cases and the histology was compatible with PBC in both cases[5]. The authors of these studies concluded that, before the advent of any clinical or biochemical indications, individuals positive for M2 did have PBC.

In several autoimmune diseases, autoantibodies are detectable in the sera of patients several years before the onset of the disease. A better example is PBC. The appearance of M2 antibodies early in the course of the disease raises the possibility that they may precede and perhaps contribute to target organ damage. That M2 antibodies are present after transplantation in the presence of normal allograft histology is consistent with this suggestion. Recent evidences have shown that M2 antibodies to intracellular targets may act directly by penetrating the biliary epithelial cells, the target of PBC and interfering with cellular function[1,36].

According to James *et al*.[21], there is a widespread impression that the number of patients with PBC is increasing, although its prevalences vary widely. The prevalence of PBC rose from 201.9 per 10^6 in the adult population and 541.4 per 10^6 women over 40 in 1987 to 334.6 per 10^6 adults and 939.8 per 10^6 women over 40 in 1994 in northern England. Due to a lack of available diagnostic equipments, there are no reliable data relating to the epidemiology of PBC in China at present, and it is presumed that PBC is rare in China. However, we checked 10 patients with cirrhosis of liver hospitalized in January, February and April of 2000 whose serum immunological studies showed there was not any sign of viral infections, and the reason for their liver cirrhosis seemed unclear. But 7 of the 10 patients were found to be M2 positive by the detailed studies of the Euroimmun Research Center (Germany). In the past two years since we established the specific immunological methods for the diagnosis of PBC, over 300 patients have been assayed,125 of them have been determined to be M2-positive. Our recent research and related domestic reports in 2001 indicate PBC is probably not so rare in China as it was thought before[19,20,37].

The three major autoantigens of M2 antibodies, i.e, BCOADC-E2, PDC-E2 and OGDC-E2 have no cross-reactivity. The M2 antibodies in serum from a confirmed patient may be reactive with one or more of them. There does not appear to be any clinical correlation with the pattern of M2 reactivity. Consequently, the use of recombinant hybrid molecule offers a rapid, simple, and sensitive ELISA for the immunodiagnosis of PBC. AMA screening using standard IIF method in a population of several thousand patients is not possible because of the economic costs. On the contrary, our methodology allows the screening of a large series of population at a minimal cost. In summary, this is one of the first studies aimed at assessing the prevalence of asymptomatic PBC in a large sample of Chinese population. Our data suggest that asymptomatic PBC in a certain Chinese population is much more common than has been supposed.

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