BRIEF ARTICLE

Primary biliary cirrhosis-specific autoantibodies in first degree relatives of Greek primary biliary cirrhosis patients

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Abstract

AIM: To determine the prevalence and significance of primary biliary cirrhosis (PBC)-specific autoantibodies in first-degree relatives (FDRs) of Greek PBC patients.

METHODS: The presence of antimitochondrial antibodies (AMA) and PBC-specific antinuclear antibodies (ANA) were determined using indirect immunofluorescence assays, dot-blot assays, and molecularly based enzyme-linked immunosorbent assays in 101 asymptomatic for liver-related symptoms FDRs of 44 PBC patients. In order to specify our results, the same investigation was performed in 40 healthy controls and in a disease control group consisting of 40 asymptomatic for liver-related symptoms FDRs of patients with other autoimmune liver diseases namely, autoimmune hepatitis-1 or primary sclerosing cholangitis (AIH-1/PSC).

RESULTS: AMA positivity was observed in 19 (only 4 with abnormal liver function tests) FDRs of PBC patients and none of the healthy controls. The prevalence of AMA was significantly higher in FDRs of PBC patients than in AIH-1/PSC FDRs and healthy controls [18.8%, 95% confidence interval (CI): 12%-28.1% *vs* 2.5%, 95% CI: 0.1%-14.7%, *P* = 0.01; 18.8%, 95% CI: 12%-28.1% *vs* 0%, 95% CI: 0%-10.9%, *P* = 0.003, respectively]. PBC-specific ANA positivity was observed in only one FDR from a PSC patient. Multivariate analysis showed that having a proband with PBC independently associated with AMA positivity (odds ratio: 11.24, 95% CI: 1.27-25.34, *P* = 0.03) whereas among the investigated comorbidities and risk factors, a positive past history for urinary tract infections (UTI) was also independently associated with AMA detection in FDRs of PBC patients (odds ratio: 3.92, 95% CI: 1.25-12.35, *P* = 0.02).

CONCLUSION: In FDRs of Greek PBC patients, AMA prevalence is significantly increased and independently associated with past UTI. PBC-specific ANA were not detected in anyone of PBC FDRs.

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INTRODUCTION

Primary biliary cirrhosis (PBC) is an autoimmune cholestatic liver disease of unknown cause which predominantly affects women and is characterized by immune-mediated destruction of the intrahepatic bile ducts, gradually leading to fibrosis, cirrhosis, and eventually, liver failure[1].

The disease is now diagnosed earlier than it was in the past as attested by the fact that more than 60% of PBC patients are asymptomatic at diagnosis as opposed to fewer than 40% a decade ago[2]. Early detection of PBC seems important, since patients with PBC, albeit asymptomatic, have reduced overall survival compared to controls[3], whereas timely treatment with ursodeoxycholic acid before the development of late-stage disease may normalize life expectancy[4,5].

The pathogenesis of PBC remains elusive, although it is generally considered to involve a combination of genetic and environmental factors[6]. The observed differences in PBC prevalence between populations living in different geographical regions but sharing similar genetic backgrounds support that environmental factors play a pivotal role in disease etiology[7,8]. Epidemiologic evidence suggests several non-genetic risk factors to be associated with PBC, including a history of urinary tract infections (UTI), past smoking and use of hormone replacement therapy[9]. Additionally, experimental data have proposed xenobiotics and several infectious agents, such as *Escherichia coli* and *Novosphingobium aromaticivorans* as initiating factors in the development of PBC[10].

Conversely, genetic factors also play an important role in conferring susceptibility to PBC, as indicated by the high concordance rate among monozygotic twins and the increased prevalence of PBC and other autoimmune disorders in patients' families[11,12]. During the past 15 years, several studies have reported familial PBC prevalence to range between 1.3% and 9%[13-18]. In addition to clustering of PBC and other autoimmune diseases in the families of PBC patients, the presence of antimitochondrial antibodies (AMA) aggregates in first-degree relatives (FDRs) of afflicted individuals as well, with AMA prevalence ranging between 1% and 13.1%[16,19-23]. Circulating AMA against the 2-oxo-acid dehydrogonase components of inner mitochondrial multi-enzyme complexes are considered the serological hallmark of PBC[1,24]. The main antigenic targets of AMA include the E2 subunits of the pyruvate dehydrogonase complex (PDC-E2), the branched-chain

2-oxo-acid dehydrogonase complex (BCOADC-E2), the oxoglutarate dehydrogenase complex (OGDC-E2), and the E1a subunit and the E3 binding protein of PDC[24]. AMA are present in 90%-95% of patients and are often detectable years before clinical signs appear[1,24]. Indeed, AMA detection in otherwise asymptomatic individuals with normal liver function tests may be associated with PBC-compatible histology in 40% of cases and eventual progression to clinically apparent disease[25,26].

In addition to AMA, previous studies have shown that antinuclear antibodies (ANA) giving a multiple nuclear dot (MND) or a rim-lime/membranous (RL/M) pattern by indirect immunofluorescence (IIF) are found in up to 65% of PBC patients if IgG isotype specific antibodies are investigated[27-29]. These antibodies seem to be disease specific (ANA-PBC specific) and have prognostic significance[27-29]. However, to the best of our knowledge these antibodies have not yet been investigated in asymptomatic for liver-related symptoms FDRs of PBC patients.

Accordingly, the aim of the present study was to determine the prevalence of AMA and ANA-PBC specific antibodies in asymptomatic for liver-related symptoms FDRs of Greek patients with PBC in comparison with asymptomatic FDRs from patients with other autoimmune liver diseases (disease control group) and healthy controls. To the best of our knowledge a combination of sensitive diagnostic techniques was used for the first time in order to improve the overall performance. In addition, we investigated the association of these antibodies with known comorbidities and reported risk factors for PBC as similar data on these issues is scarce in FDRs of PBC patients.

MATERIALS AND METHODS

Study population

From a cohort of 140 well-defined Greek PBC patients, 44 gave informed consent to participate in the study. Upon consent, patients were asked to provide information about their FDRs, i.e. parents, siblings and children. Subsequently, FDRs were contacted and were asked to participate in the study by attending an interview, physical examination and donation of blood. Accordingly, 101 FDRs of these 44 PBC patients were finally investigated. The diagnosis of PBC was based on the criteria published in our previous reports[30-33]. In brief, PBC patients met the following criteria: (1) positivity for AMA (positive titre ≥ 1/40) either by IIF on in-house fresh rodent tissue substrates confirmed by Western blot using in-house mitochondrial subtraction of rat livers, or by enhanced performance M2 enzyme-linked immunosorbent assay [M2 EP (MIT3) ELISA, QUANTA LiteR, INOVA Diagnostics, San Diego, CA], which was shown to have higher sensitivity compared to the conventional anti-M2[33]; (2) elevated cholestatic enzymes for more than 6 mo; and (3) histologic lesions consistent with PBC.

The disease control group consisted of 40 FDRs of Greek patients with well-defined autoimmune hepatitis-type 1 (AIH-1) or primary sclerosing cholangitis (PSC)

who were recruited through an identical process after an informed consent[34]. Their corresponding probands were 11 AIH-1 patients and 7 PSC patients.

All FDRs from the PBC patients and disease control patients underwent medical history and physical examination by a single investigator. The medical history was based on the US National Health and Nutrition Examination Survey questionnaire (NHANES 1999-2000)[35] with special attention to the environmental, familial and medical factors previously reported to associate with PBC[9]. Therefore, the questionnaire included data regarding demographics, smoking and alcohol consumption and past history of the FDRs. In particular, a past history of autoimmune diseases and other comorbidities reported to have increased prevalence in patients with PBC like Sjogren's syndrome, Hashimoto's thyroiditis, *etc*. was recorded in detail. From each subject serum samples were obtained and stored at -80 °C, until assayed. Finally, a healthy control group consisted of 40 age and sex matched blood donors were also investigated. The study protocol was approved by the Ethical Committee of the Larissa Medical School, University of Thessaly, Larissa, Greece.

Detection of autoantibodies
IIF-Rodent tissues: AMA were detected by IIF on an in-house rat multiorgan substrate panel that included kidney, liver, and stomach using standard protocols[36-38]. The initial screening dilution was 1/40, and positive samples were further diluted in order to determine the maximum titer. Anti-total human Ig-fluorescein isothiocyanate conjugate was used (IgG, A, M, κ and λ; Dako Ltd.; High Wycombe, Bucks, United Kingdom) according to our laboratory practice[24,31,36]. AMA titers > 1/40 were considered positive.

IIF-HEp-2cells: A commercially available IIF assay was used for the semi-quantitative determination of ANA (NOVA Lite HEp-2, INOVA Diagnostics, San Diego, CA, positive titer > 1/40).

Dot blot assay: In order to confirm AMA-reactivity and to detect other liver-related autoantibodies, all sera were further examined using a commercial line (dot) blot assay (Euroline Liver Profile, Euroimmun AG, Lübeck, Germany), according to manufacturer's instructions. The Euroline Profile assay consists of membrane strips coated with thin parallel lines of purified, biochemically characterized antigens, allowing the detection of AMA, antibodies against liver kidney microsomes type 1, antibodies against liver cytosol type 1, and antibodies against soluble liver antigen/liver-pancreas antigen.

M2 ELISA: IgG-isotype specific AMA detection was also performed with an enhanced performance M2 ELISA, which utilizes a recombinant antigen (MIT3) containing the three immunodominant mitochondrial antigenic epitopes, namely PDC-E2, BCOADC-E2 and OGDC-E2

(INOVA Diagnostics, San Diego, CA), as we have described previously[33].

Utilizing all methods for the detection of AMA we have calculated the cumulative AMA reactivity of the study population, in which serum samples were considered positive if they fulfilled one of the following criteria: (1) they were AMA positive by IIF on rat multiorgan substrate at a dilution > 1/40 accompanied by AMA reactivity either in the dot blot assay or the enhanced MIT3-based ELISA; and (2) they were AMA positive on M2 MIT3-based ELISA irrespective of the AMA titer obtained by the IIF assay.

Anti-sp100 and anti-gp210 ELISAs: Semi-quantitative detection of anti-sp100 and anti-gp210 antibodies of the IgG class was performed with commercially available specific ELISAs according to the manufacturer's instructions (QUANTA Lite sp100 and gp210, INOVA Diagnostics, San Diego, CA). Serum samples were tested in duplicate and considered positive for PBC-specific ANA only when fulfilled the following criteria: MND or RL/M reactivity by IIF on Hep2 cells accompanied by positivity against anti-sp100 or anti-gp210, respectively, by using the molecularly based assays mentioned above (ELISAs)[24].

Statistical analysis
Data normality was analyzed using the Kolmogorov-Smirnov test. Continuous, normally distributed variables are presented as mean ± SD, and non-normally distributed variables as median (interquartile range). Categorical and ordinal data are presented as frequencies and/or percentages. Student's *t*-test, Mann-Whitney *U*-test, Kruskal-Wallis test, χ^2 test, Fischer's exact test and binary logistic regression analysis were also used, where applicable. *P* values of < 0.05 were considered statistically significant.

RESULTS
Demographic characteristics
The baseline demographic characteristics of the study population are summarized in Table 1. In total, the FDRs group consisted of 141 participants (61% children, 24.1% siblings and 14.9% parents).

Since a significant percentage of the FDRs group consisted of descendants of patients with autoimmune liver diseases, the mean age in the proband group was higher than the FDRs group. However, this difference was statistically significant only in PBC probands compared to PBC FDRs (58.8 ± 11.8 *vs* 40.8 ± 17.9, *P* < 0.01). Moreover, there were no statistically significant differences in age between the PBC FDRs, the AIH-1 FDRs and the PSC FDRs group.

Clinical disorders in FDRs
The prevalence of clinical disorders reported by FDRs of patients with autoimmune liver diseases is shown in Table 2. There were no statistically significant differences

Table 1 Demographic characteristics of the study population

	Total	Male	Female	Age (yr)
PBC probands	44	4 (9.1)	40 (90.9)	58.8 ± 11.8
PBC FDRs	101	45 (44.6)	56 (55.4)	40.8 ± 17.9
Parents	9 (8.9)	5 (55.6)	4 (44.4)	72.8 ± 8.8
Children	69 (68.3)	30 (43.5)	39 (56.5)	31.3 ± 10.6
Siblings	23 (22.8)	10 (43.5)	13 (56.5)	56.7 ± 10.7
AIH-1 probands	11	2 (18.2)	9 (81.8)	48.8 ± 22.0
AIH-1 FDRs	21	12 (57.1)	9 (42.9)	37.2 ± 14.0
Parents	3 (14.3)	1 (33.3)	2 (66.7)	46.0 ± 19.3
Children	13 (61.9)	7 (53.8)	6 (46.2)	36.6 ± 10.3
Siblings	5 (23.8)	4 (80)	1 (20)	33.6 ± 20.0
PSC probands	7	4 (57.1)	3 (42.9)	40.7 ± 9.5
PSC FDRs	19	9 (47.4)	10 (52.6)	49.9 ± 21.0
Parents	9 (47.4)	4 (44.4)	5 (55.6)	67.4 ± 9.4
Children	4 (21.1)	2 (50)	2 (50)	17.8 ± 2.2
Siblings	6 (31.6)	3 (50)	3 (50)	45.0 ± 6.1
Healthy controls	40	17 (42.5)	23 (57.5)	43.1 ± 9.8

Data are shown as *n* (%) or mean ± SD. PBC: Primary biliary cirrhosis; AIH-1: Autoimmune hepatitis-1; PSC: Primary sclerosing cholangitis; FDRs: First-degree relatives.

Table 2 Surgical history and comorbidities in first degree relatives of patients with autoimmune liver diseases and healthy controls *n* (%)

	PBC FDRs (*n* = 101)	AIH-1 and PSC FDRs (*n* = 40)	Healthy (*n* = 40)
History of comorbidities			
Sjögren's syndrome	7 (6.9)	1 (2.5)	0
Raynaud's syndrome	8 (7.9)	2 (5)	0
Autoimmune thyroiditis	4 (3)	0	0
Rheumatoid arthritis	1 (1)	0	0
Systemic lupus erythematosous	0	1 (1)	0
Asthma	3 (3)	4 (10)	0
Atopic dermatitis	2 (2)	1 (2.5)	0
Vitiligo	1 (1)	0	0
Psoriasis vulgaris	1 (1)	0	0
Erythema nodosum	1 (1)	0	0
Type 1 diabetes mellitus	1 (1)	1 (2.5)	0
Idiopathic thrombocytopenic purpura	1 (1)	0	0
Osteoporosis	4 (4)	3 (7.5)	0
Urinary tract infections	33 (32.7)[a]	11 (27.5)	6 (15)
Hypercholesterolemia	10 (9.9)	7 (17.5)	4 (10)
Hypertension	6 (5.9)	5 (12.5)	3 (7.5)
History of surgery			
Tonsillectomy	14 (13.9)	3 (7.5)	3 (7.5)
Appendectomy	18 (17.8)	7 (17.5)	5 (12.5)
Cholecystectomy	6 (5.9)	1 (2.5)	1 (2.5)
Inguinal hernia	14 (13.9)[b,c]	0	1 (2.5)

[a]*P* < 0.05 *vs* healthy controls; [b]*P* = 0.01 *vs* autoimmune hepatitis-1 (AIH-1)/primary sclerosing cholangitis (PSC) first-degree relatives (FDRs); [c]*P* = 0.05 *vs* healthy controls. PBC: Primary biliary cirrhosis.

between FDRs of patients with PBC and FDRs of patients with AIH-1 or PSC, with the exception of a higher reported prevalence of inguinal hernia and subsequent surgical repair in PBC FDRs (Table 2). Of note, PBC FDRs reported significantly higher prevalence of UTI compared to healthy controls [32.7%, 95% confidence interval (CI): 23.8%-42.8% *vs* 15%, 95% CI: 6.3%-30.5%,

Table 3 Autoantibody prevalence in first degree relatives of patients with primary biliary cirrhosis, compared to autoimmune hepatitis-1 and primary sclerosing cholangitis first degree relatives and healthy controls *n* (%)

Autoantibody	PBC FDRs (*n* = 101)	AIH-1 and PSC FDRs (*n* = 40)	Healthy controls (*n* = 40)	*P*[1]	*P*[2]
AMA (IIF)	10 (9.9)	1 (2.5)	0	NS	< 0.001
1:80	8 (7.9)	1 (2.5)	-		
1:160	1 (1)	-	-		
1:320	1 (1)	-	-		
IgG MIT3-based ELISA	13 (12.9)	0	0	0.02	0.02
AMA positivity[3]	19 (18.8)	1 (2.5)	0	0.01	0.003
RL/M pattern	8 (7.9)	1 (2.5)	0	NS	NS
1:80	8 (7.9)	1 (2.5)	-		
MND pattern	9 (8.9)	5 (12.5)	0	NS	0.06
1:80	7 (6.9)	3 (7.5)	-		
1:160	1 (1.0)	-	-		
1:320	-	2 (5.0)	-		
1:640	1 (1.0)	-	-		
PBC-specific ANA[3] positivity	0	1 (2.5)	0	NS	NS

[1]Comparison between primary biliary cirrhosis (PBC) first degree relatives (FDRs) and autoimmune hepatitis-1 or primary sclerosing cholangitis (AIH-1/PSC) FDRs; [2]comparison between PBC FDRs and healthy controls; [3]for definition of antimitochondrial antibodies (AMA) and PBC-specific antinuclear antibodies (ANA) positivity please see text (materials and methods section). IIF: Indirect immunofluorescence; ELISA: Enzyme-linked immunosorbent assay; RL/M: Rim-lime/membranous; MND: Multiple nuclear dot; NS: Not significant.

P < 0.05] (Table 2). Binary logistic regression analysis demonstrated that only a past history of UTI was independently associated with PBC FDRs (odds ratio: 2.89, 95% CI: 1.1-7.6, *P* = 0.03).

Autoantibody prevalence in FDRs
According to the above-mentioned criteria, AMA were detected in 20 FDRs of patients with autoimmune liver diseases (14.2%, 95% CI: 9.1%-21.3%) and none of the healthy controls (0%, 95% CI: 0%-10.9%, *P* = 0.008). The prevalence of AMA was also significantly higher in FDRs of patients with PBC compared to FDRs of patients with AIH-1 or PSC (18.8%, 95% CI: 12%-28.1% *vs* 2.5%, 95% CI: 0.1%-14.7%, *P* = 0.01; Table 3). PBC-FDRs had significantly increased AMA prevalence compared to healthy controls (18.8%, 95% CI: 12%-28.1% *vs* 0%, 95% CI: 0%-10.9%, *P* = 0.003).

ANA reactivity on Hep-2 cells is presented in Table 3. Specifically, antibodies giving RL/M pattern of ANA were observed in 7.9% (95% CI: 3.7%-15.5%) of PBC-FDRs. RL/M pattern of ANA was also observed in one PSC-FDR and in none of the controls; however, this difference did not approach significance. MND pattern of ANA tended to be detected in more AIH-1/PSC-FDRs (12.5%, 95% CI: 4.7%-27.6%) and PBC-FDRs (8.9%, 95% CI: 4.4%-16.7%) compared to none of healthy controls (*P* = 0.05 and *P* = 0.06, respectively). According to the predefined criteria of the study, PBC-specific ANA positivity was only observed in one FDR of a PSC patient.

Table 4 Characteristics of first degree relatives according to antimitochondrial antibodies positivity

	AMA positive (*n* = 20)	AMA negative (*n* = 121)	P
Demographics			
Female	12 (60)	63 (52.1)	NS
Age (yr)	47.8 ± 17.8	40.4 ± 18.0	NS
Proband disease (PBC)	19 (95)	87 (67.8)	0.012
Educational level, %			NS
Primary	41.70	26.50	
Secondary	33.30	39.30	
Higher	25	34.20	
Body mass index (kg/m^2)	25.7 ± 3.9	25.7 ± 5.0	NS
Smoking and alcohol consumption			
Smoking	8 (40)	30 (27.5)	NS
Daily alcohol intake (g)	19.8 ± 38.2	17.7 ± 43.5	NS
Medical history			
Urinary tract infection	11 (55)	33 (27.3)	0.01
Number of UTI events (range)	4 (1-5)	1 (1-2.5)	NS
Sicca symptoms	4 (20)	19 (15.7)	NS
Autoimmune disorders	3 (15)	5 (4.1)	NS
Autoimmune thyroid disease	2 (10)	2 (1.7)	NS
Diabetes	1 (5)	3 (2.5)	NS
Osteoporosis	0 (0)	7 (5.8)	NS
Tonsillectomy	4 (20)	13 (10.7)	NS
Appendectomy	3 (15)	22 (18.2)	NS
Inguinal hernia	2 (10)	12 (9.9)	NS
Laboratory values (U/L)			
AST	29.6 ± 11.4	23.6 ± 9.7	NS
ALT	32.6 ± 25.5	26.1 ± 26.4	NS
γ-GT	29.6 ± 24.6	28.9 ± 37.7	NS
ALP	76.0 ± 23.3	73.5 ± 35.7	NS

Data are shown as *n* (%) or mean ± SD. UTI: Urinary tract infections; AMA: Antimitochondrial antibodies; PBC: Primary biliary cirrhosis; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γ-GT: Gamma-glutamyl transpeptidase; ALP: Alkaline aminophosphatase; NS: Not significant.

FDR characteristics associated with AMA positivity

Demographic and clinical characteristics according to the presence of AMA are presented in Table 4. AMA-positive FDRs were predominantly female (60%). Age, level of education, smoking habits, body mass index, and daily alcohol consumption were not significantly associated with AMA-positivity. However, a medical history of UTI was significantly more frequently reported in AMA-positive FDRs, compared to AMA-negative FDRs, whereas a trend towards a higher number of UTI recurrences in AMA-positive FDRs was observed (Table 4). Binary logistic regression revealed having a proband with PBC or a positive history for UTI were both independently associated with AMA positivity (odds ratio: 11.24, 95% CI: 1.27-25.34, *P* = 0.03 and odds ratio: 3.92, 95% CI: 1.25-12.35, *P* = 0.02, respectively).

Clinical and laboratory manifestations of PBC in AMA-positive FDRs

Of the 20 FDRs, who were determined positive for AMA reactivity, 5 (25%) presented with abnormal liver enzymes and their characteristics are shown in Table 5. Male FDRs with AMA were asymptomatic either for liver- or extrahepatic-related symptoms whereas one female

Table 5 Characteristics of antimitochondrial antibodies-positive relatives with abnormal liver enzymes

	Age (yr)	Proband disease	Relationship to the proband	AST/ ALT (U/L)	ALP/ γ-GT (U/L)	Symptoms
Patient A	44	PBC	Son	37/45	102/122	No
Patient B	47	PBC	Brother	23/18	65/104	No
Patient C	40	PBC	Son	37/90	67/154	No
Patient D	24	PBC	Daughter	60/165	136/61	Sicca + Hashimoto's thyroiditis
Patient E	52	PSC	Sister	53/103	91/94	Hashimoto's thyroiditis

AST: Aspartate aminotransferase (upper limit: 35 U/L); ALT: Alanine aminotransferase (upper limit: 40 U/L); γ-GT: Gamma-glutamyl transpeptidase (upper limit: 37 U/L); ALP: Alkaline aminophosphatase (upper limit: 104 U/L); PBC: Primary biliary cirrhosis; PSC: Primary sclerosing cholangitis.

suffered from Sicca syndrome and two from Hashimoto thyroiditis. Physical examination was unremarkable in all 5 subjects. To date, none of these subjects have agreed to undergo liver biopsy.

DISCUSSION

The data reported herein demonstrate that FDRs of patients with PBC have increased prevalence of AMA compared to healthy controls, whereas the presence of PBC-specific ANA was similar among all groups. During the seventies, two studies investigating the autoantibody prevalence in relatives of patients with PBC reported AMA detection in approximately 7.5% of the screened subjects[19,20]. In a subsequent study, Caldwell *et al*[21] reported a somewhat lower prevalence of 4.9% in 41 FDRs of 27 PBC probands. Using IIF, Floreani *et al*[16] observed AMA positivity in 6.1% of 115 relatives of unspecified relationship to 30 PBC probands. Conversely, in a 2004 study from Brazil, AMA prevalence in FDRs was estimated to be as low as 1%[22]. Taken together, these studies illustrate that AMA prevalence in PBC FDRs varies significantly according to the method used for AMA detection and their prevalence in the general population from which the study population is derived. Indeed, several studies from Europe have reported AMA prevalence in the general population to range between 0.5% and 0.9%, while in studies from China and Japan it was lower (range: 0.16% to 0.64%), demonstrating again slight variances according to methodology used and the origin of the populations studied[39-42].

In the most comprehensive study concerning AMA prevalence in FDRs of PBC patients conducted in Minnesota, an area with one of the highest reported PBC prevalence[43], Lazaridis *et al*[23] found AMA positivity in 13.1% of the FDRs and 1% of healthy controls. AMA prevalence in FDRs of PBC patients in our study was found to be slightly higher (18.8%) compared to that recorded in all the abovementioned reports[16,19-23]. In particular, taking into account that first, the proportion of female FDRs

in the study of Lazaridis *et al*[23] was higher (64%-68.5%) compared to the present study, and second, that their population consisted predominantly of older FDRs, the actual difference in AMA prevalence in FDRs of Greek and US patients with PBC might be even higher.

One possible explanation for the higher AMA prevalence in FDRs of Greek PBC patients compared to the previous reports[16,19-23] is that we used a combination of diagnostic techniques for AMA detection which may improve the overall sensitivity. Indeed, to the best of our knowledge this is the first study to utilize a combination of IIF, MIT3-based ELISA and dot-blot for the detection of AMA in FDRs of PBC patients. It is well known that almost 90% of AMA-positive PBC sera react to PDC-E2, while approximately 10% of them react only to BCOADC-E2 and/or OGDC-E2 indicating that, as we have previously demonstrated, testing for AMA by the first generation commercial M2-ELISAs (using only the PDC-E2 as the primary substrate) or using only IIF would result in low sensitivity of AMA testing[33]. The latter has been shown recently by our group since up to 12.6% of AMA-negative PBC patients by IIF were MIT3-ELISA positive whereas 4.8% of MIT-3 negative PBC sera were AMA positive by IIF[33].

An alternative explanation for the relatively high rates of AMA positivity in FDRs of Greek PBC patients observed in our study could be attributed to the background of the study population. Although no published data on PBC prevalence exist for Greece, subjects of Greek descent have been shown to carry the highest prevalence of PBC in an Australian study[7]. Of course, the participation of a relatively low number of patients' families (44 families out of 140 patients) may have led to selection bias, which theoretically could influence our analysis.

Of interest, AMA prevalence was significantly higher in FDRs reporting a past history of UTI with a trend towards a higher number of UTI recurrences in AMA-positive FDRs. This finding was stable even after multivariate analysis suggesting UTI as an independent factor for AMA detection among FDRs of PBC patients. Epidemiological data have suggested past history of UTI as a risk factor for PBC development[9] whereas patients with recurrent UTI have an increased incidence of AMA seropositivity[44]. Recurrent UTI have been suggested to increase the exposure to microbial antigens like *Escherichia coli* which may then induce the development of PBC in genetically susceptible individuals through molecular mimicry mechanisms[45].

The second major finding of this study is that FDRs of Greek patients with PBC do not have increased prevalence of PBC-specific ANA. Using HEp2 cells as substrate, ANA in PBC patients are heterogeneous[24,46]; however, ANA presenting with the MND or rim-like membranous (RLM) pattern by IIF have proven to be clinically significant for PBC[24,46]. The molecular targets of ANA with the MND pattern in PBC are mainly the nuclear body protein sp100 and less often the promyelocytic leukemia protein, small ubiquitin-related modifiers and more recently SP140 antigen, whereas the molecular

targets of the RLM pattern are structural components of the nuclear envelope primarily gp210, nucleoporin p62 and lamin B receptor[24,46-48]. A possible limitation of our study is that we have determined reactivities only against the two most common molecular targets of PBC-specific ANA, namely anti-gp210 and anti-sp100, which are highly specific for PBC and have been found in up to 65% of PBC patients if IgG-isotype specific antibodies (IgG1, 2, 3 and 4) are investigated[24,27-29,46,49].

According to the available evidence, there are significant differences between AMA- and ANA-reactivity in patients with PBC, since AMA may appear several years before the clinical or histological signs of disease, whereas PBC-specific ANA are more frequently found in those patients with advanced disease[24,27,28,46,50]. Moreover, the detection of PBC-specific ANA seems to confer prognostic information, because when ANAs become detectable during the course of disease, a higher rate of disease progression could be expected[24,46,49,51,52]. Therefore, the lack of PBC-specific ANA in AMA-positive FDRs might suggest a pre-clinical stage of the disease. Furthermore, the detection of PBC-specific ANA in FDRs who exhibit AMA-positivity may indicate further diagnostic evaluation. According to a cohort study by Invernizzi *et al*[52], antibodies against the nuclear pore complex (anti-NPCs), may aid prognostic evaluation in patients presenting at early PBC stages, i.e., those with normal total bilirubin levels at presentation. Actually, the contribution of anti-NPC detection in a prognostic model incorporating the Mayo score and the histological stage was greater in early PBC cases.

In conclusion, using a combination of tests for AMA detection, we have provided evidence that AMA prevalence in asymptomatic for liver-related symptoms FDRs of Greek patients with PBC is significantly increased compared to FDRs of Greek patients with other autoimmune liver diseases and healthy controls. This prevalence is in fact the highest reported to date among FDRs of PBC patients and it is significantly associated with past history of UTI. Further large-scale multicentre prospective studies with the use of combination techniques for AMA detection are needed in order to precisely define the AMA prevalence in FDRs from PBC patients and assess whether these subjects will develop the clinical and biochemical spectrum of PBC in the future. We are currently undertaking such follow-up in our AMA positive FDRs from PBC patients. PBC-specific ANA are not detected in FDRs of PBC patients. Nevertheless, in our opinion the sequential determination of the latter antibodies as a marker of progression from a pre-clinical to an early clinical stage should be further evaluated.

COMMENTS

Background

Primary biliary cirrhosis (PBC) is an autoimmune liver disease characterized usually by an insidious or even silent onset with long asymptomatic course which can eventually lead to cirrhosis and liver failure. The laboratory hallmark of the disease is the detection of antimitochondrial antibodies (AMA). Early diagnosis is crucial for timely treatment and improvement of the quality of life and

survival. Among several factors, genetic predisposition is important in conferring susceptibility to PBC. Indeed, during the past 15-20 years, several studies have reported familial clustering of PBC whereas the presence of AMA seems to aggregate in first-degree relatives (FDRs) of afflicted individuals as well, with AMA prevalence among FDRs ranging between 1% and 13.1%.

Research frontiers

The research hotspot is to estimate the prevalence and associations of AMA along with PBC-specific ANA with known risk factors of PBC development in asymptomatic FDRs of Greek patients with PBC. This could be very important in an attempt to identify new asymptomatic PBC cases and potentially alter the disease course by the prompt initiation of the indicated therapy.

Innovations and breakthroughs

In the present study, the authors estimated the prevalence and significance of AMA in FDRs of PBC patients using for the first time a combination set of sensitive diagnostic techniques (indirect immunofluorescence, dot-blot assays, and molecularly based enzyme-linked immunosorbent assays) in order to improve the overall performance of AMA detection. In addition, they assessed for the first time, the significance of PBC-specific ANA in the same population since these autoantibodies are known prognostic factors for PBC outcome and progression.

Applications

Based on their results the authors believe that further large-scale multicentre prospective studies with the use of combination techniques for AMA detection (not only indirect immunofluorescence) are needed in order to precisely define the AMA prevalence in FDRs of PBC patients and assess whether these subjects will develop the clinical and biochemical spectrum of PBC or not in the future.

Terminology

PBC is an autoimmune cholestatic liver disease of unknown etiology with clustering in families of PBC patients whereas AMA detection is higher in FDRs of PBC patients compared to healthy population. The use of combination tests for AMA detection in FDRs of PBC patients seems to be the design of choice in order to identify asymptomatic individuals who potentially will develop the disease since the present study showed the highest incidence of AMA in FDRs of PBC ever reported using this methodology. This is of particular importance especially among those with a past history of urinary tract infection.

Peer review

This study described the prevalence and significance of AMA and PBC-specific ANA in FDRs of Greek PBC patients. The authors concluded that AMA prevalence in this group of subjects is significantly increased compared to healthy and FDRs of patients with other autoimmune liver diseases like autoimmune hepatitis-1 or primary sclerosing cholangitis. In addition, AMA detection in FDRs of PBC patients was independently associated with past history of urinary tract infections. To the contrary, PBC-specific ANA were not detected in PBC FDRs. Therefore, the study provides significant data, which is of great importance, as it includes original and useful data for these otherwise healthy subjects (FDRs of PBC patients).

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