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High Frequency of Antiphospholipid Antibodies in Primary Biliary Cirrhosis

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Aim: To evaluate, retrospectively, the frequency of autoantibodies of antiphospholipid syndrome (APLS) in Tunisian patients with primary biliary cirrhosis (PBC). *Patients and methods:* We analyzed 80 PBC sera and 80 sera from blood donors. ELISA was used to determine the frequency of antibodies against cardiolipin (aCL IgG, IgA, and IgM) and beta 2 glycoprotein I (aβ2GPI IgG, IgA, and IgM). *Results:* The frequency of antiphospholipid antibodies (aCL and/or aβ2GPI) was significantly higher in PBC patients than in controls (70 vs. 5%, $P < 10^{-6}$). The frequency of aCL antibodies (IgG, IgA or IgM) was significantly higher in PBC patients than in the control group (23.7 vs. 3.7%, $P = 0.0005$). The frequencies of aCL IgA and aCL IgM in PBC patients' sera were significantly higher than those in the control group (10 vs. 0%, $P = 0.003$ and 20 vs. 2.5%, $P = 0.001$, respectively). Two patients of eighty (2.5%) had aCL IgG, aCL IgA and aCL IgM. The frequency of aβ2GPI antibodies (IgG, IgA, or IgM) was significantly higher in PBC patients than in the control group (70 vs. 1.2%, $P < 10^{-6}$). The frequencies of aβ2GPI IgG, aβ2GPI IgA, and aβ2GPI IgM in PBC patients' sera were significantly higher in patients than in the control group (12.5 vs. 0%, $P = 0.003$; 62.5 vs. 1.2%, $P < 10^{-6}$; and 21.2 vs. 0%, $P < 10^{-4}$, respectively). *Conclusion:* Autoantibodies related to APLS (aCL and aβ2GPI) were present in the majority of patients with PBC, reflecting the ability of these antibodies to engage mediators of damage. J. Clin. Lab. Anal. 29:32–36, 2015.

Key words: primary biliary cirrhosis; antiphospholipid antibodies; anticardiolipin antibodies; anti-beta 2 glycoprotein I antibodies; Tunisia

INTRODUCTION

Primary biliary cirrhosis (PBC) is a slowly progressive autoimmune disease of the liver that primarily affects women. In terms of pathology, PBC is characterized by portal inflammation and immune-mediated destruction of the intrahepatic bile ducts (1). The most characteristic feature of PBC is the presence of circulating antimitochondrial antibodies (AMAs) that are directed against components (collectively named M2) of the inner mitochondrial multienzyme 2-oxoacid dehydrogenase complexes (2).

The etiology of the disease remains elusive, although genetic, epigenetic, environmental, and infectious factors have been considered important for the induction of the disease in genetically prone individuals (3, 4). Many autoantibodies, related to other autoimmune diseases, have been detected in PBC patients' sera (5), such as antibodies to nuclear antigen (6), thyroid gland (7), *Saccharomyces cerevisiae* (8), and serological markers of celiac disease (9) and of rheumatoid arthritis (10).

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TABLE 1. Epidemiologic Features of Patients With PBC and of the Control Group

	PBC ($n = 80$)	Control group ($n = 80$)
Sex ratio (F/M)	12 (74/6)	12 (74/6)
Mean age	58 years	21 years 4 months
Age range	22–85 years	17–45 years

The association between PBC and antiphospholipid (aPL) antibodies was previously described (5, 11–14). Among these previous studies, only one has done the three isotypes of aCL (anticardiolipin) antibodies and the three isotypes of aβ2GPI (anti-beta 2 glycoprotein I) antibodies. On the other hand, Gabetta et al. demonstrated that aβ2GPI IgA were associated with clinical and biochemical markers of disease severity in PBC (14). So, the aim of our study was to evaluate, retrospectively, the frequency of aPL antibodies (aCL IgG, aCL IgA, aCL IgM, aβ2GPI IgG, aβ2GPI IgA, and aβ2GPI IgM) in a large series of Tunisian patients with PBC.

PATIENTS AND METHODS

Patients

In our retrospective multicentric study, sera from 80 PBC patients, with positive AMAs, (74 females and 6 males, mean age 58 years; range 22–85 years) were included from the database of our laboratory. Sera were collected between 1997 and 2010 from four hospitals in the center of Tunisia. In all patients, the diagnosis of PBC was based on liver histology and/or liver biochemistry, and AMA positivity (15).

Sera of 80 sex-matched blood donors were served as normal controls (Table 1). These controls are not age-matched with patients because blood donors are young but mean age of PBC patients was 58 years. All sera were stored at −80 °C until use. The study was approved by the local ethics committee and all patients gave their informed consent.

Methods

AMAs

AMAs were detected by indirect immunofluorescence on cryostat sections of rat liver, kidney, and stomach as we described previously (16). Briefly, sections were incubated with sera diluted 1:100 in phosphate-buffered saline (PBS) for 30 min in a humidified chamber. After three washes in PBS, the sections were incubated for 30 min with fluorescein isothiocyanate conjugated anti-human IgG antibodies (Bio-Rad®, Marnes-La Coquette, France) used as secondary antibody (diluted 1:10). The immunofluorescence patterns were assessed under a fluorescence microscope. The typical "granular" positivity within the cytoplasm of cells in kidney, stomach, and hepatocytes is considered as M2 AMA reactivity.

aCL antibodies assays

Serum samples were evaluated for aCL IgG, IgA, and IgM by using a commercial enzyme-linked immunosorbent assay (ELISA; Orgentec Diagnostika®, Mainz, Germany) as we described previously (17). Results were expressed as arbitrary units with a cutoff for positivity of 10 U/ml for IgA and IgG, and 7 U/ml for IgM following the manufacturer's instructions.

aβ2GPI antibodies assays

Determinations of aβ2GPI IgG, IgA, and IgM were carried out with a commercial ELISA (Orgentec Diagnostika®) using a purified human β2GPI as we described previously (18). Results were expressed as arbitrary units with a cutoff for positivity of 8 U/ml, following the manufacturer's instructions.

Statistical Analysis

The comparison of frequencies of aPL antibodies was performed using Chi-square or Fisher's exact test. A P-value less than 0.05 was considered significant. All calculated P-values are two-tailed.

RESULTS

aCL and aβ2GPI antibodies' frequencies are summarized in Table 2. The frequency of aPL antibodies (aCL or aβ2GPI) in PBC patients was significantly higher than that in the control group (70 vs. 5%, $P < 10^{-6}$). O 56 patients who had aPL antibodies, 16 had high levels (>40 U/ml).

Frequencies of aCL IgG, IgA, and IgM

Nineteen patients of eighty had aCL (23.7%) antibodies. aCL (IgG, IgA, or IgM) antibodies were significantly more frequent in PBC patients than in the control group (23.7 vs. 3.7%, $P = 0.0005$). The frequencies of aCL IgA and aCL IgM in PBC patients' sera were significantly higher than those in the control group (10 vs. 0%, $P = 0.003$ and 20 vs. 2.5%, $P = 0.001$, respectively). The frequency of aCL IgG in PBC patients was not statistically different from that of the control group (3.7 vs. 1.2%).

Two patients of nineteen had three isotypes of aCL antibodies, but none of the control group had these three

TABLE 2. Frequency of aCL and aβ2GPI Antibodies in Patients With PBC and in Control Group

Autoantibodies	PBC patients ($n = 80$)	Control group ($n = 80$)	P
aPL (aCL et/ou aβ2GPI)	70% (56/80)	5% (4/80)	$<10^{-6}$
aCL IgG, aCL IgA, or aCL IgM	23.7%[a] (19/80)	3.7% (3/80)	0.0005
aCL IgG	3.7% (3/80)	1.2% (1/80)	NS
aCL IgA	10%[b] (8/80)	0%	0.003
aCL IgM	20% (16/80)	2.5% (2/80)	0.001
aβ2GPI IgG, aβ2GPI IgA, or aβ2GPI IgM	70%[a] (56/80)	1.2% (1/80)	$<10^{-6}$
aβ2GPI IgG	12.5% (10/80)	0	0.003
aβ2GPI IgA	62.5%[b] (50/80)	1.2% (1/80)	$<10^{-6}$
aβ2GPI IgM	21.2% (17/80)	0%	$<10^{-4}$

[a]Comparison between aCL (IgG, IgA, or IgM) and aβ2GPI (IgG, IgA, or IgM; $P < 10^{-6}$) antibodies.
[b]Comparison between aCL IgA and aβ2GPI IgA ($P < 10^{-6}$).

isotypes. Four patients had two isotypes and thirteen patients had one isotype.

Frequencies of aβ2GPI IgG, IgA, and IgM

Fifty-six patients of eighty had aβ2GPI (70%) antibodies. The frequency of aβ2GPI antibodies (IgG, IgA, or IgM) was significantly higher in PBC patients than in the control group (70 vs. 1.2%, $P < 10^{-6}$). aβ2GPI IgG, aβ2GPI IgA, and aβ2GPI IgM were significantly more frequent in PBC patients than in control group (12.5 vs. 0%, $P = 0.003$; 62.5 vs. 1.25%, $P < 10^{-6}$; 21.2 vs. 0%, $P < 10^{-4}$, respectively). Of 56 patients with aβ2GPI antibodies, 38 had one isotype, 13 had two isotypes, and 4 had three isotypes of aβ2GPI.

Comparison between aCL and aβ2GPI antibodies

In PBC patients, the frequency of aβ2GPI antibodies (IgG, IgA, or IgM) was significantly higher than that of aCL antibodies (IgG, IgA, or IgM; 70 vs. 23.7%, $P < 10^{-6}$). aβ2GPI IgA was significantly more frequent than IgA aCL (62.5 vs. 10%, $P < 10^{-6}$).

DISCUSSION

aPL antibodies have been primarily described in systemic lupus erythematosus and in antiphospholipid syndrome (APLS) (19). Then, these autoantibodies have also been identified in patients with PBC (12). Furthermore, the presence of serum aPL in PBC could be of interest, as some kind of vasculopathy of the small vessels surrounding the bile ducts has been postulated to play a role in the pathogenesis of PBC (20).

This study demonstrated a significantly higher prevalence of aPL antibodies (70%) in a cohort of 80 AMA-positive PBC patients compared to the healthy group (5%). The frequency of aPL antibodies in our PBC patients was lower than that found by Klein et al. (93%). This discrepancy could be explained by the fact that we included 80 PBC patients compared to 14 patients in the study of Klein et al. (11). On the other hand, in the study of Klein et al., sera were tested for aPL antibodies by an in-house ELISA using cardiolipin, β2GPI, phosphatidylserine, and thromboplastine as antigen, but in our study we tested only cardiolipin and β2GPI (11).

We found that the frequency of aCL antibodies in patients with PBC was significantly higher than that in normal population (23.7 vs. 3.7%, $P = 0.0005$). Our aCL frequency was significantly lower than that of a previous study about 99 patients with PBC (45.5%) (13), and higher than that, found by Klein et al., about 14 PBC patients (14%) (11). On the other hand, Agmon-Levin et al. (5) analyzed the frequency of aCL antibodies of only two isotypes (IgG and IgM) in a cohort of 69 AMA-positive PBC patients, and they did not found any positivity. In our study, we have done not only IgG and IgM but also aCL IgA, which was significantly more frequent in patients than in control group. Our frequency of aCL IgA

TABLE 3. Frequency of aPL Antibodies in Patients With PBC in Literature

Authors	Number of patients	aPL antibodies (%)	aCL IgG (%)	aCL IgA (%)	aCL IgM (%)	aβ2GPI IgG (%)	aβ2GPI IgA (%)	aβ2GPI IgM (%)
Klein et al. (11)	14	93 (aCL, aβ2GPI, thromboplastine	7	7	0	60	29	36
von Landenberg et al. (12)	51				75			59
Zachou et al. (13)	99		27.3		27.3	2		
Agmon-Levin et al. (5)	69		0		0	15		15
Gabeta et al. (14)	96			13.5			27	
Our study	80	70	3.7	10	20	12.5	62.5	21.2

(10%) was similar to that found by Gabetta et al. (13.5%) and Klein et al. (7%) (11, 14).

The question arises whether the association between PBC and aCL antibodies is explained by cross-reactivity between aCL antibodies and AMAs. In fact, it is known that mitochondria contain cardiolipin (21). Meroni et al. found that mitochondrial membranes were able to absorb AMA-M5 fluorescence completely and were also able to inhibit, in a dose-dependent manner, aCL activity. These authors concluded that AMA type M5 cross-reacts with cardiolipin (22) while, in PBC, we have AMA type 2 and not M5.

In this study, the frequency of aβ2GPI antibodies (IgG or IgA or IgM) was 70%. This high frequency was due to that of the aβ2GPI IgA (62.5%). Even in our previous study on SLE (18), IgA was the most frequent isotype of aβ2GPI. On the other hand, it has been reported that IgA is the dominant isotype of aPL antibodies in Afro-Caribbeans (23) and also in Afro-Americans (24). Klein et al. found that IgG was the predominant isotype with a frequency of 60% similar to that of our predominant isotype, which is IgA (62.5%) (11). Gabeta et al. demonstrated that aβ2GPI IgA antibodies were associated with clinical and biochemical markers of disease severity in PBC (14).

A literature research revealed some case reports of coexisting PBC and APLS (25, 26). Hoffman et al. described a 47-year-old female who was admitted for severe pain of 1-month duration in the third and fourth toes of the right foot, culminating in gangrene. Laboratory findings revealed liver enzyme abnormalities and positive antimitochondrial and aPL antibodies. Therefore, a diagnosis of APLS associated with PBC was done (25). Gupta et al. reported a case of an association between PBC and APLS and recommended that PBC patients should undergo screening tests for APLS, considering the high risk of life-threatening thrombosis (26). In the study of Efe et al., APLS was identified in 1 patient among 31 with autoimmune hepatitis/PBC overlap syndrome. These authors demonstrated in their study that hepatic and extrahepatic autoimmune diseases may occur in the same patient and explained this association by "mosaic of autoimmunity" (27).

In this study, we had included 80 patients with PBC. These patients were diagnosed between 1997 and 2010. The high frequency of PBC in the center of Tunisia could be explained by an environment factor that triggers PBC in patients with genetic predisposition. Among these environment factors is *S. cerevisiae.* It is known that the staple food in Tunisia is bread. In fact, Rinaldi et al. demonstrated that bread baking increases the risk to have autoimmune diseases (28). *S. cerevisiae* could be considered as an environment factor to many autoimmune diseases. Indeed, we demonstrated a high frequency of anti-*S. cerevisiae* antibodies in many autoimmune diseases (8, 18, 29–31).

We could also explain the high frequency of aβ2GPI antibodies in our PBC patients by the fact that aβ2GPI antibodies had a cross-reactivity with *S. cerevisiae* as demonstrated by Krause et al. (32). In the same way, we have previously demonstrated a higher frequency of ASCA in PBC patients than in the general population (8).

In conclusion, our study demonstrated a high frequency of aPL antibodies, and especially aβ2GPI, in PBC Tunisian patients. A long-term prospective study is needed to address whether this finding is of clinical importance in PBC patients.

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CONFLICT OF INTEREST

None of the authors have conflicts of interest to declare.

REFERENCES

1. Bogdanos DP, Gershwin ME. What is new in primary biliary cirrhosis? Dig Dis 2012;30(Suppl 1):20–31.
2. Gershwin ME, Rowley M, Davis PA, Leung P, Coppel R, Mackay IR. Molecular biology of the 2-oxo-acid dehydrogenase complexes and anti-mitochondrial antibodies. Prog Liver Dis 1992;10:47–61.
3. Uibo R, Kisand K, Yang CY, Gershwin ME. Primary biliary cirrhosis: A multi-faced interactive disease involving genetics, environment and the immune response. APMIS 2012;120:857–871.
4. Svyryd T, Hernandez-Molina G, Vargas F, Sanchez-Guerrero J, Segovia DA, Mutchinick OM. X chromosome monosomy in primary and overlapping autoimmune diseases. Autoimmun Rev 2012;11:301–304.
5. Agmon-Levin N, Shapira Y, Selmi C, Barzilai O. A comprehensive evaluation of serum autoantibodies in primary biliary cirrhosis. J Autoimmun 2010;34:55–58.
6. Hall S, Axelsen PH, Larson DE, Bunch TW. Systemic lupus erythematosus developing in patients with primary biliary cirrhosis. Ann Intern Med 1984;100:388–389.
7. Nakamura H, Usa T, Motomura M, et al. Prevalence of interrelated autoantibodies in thyroid diseases and autoimmune disorders. J Endocrinol Invest 2008;31:861–865.
8. Sakly W, Jeddi M, Ghedira I. Anti-*Saccharomyces cerevisiae* antibodies in primary biliary cirrhosis. Dig Dis Sci 2008;53:1983–1987.
9. Kingham JG, Parker DR. The association between primary biliary cirrhosis and coeliac disease: A study of relative prevalences. Gut 1998;42:120–122.
10. Caramella C, Avouac J, Sogni P, Puéchal X, Kahan A, Allanore Y. Association between rheumatoid arthritis and primary biliary cirrhosis. Joint Bone Spine 2007;74:279–281.
11. Klein R, Goller S, Bianchi L. Nodular regenerative hyperplasia (NRH) of the liver: A manifestation of "organ-specific antiphospholipid syndrome?" Immunobiology 2003;207:51–57.

12. von Landenberg P, Baumgartner M, Schoelmerich J, Lackner KJ, Klein R. Clinical relevance of antiphospholipid antibodies in primary biliary cirrhosis. Ann NY Acad Sci 2005;1051: 20–28.
13. Zachou K, Liaskos C, Rigopoulou E, Gabeta S, et al. Presence of high avidity anticardiolipin antibodies in patients with autoimmune cholestatic liver diseases. Clin Immunol 2006;119:203–212.
14. Gabeta S, Norman GL, Gatselis N, et al. IgA anti-ß2GPI antibodies in patients with autoimmune liver diseases. J Clin Immunol 2008;28:501–511.
15. Kaplan MM. Primary biliary cirrhosis. N Engl J Med 1996;335:1570–1580.
16. Bargou I, Mankaï A, Jamaa A, et al. Detection of M2 antimitochondrial antibodies by dot blot assay is more specific than by enzyme linked immunosorbent assay. Pathol Biol 2008;56: 10–14.
17. Mankaï A, Achour A, Thabet Y, Manoubia W, Sakly W, Ghedira I. Anti-cardiolipin and anti-beta 2-glycoprotein I antibodies in celiac disease. Pathol Biol 2012;60:291–295.
18. Mankaï A, Sakly W, Thabet Y, Achour A, Manoubi W, Ghedira I. Anti-*Saccharomyces cerevisiae* antibodies in patients with systemic lupus erythematosus. Rheumatol Int 2013;33:665–669.
19. Asherson RA, Khamashta MA, Ordi-Ros J, et al. The "primary" antiphospholipid syndrome: Major clinical and serological features. Medicine (Baltimore) 1989;68:366–374.
20. Abe M, Masumoto T, Ninomiya T, et al. Hyperplastic liver nodules associated with early-stage primary biliary cirrhosis mimicking hepatocellular carcinoma. Dig Dis Sci 2000;45:1563–1567.
21. Ernster L, Schatz G. Mitochondria: A historical review. J Cell Biol 1981;91:227s–255s.
22. Meroni PL, Harris EN, Brucato A, et al. Anti-mitochondrial type M5 and anti-cardiolipin antibodies in autoimmune disorders: Studies on their association and cross-reactivity. Clin Exp Immunol 1987;67:484–491.
23. Molina JF, Gutierrez-Ureña S, Molina J, et al. Variability of anticardiolipin antibody isotype distribution in 3 geographic populations of patients with systemic lupus erythematosus. J Rheumatol 1997;24:291–296.
24. Diri E, Cucurull E, Gharavi AE, et al. Antiphospholipid (Hughes') syndrome in African-Americans: IgA aCL and abeta2 glycoprotein-I is the most frequent isotype. Lupus 1999;8:263–268.
25. Hoffman M, Burke M, Fried M, Turner D, Yosipov Y, Yust I. Primary biliary cirrhosis associated with antiphospholipid syndrome. Isr J Med Sci 1997;33:681–686.
26. Gupta V, Balar B, Gbadehan E, Orleans LK, Ozick LA. A rare association of primary biliary cirrhosis with antiphospholipid [corrected] antibody syndrome. Dig Dis Sci 2007;52:3530–3531.
27. Efe C, Wahlin S, Ozaslan E, et al. Autoimmune hepatitis/primary biliary cirrhosis overlap syndrome and associated extrahepatic autoimmune diseases. Eur J Gastroenterol Hepatol 2012;24:531–534.
28. Rinaldi M, Perricone R, Blank M, Perricone C, Shoenfeld Y. Anti-*Saccharomyces cerevisiae* autoantibodies in autoimmune diseases: From bread baking to autoimmunity. Clin Rev Allergy Immunol 2013.
29. Toumi D, Mankaï A, Belhadj R, Ghedira-Besbes L, Jeddi M, Ghedira I. Anti-*Saccharomyces cerevisiae* antibodies in coeliac disease. Scand J Gastroenterol. 2007;42:821–826.
30. Sakly W, Mankaï A, Sakly N, et al. Anti-*Saccharomyces cerevisiae* antibodies are frequent in type 1 diabetes. Endocr Pathol 2010;21:108–114.
31. Mankaï A, Thabet Y, Manoubi W, Achour A, Sakly W, Ghedira I. Anti-*Saccharomyces cerevisiae* antibodies are elevated in Graves' disease but not in Hashimoto's thyroiditis. Endocr Res 2013;38:98–104.
32. Krause I, Blank M, Cervera R, et al. Cross-reactive epitopes on beta2- glycoprotein-I and *Saccharomyces cerevisiae* in patients with the antiphospholipid syndrome. Ann NY Acad Sci 2007;1108:481–488.