Japanese Journal of Gastroenterology and Hepatology

Research Article ISSN 2435-1210 |Volume 6

Anti-Ribosomal P (Anti-P) Antibodies in Autoimmune Hepatitis Patients

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Received: 01 May 2021
Accepted: 22 May 2021
Published: 27 May 2021

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Keywords:

Autoimmune hepatitis; Anti-ribossomal P protein antibody; Autoantibodies

Citation:

Gallo C. Anti-Ribosomal P (Anti-P) Antibodies in Autoimmune Hepatitis Patients. Japanese J Gstro Hepato. 2021; V6(14): 1-4

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Abbreviations:

AIH: Autoimmune hepatitis; Anti-P: Anti-ribosomal P protein antibody; ANA: Antinuclear antibodies; SMA: Smooth muscle antibodies; LKM1: Anti-liver kidney microsomal antibody type 1; HLA: histocompatibility leucocyte antigen; SLE: Systemic lupus erythematous; ELISA: Enzyme-linked immunosorbent assay; CLIA: Chemiluminescence assay

1. Abstract

1.1. Introduction: A few studies have investigated the occurrence of anti-ribosomal P antibody (Anti-P) in autoimmune hepatitis (AIH), based on the partial overlap of clinical and pathological features of AIH and systemic lupus erythematosus (SLE), for which anti-P is a diagnostic biomarker. In face of the controversial results obtained, this study aimed to evaluate the frequency of anti-P determined by two different immunoassays in a cohort of AIH patients.

1.2. Methods: One-hundred seventy-seven patients with AIH diagnosis were screened, and 142 were analyzed for the presence of anti-P antibodies. Samples were analyzed by two different immunoassays, namely enzyme-linked immunosorbent assay (ELISA) and chemiluminescence (CLIA). Positive samples were submitted to western blot assay (WB). A comparison was done with a group of 60 SLE patients.

1.3. Results: Anti-P was found in 5/142 AIH patients (3.5%) using CLIA. No AIH patient was anti-P-positive using ELISA. Among the five positive AIH samples by CLIA, one was negative, two weakly positive, and two were anti-P-positive in WB. Anti-P was found in 10/60 SLE patients (16.7%) and presented higher CLIA units than in AIH samples.

1.4. Conclusion: Anti-P antibody was confirmed to occur in AIH at low frequency with serum levels lower than those observed in SLE. This marker seems not to be useful for the management of patients with AIH.

2. Introduction

Autoantibodies are a hallmark of autoimmune liver diseases [1]. Autoimmune hepatitis (AIH) is the emblematic member of these entities and is largely distributed worldwide, with a prevalence varying from 8.0-18.3/100,000 [2, 3], in a proportion of 3.6 females/male Despite wide distribution among races, Caucasians are predominantly affected. Clinically, a spectrum of aspects can be expected, ranging from acute hepatitis presentation to chronic or overt liver disease, and one fourth of the patients are diagnosed already with advanced stage of liver fibrosis [4].

The gold standard diagnostic features remain the histopathological findings in liver biopsy, characterized by interface hepatitis, lymphoplasmcytic infiltrate, and rosette formation [5]. Autoantibodies are

also important in the diagnosis, mainly represented by anti-smooth muscle antibodies - SMA (actin-F fraction), antinuclear antibodies (ANA), anti-liver and kidney microsomal antibodies type-1 (LKM1), and anti-soluble liver antigen (SLA-LP) antibodies, sometimes in association with other autoantibodies at lower frequency [1].

Disruption in regulatory T cell balance leading to increased production of Th17 [6], cytotoxicity, apoptosis, necroptosis, and antibody production causes perennial stimulus of the inflammatory cascade that finally leads to hepatic tissue lesion, consequently evolving to fibrosis and cirrhosis [5, 7]. Part of these mechanisms, as well as some clinical features, are remarkably similar in AIH and other systemic autoimmune diseases, particularly systemic lupus erythematous (SLE).

One of the important biomarkers in SLE, associated with specific disease features, is the anti-ribosomal P protein antibody (anti-P). This autoantibody is found in 6 to 46% of SLE patients and is associated with specific manifestations of disease, such as type V nephritis, hepatitis, and neuropsychiatric involvement [8]. Based on several similar features of these two autoimmune diseases, the possibility that anti-P autoantibody could also be present in AIH not associated to SLE was considered. In fact, Calich et al [9]. found anti-P in nine of 93 (9.7%) patients with non-SLE associated AIH, and anti-P antibody was associated with higher frequency of cirrhosis in the long-term follow-up of these patients. However, these findings have not been confirmed in other studies [10-12]. Due to these controversial findings, the aim of this study was to evaluate the frequency of anti-P antibodies in patients with AIH.

3. Material and Methods

3.1. Study Design

This is a prospective study to evaluate the presence of anti-ribosomal P antibody (anti-P) in patients diagnosed with autoimmune hepatitis. The study has been conducted at the Gastroenterology and Hepatology Division, Federal University of São Paulo, Sao Paulo, Brazil, and was approved by the Institutional Ethics Committee under the code 1.524.672.

3.2. Patients

Patients with autoimmune hepatitis diagnosis based on the criteria of the International Autoimmune Hepatitis Group [13] were consecutively recruited from 2015 to 2019. Patients with overlap syndrome and other associated causes of liver disease (HBV, HCV, alcohol, NASH) were excluded. A cohort of 60 patients with confirmed diagnosis of SLE, followed at the Rheumatology Division of the same University, were included as a control group.

3.3. Methods

Gender, age, AIH classification, autoantibody positivity, and fibrosis stage at presentation were recorded. The fibrosis stage was determined by histological analysis of liver biopsies, or by evident clinical signs of cirrhosis.

The following autoantibodies were determined: antinuclear antibodies (ANA), using standard indirect immunofluorescence on HEp-2 cells (HEp-2 IFA); and anti-smooth-muscle (SMA), anti-mitochondrial (AMA), and LKM-1, using indirect immunofluorescence (IIF) with labeled anti-human antibody in rodent tissue [1].

Two different methods were used to evaluate the presence of anti-P antibodies and the concordance between them was analyzed: chemiluminescence (CLIA) and enzyme-linked immunosorbent assay (ELISA). For CLIA, QUANTA Flash® Ribosomal P kit (Inova Diagnostics, San Diego CA, USA) was used according to the instructions of the manufacturer. Paramagnetic beads coated with the antigenic determinants were incubated with patient serum. After appropriate washing, isoluminol-conjugated anti-human IgG was added to the beads. Following washing as before, activating buffer was added, changing isoluminol to luminol, and the luminescence produced was measured as relative light units - chemiluminescent units (CU) - by an optical system BIO-FLASH (Biokit, Werfen Medical LTDA, Barcelona, Spain).

For ELISA, QUANTA LiteTM Ribosome P kit (Inova Diagnostics, San Diego, CA, USA) was used according to the instructions of the manufacturer. Patient serum was added to microplate wells coated with P dominant epitope leading to autoantibody binding. After washing, enzyme-conjugated anti-human IgG was added to the microplates. After washing as before, enzyme substrate and chromogenic indicator were added to the wells, and the color of the reaction was read by spectrophotometry.

Samples positive in CLIA were further processed in Western Blot (WB) as previously reported [1]. Briefly, rabbit reticulocyte lysate was separated in 12% polyacrylamide gel electrophoresis and transferred to nitrocellulose sheets. Individual serum samples diluted 1:100 in 5% slim milk in 0.05% Tween 20 phosphate buffered saline (MT-PBS) were incubated with vertical nitrocellulose strips for 30 minutes at room temperature. Following washing with T-PBS, strips were incubated with peroxidase-conjugated goat anti-human IgG diluted 1:20, 000 in MT-PBS for 30 minutes. After washing as before, strips were incubated with H_2O_2 and chromogenic solution until optimal development of color. The reaction was interrupted with 1M H_2SO_4.

3.4. Statistical Analysis

Results are presented as descriptive analysis to identify the frequency of positive results in the groups. The Chi-square test was used to compare AIH patients with SLE patients. A reference level of 5% ($p<0.05$) was considered statistically significant. The analysis was conducted with statistical software IBM SPSS (v23) and free software Open Epi 3.0.

4. Results

During the period of study, 177 patients with AIH diagnosis were recruited. Thirty-five patients were excluded due to imprecision in diagnosis, lack of data, or association with other causes of liver disease, rendering a final cohort of 142 subjects. The general characteristics

of the subjects can be seen in (Table 1).

CLIA for anti-P was performed in all AIH patients and was positive in five (3.5%), whereas ELISA for anti-P was evaluated in 103 AIH patients, including the five positive by CLIA, and was negative in all of them. The five anti-P-positive samples were tested in WB assay. One sample was negative (20.8 CU in CLIA), two weakly positive (23.3 and 25.8 CU in CLIA), and two strongly positive (33.9 and 42.8 CU in CLIA). As for the SLE, 10 out of 60 (17.7%) patients had anti-P detected in CLIA (Table 2).

Table 1: Demographic, clinical, and laboratory characteristics of the 142 AIH patients included in the study.

Characteristic	N = 142
Age median and range (years)	47 (18 – 76)
Female gender, n (%)	129 (89.5)
Associated autoimmune diseases, n (%)	14 (10)
Type of AIH, n (%)	
Type 1, n (%)	135 (93.8)
Type 2, n (%)	9 (6.2)
Advanced liver fibrosis, n (%)	61 (42)
HEp-2 IFA positive, n (%)	119 (82.6)
SMA positive, n (%)	62 (43)
Anti-LKM-1 positive, n (%)	7 (5.2)
AMA positive, n (%)	11 (7.7)
IgG levels (mg/dL)	773-5307 (median 1696)

AIH: autoimmune hepatitis; IFA: indirect immunofluorescence assay; SMA: smooth muscle antibody; LKM-1: liver-kidney microsomal antibody type 1; AMA: antimitochondrial antibody; IgG: immunoglobulin type G

Table 2: Anti-P antibodies in patients with AIH and SLE

Anti-P assay	AIH patients			SLE patients		%	p
	Positive	Negative	%	Positive	Negative		
CLIA	5	137	3.5	10	50	16.7	<0.001
ELISA	0	103	0	-	-	-	

AIH: autoimmune hepatitis; SLE: systemic lupus erythematosus; anti-P: Anti-P: anti-ribosomal P protein antibody; ELISA: enzyme-linked immunosorbent assay; CLIA: chemiluminescence assay

The number of anti-positive AIH was too low to allow statistical comparison with anti-negative patients, but some characteristics of these patients should be pointed out. Four of the five anti-P-positive patients were cirrhotic, and one had score F2. Among AIH patients, CU values ranged from 20.8 to 42.8 CU (median 25 CU), and among SLE patients, from 31.7 to 60.3 (median 33.5 CU).

5. Discussion

New markers for diagnosis and disease progression are extremely needed in AIH patients to assist establishing correct diagnosis, evaluating therapy interventions, defining treatment withdrawal, and estimating long-term survival. Furthermore, better understanding of triggers of the autoimmune process is also needed [7, 8].

Pathogenesis of autoimmune diseases rests over three main pillars: genetic susceptibility, environmental triggers, and impairment of tolerance mechanisms, which ultimately leads to self-reactive T cell activation. Cytokines also play an important role in the autoimmune process and consequent tissue damage. In SLE models, immuno-complex mediated inflammation is driven by pro-inflammatory cytokines, mainly produced in hepatocytes. Increased serum levels of several cytokines in SLE patient strongly suggest their involvement in disease pathogenesis. Disruption in the immune system balance has been also demonstrated in AIH, driven by IL4, IL10, TGF-beta and IL17, besides regulatory T cells (Treg) alterations, confirming the similarities in the pathogenic mechanisms of both diseases [6, 1].

Due to these immunological and clinical similarities between AIH and SLE, the possibility of anti-P as a potential biomarker in AIH has been raised. This hypothesis has been investigated, but divergent results have been reported. Some authors have described a positive association with more severe forms of AIH, and others argued methodological differences driving contradictory results [9-12]. Considering that AIH is associated with peculiar immunogenetic patterns (DR3, DR4, DR7, DR13) in different populations worldwide, we sought to conduct a study in a cohort of Brazilian patients with AIH in order to confirm previous results in our population using different methods [9].

In order to investigate the presence and impact of anti-P antibodies in patients with AIH, CLIA and ELISA anti-P immunoassays were performed in 142 sequentially selected patients, and the results were compared to those obtained in a group of SLE patients, where 10-20% frequency of anti-P is expected, reaching 40% in severe forms of the disease, according to some authors [1].

Results confirmed the occurrence of anti-P in AIH patients, although at a very low frequency (3.5%). For the sake of comparison, 60 SLE patients were assayed in the anti-P CLIA, and 10 (16.7%) turned out to be positive. This frequency is in the expected range for SLE and demonstrates appropriate performance of the CLIA

assay in this study. Interestingly, the positive anti-P results obtained using CLIA were not confirmed in the ELISA anti-P test but were confirmed in 4/5 cases in western blot analysis. The herein observed low frequency of anti-P antibodies in AIH confirms previous reports that this autoantibody specificity is possible in this autoimmune liver disease [11, 12], albeit at a considerably lower frequency in comparison to SLE.

There are studies comparing different techniques to detect anti-P antibodies and, despite the possibility of false-negative results of indirect immunofluorescence, high sensitivity in ELISA and immunoblotting have been reported, and these methods seem to have a reasonable correlation index [11, 2, 2]. The observed differences can be partially attributed to the fact that anti-P antibodies detection is highly dependent on the antigenic epitopes available in each assay platform. Anti-P antibodies are known to react with a conserved epitope at the carboxy-terminal domain of the three main ribosomal autoantigens P0, P1, and P2. This short immunodominant epitope is widely used in solid-phase immunoassays, such as ELISA, CLIA, and line blot. In the present study, the observed anti-P reactivity of AIH patients was confirmed using CLIA and WB, but not in ELISA. Discordant results in different solid-phase immunoassays are relatively common, especially with samples with low reactivity.

It is also possible that in AIH the finding of anti-P antibodies at a low frequency could be related to the inflammatory nature of this liver disease, ultimately exposing anti-P target epitopes, characterizing an epiphenomenon.

This study has some limitations. Although the number of patients is larger than other studies evaluating anti-P reactivity in AIH patients, data regarding the presence and role of this autoantibody in SLE patients are derived from larger casuistics. Furthermore, the groups with AIH and SLE were not paired according to ant variable.

In conclusion, the present study has shown that although anti-P antibodies can be detected in AIH patients, they seem not to be related to any special feature. They are present in low number of patients and not related to any aspect of the disease. According to our data anti-P has no major implication in phisiopathology of AIH neither seems to be useful as a relevant biomarker for this disease.

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