Autoimmunity Reviews 18 (2019) 102389

Contents lists available at ScienceDirect

Autoimmunity Reviews

journal homepage: www.elsevier.com/locate/autrev

Association of solid-phase assays to the indirect immunofluorescence in primary biliary cholangitis diagnosis: Results of an Italian multicenter study

ARTICLE INFO

Keywords:
Primary biliary cholangitis
Anti-mitochondrial antibodies
Antinuclear antibodies
Indirect immunofluorescence
ELISA
Immunoblotting

Dear Editor,

The aim of this letter is to highlight the crucial role of new antigen-specific tests for better diagnostic strategy in the field of primary biliary cholangitis (PBC) along with the optimal agreement among solid-phase assays (ELISA and Immunoblotting).

PBC is a relatively rare multifactorial chronic cholestatic liver disease characterized by immune-mediated destruction of intrahepatic bile ducts and by the presence of increased values of specific anti-mitochondrial antibodies (AMA) in plasma [1,2]. The diagnosis is currently based on evidence of elevated values of alkaline phosphatases (ALP) and positivity to AMA and/or to specific Anti Nucleus Antibodies (ANA) [1]. AMA positivity is typically observed in over 90% PBC patients, [3]. Antinuclear antibodies (ANA) are present in approximately 50–70% of patients with PBC. Speckled, multiple nuclear dot (MND), rim-like/membranous (RL/M) and anti-centromere (ACA) are the most frequently detectable ANA patterns in PBC patients [4]. Among ANA, MND (i.e., reflecting anti-sp100 reactivity) and RL/M (i.e., reflecting anti-gp210 reactivity) are specific for PBC and positive in approximately 30–50% of PBC patients, thus reflecting an evidently low diagnostic sensitivity [3,4]. The detection of these autoantibodies allows confirming the diagnosis of PBC in AMA-negative patients, even if the presence of PBC sera AMA negative carries a significant risk of misclassification [5]. AMA, MND and RL/M have originally been detected as immunofluorescent patterns. The identification of relevant target auto-antigens such as E2 component of pyruvate dehydrogenase (PDH), sp100 and gp210, has allowed developing ELISA-based diagnostic assays along with specific immunoblotting tests based on recombinant or purified antigens [6–8]. Moreover, molecular testing allows detecting antibodies against nuclear protein (gp210) and nuclear body protein (sp100) more accurately and objectively than using IIF on Hep2 cells [3,9,10]. Therefore, the assessment of antinuclear antibodies in PBC seems promising, but its clinical impact remains uncertain. This background of uncertainty has prompted us to investigate whether (i) the identification of AMA and PBC-specific anti-gp210 and anti-sp100 by solid-phase assays (immunoblotting and ELISA assays) would increase the diagnostic sensitivity compared to immunofluorescence techniques and (ii) the use of panels of autoantibodies would permit serological confirmation of PBC in AMA negative patients compared to a single test, thus reducing the misclassification rate.

We designed a multicenter study aimed to recruit patients with PBC or suspected PBC according to validated international criteria [2] from the Hospitals of Parma, Modena, Reggio Emilia and Piacenza between January 2014 and March 2017. These reference centers were chosen for their experience with AMA-Indirect Immunofluorescence (IIF) testing and for the high number of tests annually performed in each center. The local Ethical Committees approved the study protocol, which was carried out in accordance with the Declaration of Helsinki.

Anti-nuclear antibodies (ANA) and anti-mitochondrial antibodies (AMA), PBC Screen, MIT3, M2, gp210 and sp100 antigens were assayed in all serum samples. ANA and AMA were assessed by IIF (Alphadia, Wavrem, Belgium, provided by Alifax, Padova, Italy) on Hep-2 cells and on section of rat kidney, stomach and liver, respectively. All serum samples were tested according to manufacturer's instruction, with *Multiple Immunodot Liver profile 7 Ag*® (Alphadia, Wavrem, Belgium, provided by Alifax, Padova, Italy). This immunoblot contains the PBC-associated antigens M2/native PDC (E1, E2, E3 subunits of Pyruvate Dehydrogenase Complex, purified from bovine heart), gp120 (recombinant human) and sp100 (recombinant human), as well as four autoimmune hepatitis specific antigens (LKM1, LC1, SLA and F-actin). Four ELISA kits were used (Inova, San Diego, USA): QUANTA Lite® MIT3 (immunodominant portion of PDC-E2, BCOAD-E2, OGDC-E2), QUANTA Lite® gp210, QUANTA Lite® sp100 and QUANTA Lite® PBC screen IgG/IgA (purified recombinant antigen MIT3 and purified fragment of gp210 and sp100). The diagnostic performance was calculated for each CBP-associated autoantibody. Cohen's kappa with 95% confidence interval (95% CI) was used to assess the agreement of anti-mitochondrial autoantibodies, anti-sp100 and anti-gp210 with different analytical techniques (i.e., IIF, ELISA, Immunoblotting). The degree of agreement was graded accordingly to Altman et al. [11].

The final study population consisted of 165 patients diagnosed with PBC (mean age, 65.5 years; range 36–89 years; male/female ratio, 1:9). *The autoantibody profiles* of the 165 PBC patients are shown in Table 1. Overall, AMA test was found to be positive in 147 (89.1%) PBC patients using the IIF assay. IIF positivity on HEp 2 cells was instead found in 22 (13.3%) and 23 (13.9%) PBC patients regarding MND and RL/M

https://doi.org/10.1016/j.autrev.2019.102389
Received 5 June 2019; Accepted 9 June 2019
Available online 11 September 2019
1568-9972/

Table 1
Prevalence of PBC specific antibodies detected with different methods in 165 PBC patients.

	N° positive patients (%)
Indirect immunofluorescence	
AMA	147 (89.1%)
Multiple nuclear dots (MND)	22 (13.3%)
Rim-like/membranous (RL/M)	23 (13.9%)
Antinuclear antibody (MND-centromere-RL/M, speckled ecc)	91 (55.1%)
ELISA	
PBC screen (IgG and/or IgA)	156 (94.5%)
Mit3 (IgG)	145 (87.9%)
sp100 (IgG)	28 (17.0%)
gp210	31 (18.8%)
Multiple Immunodot Liver Profile (IgG)	
M2/native PDC	152 (92.1%)
sp100	30 (18.2%)
gp210	27 (16.4%)

pattern, respectively. The AMA-M2 sensitivity was 92.1% with multiple Immunodot Liver profile, whilst anti-sp100 and anti-gp210 antibodies were positive in 18.2% and 16.4% patients, respectively. The PBC screen with ELISA was positive in 156 (94.5%) PBC patients, 145 (87.9%), 28 (17.0%) and 31 (18.8%) of whom tested positive for MIT3, sp100 and gp210 with specific ELISA tests, respectively (Table 1). A moderate *agreement* for anti-mitochondrial autoantibodies was observed among AMA-M2, MIT3 and IIF-AMA (kappa values comprised between 0.538 and 0.465), whilst a better agreement was observed between AMA-M2 and MIT3 (kappa value, 0.698). Unlike these findings, an almost perfect agreement was found for CBP-specific ANA between anti-sp100dot and anti-sp100 ELISA, and between anti-gp210 dot and anti-gp210 ELISA (kappa values of 0.875 for both).

The overlap of PBC specific autoantibodies identified with ELISA and multiple Immunodot Liver profile is shown in Fig. 1. Among all patients with positive ELISA tests, 22 (13.3%) displayed double reactivity for MIT3 and sp100, 20 (12.1%) displayed double reactivity for MIT3 and gp210, and 4 (2.4%) displayed reactivity for all these antigens. As concerns the Multiple Immunodot Liver profile, 23 PBC patients (13.9%) displayed double reactivity for AMA-M2 and sp100, 16 (9.7%) for AMA-M2 and gp210, 5 (3.0%) for all these antigens. Regarding *combined diagnostic value of PBC specific autoantibodies*, when the PBC screen assay was combined with IIF-AMA, the diagnostic sensitivity significantly increased from 0.89 to 0.98 ($p < 0.01$). Similarly, when Multiple Immunodot Liver profile test was combined with IIF-AMA, the diagnostic sensitivity also increased from 0.89 to 0.99 ($p < 0.01$).

Our findings suggest that the combination of solid-phase techniques with AMA IIF may be effective to improve the sensitivity (from 0.89 to over 0.98) when associated with ELISA or immunodot, as recently reported for autoimmune rheumatic diseases [12–16]. The data obtained on concordance between ELISA and immunoblotting lead to hypothesize two possible combinations of solid-phase techniques and AMA IIF. The choice of the best option depends on many factors, including expertise, technologies available to the local laboratory, prevalence of

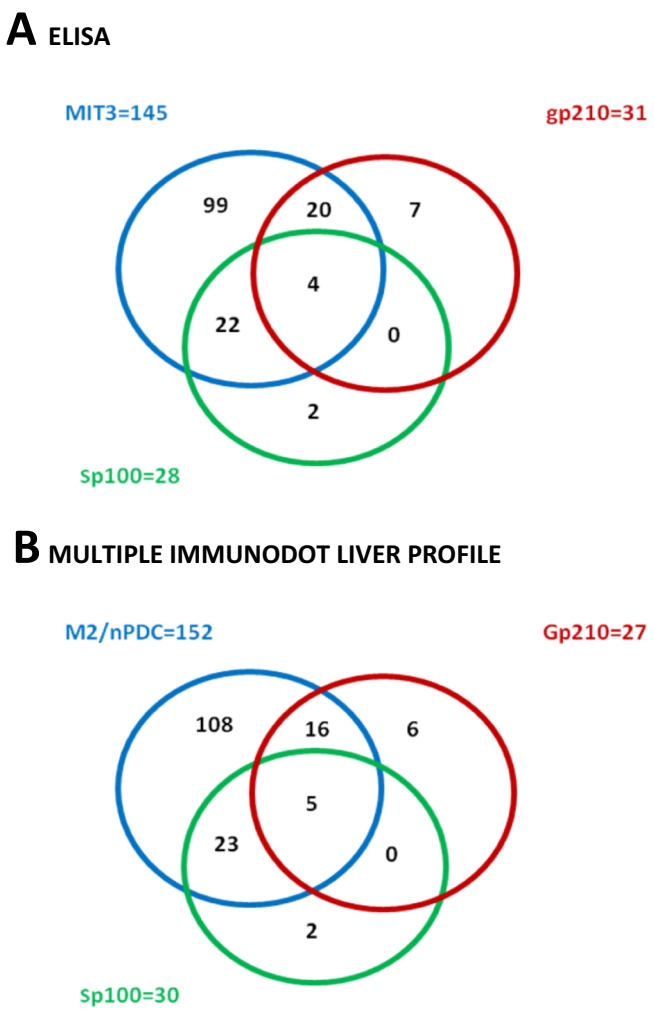

Fig. 1. Overlap of PBC specific autoantibodies in PBC samples. The serum samples were tested both by ELISA (A) and multiple Immunodot liver profile assays (B).

PBC-associated autoantibodies, level of diagnostic accuracy needed and overall expenditure. In this new scenario, the clinical governance of autoimmune diagnostics in the area of liver diseases remains crucial [17].

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