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REVIEW

Molecular diagnostic testing for primary biliary cholangitis

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ABSTRACT

Introduction: A reliable liver autoimmune serology for the diagnosis of primary biliary cholangitis (PBC) is of particular importance. Recognition of patients at early stages and prompt treatment initiation may alter the outcome, slow progression, delays liver failure, and improves survival.

Areas covered: In this review, we summarize and discuss the published data obtained from literature searches from PubMed and The National Library of Medicine (USA) and our own experience on the current and potential molecular based approaches to the diagnosis of PBC.

Expert commentary: Standardization of liver diagnostic serology and clinical governance are two major points as antimitochondrial antibodies are the diagnostic hallmark of the disease and PBC-specific antinuclear antibodies could assist in the diagnosis and estimation of prognosis. New biomarkers such as novel autoantibodies, genetic polymorphisms, metabolomic profiling, micro-RNA and epigenetics may assist to the understanding, diagnosis and management of the disease.

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1. Introduction

Primary biliary cholangitis (PBC) – previously known as primary biliary cirrhosis – is the most prevalent autoimmune liver disease, characterized by the presence of specific antibodies namely, antimitochondrial antibodies (AMA) and progressive nonsuppurative destruction and loss of the intrahepatic bile ducts, resulting in cholestasis, portal inflammation, and fibrosis that may lead to cirrhosis and liver failure [1,2]. The disease predominantly affects women typically diagnosed in their fifth and sixth decade, although younger patients have been described [1]. The course of the disease is variable and an accurate diagnosis at early stages is of major importance as prompt treatment, even at the asymptomatic phase, can slow progression, delay liver failure, and improve survival [3].

Diagnosis of PBC is based on a combination of clinical, biochemical, immunological, and eventually histological evidences; thus, it can be established by the presence of at least two of the following criteria [4]: (a) AMA-positivity, (b) unexplained elevated cholestatic enzymes for more than 6 months, and (c) compatible liver histology, specifically nonsuppurative cholangitis and interlobular bile duct injury. Of note, as many as 5–10% of PBC patients are AMA negative, but their disease appears to be identical to that of AMA-positive patients.

As AMA are detected in 90–95% of patients with PBC, they have been considered as the key diagnostic feature of the disease. Besides, AMA may be detectable in serum when patients are asymptomatic with normal liver tests. Histological evidence of PBC has been found in nearly 40% of AMA-positive asymptomatic individuals with normal liver enzymes, and long-term follow-up suggests that PBC will develop in the majority of them [5].

Besides, recent continuing studies have also shown the presence of PBC-specific antinuclear antibodies (ANA). PBC-specific ANA detection seems to be of diagnostic and prognostic value [6–9]. Finally, there is evidence that new biological markers such as novel autoantibodies [10], genetic polymorphisms [11], metabolomics [12], and microRNA (miRNA) [13] may prove of significant importance of understanding PBC pathogenesis and subsequently assist to diagnosis and management of the disease. In this concise review, we summarize and discuss the literature data and our own experience on the current and potential molecular-based approaches to the diagnosis of PBC patients.

2. AMA

The presence of AMA in PBC patients has been known for more than five decades, when for the first time Ian Mackay found in a PBC case high titers of circulating complement-fixing autoantibodies directed against the liver, kidney, and other human tissue antigens [14]. In the following years, sera from PBC patients were found to manifest a characteristic pattern when tested against animal tissues by indirect immunofluorescence (IIF) and the cytoplasmic target identified as the mitochondria. It was not until 1987, that AMA antigens were cloned and identified. In fact using a λgt11 cDNA library, Gershwin et al. identified the cDNA encoding the 70-kDa mitochondrial antigen corresponding to the E2 subunit of pyruvate dehydrogenase complex (PDC-E2) [15]. Afterward, the same group identified the other mitochondrial autoantigens as two other components of the 2-oxo-acid dehydrogenase complexes (2-OADC) [16].

Given their high sensitivity and specificity, the presence of AMA represents one of the three criteria required for a definite diagnosis of PBC [4]. The autoantibodies are mainly directed to the E2

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Table 1. Classification of PBC-specific autoantibodies and antigens.

Antibody	Antigen	
Antimitochondrial antibodies	PDC	E2 subunit (PDC-E2) E1a subunit (PDC-E1a) E1b subunit (PDC-E1b) E3-binding protein (PDC-E3BP)
	BCOADC	E2 subunit (BCOADC-E2)
	OGDC	E2 subunit (OGDC-E2)
PBC-specific antinuclear antibodies	Nuclear pore complex antigens*	gp210 NUP62 LBR
	Nuclear envelope antigens**	Nuclear body sp100 Nuclear body sp140 SUMOs PML

PDC: Pyruvate dehydrogenase complex; BCOADC: branched chain 2-oxo-acid dehydrogenase complex; OGDC: oxoglutarate dehydrogenase complex; gp210: glycoprotein 210 kDa; NUP62: nucleoporin p62; LBR: lamin B receptor; sp100: speckled 100 kDa; sp140: speckled 140 kDa; SUMOs: small ubiquitin-related modifiers; PML: promyelocytic leukemia proteins; PBC: primary biliary cholangitis.
*Rim-like perinuclear immunofluorescence pattern on HEp-2 cells.
**Multiple nuclear dot immunofluorescence pattern on HEp-2 cells.

subunits of the 2-OADC (PDC-E2, 2-oxoglutarate dehydrogenase complex [OGDC-E2], branched-chain 2-oxo-acid dehydrogenase complex [BCOADC-E2]), and to a lesser extent to the E1 and E3 subunits (Table 1). Up to 90% of AMA-M2 antibodies are directed against PDC-E2, whereas about 50% react with OGDC-E2 and BCOADC-E2 [1,2,4,17].

In regard with AMA subclassification according to the old nomenclature based on antigen subfractions (M1–M9), the M2 contains the 2-OADC antigens specific for PBC [18], while the prognostic and diagnostic significance of the other anti-M antibodies is questionable as they have been found in diverse pathological conditions (syphilis, acute myocarditis, drug-induced hepatitis, and autoimmune rheumatic diseases) [18].

2.1. IIF

IIF assay using either HEp-2 cells or cryostat sections of rat liver, kidney, and stomach as the substrate is considered to be the 'gold standard' for routine screening for AMA [2,19]. The presence of AMA displays a fine granular cytoplasmic staining of both distal and proximal renal tubules (Figure 1(a)). The distal tubules are richer in mitochondria and therefore display a more intense fluorescence. Besides, taking into account that anti-liver kidney microsomal antibodies (anti-LKM) stain only proximal renal tubules, a careful orientation of the sectioning of the kidney substrate is mandatory in order to both proximal and distal tubules fixed on cryostat sections [2,19]. AMA also stain the gastric parietal cells with a bright granular pattern, which are spared by anti-LKM antibodies, whereas anti-LKM antibodies stain hepatocytes much more brightly than do AMA [16,20] (Figure 1(b)). Therefore, frequent interpretative problems arise in laboratories using only kidney as substrate and particularly when tissue is inappropriately oriented. AMA on HEp-2 cells provide usually a granular cytoplasmic pattern [21,22] (Figure 2). The sections (including liver, kidney, and stomach) are used without further fixation or can be stored at −20°C for 4–8 weeks [19]. Equivalent sections that are commercially available are of variable quality as, to lengthen the expiry date, they are treated with fixatives, which may result in enhanced background staining that usually limits interpretation of fluorescence patterns.

The clinical association of AMA in PBC patients is still an issue of debate since the majority of the studies have failed to find any clinical association of these autoantibodies with disease progression and there is no evidence that a decrease in AMA titers during treatment is associated with therapeutic response [23], although some studies including from our group have reported that high concentrations of AMA might predict progressive PBC course and that AMA serum levels are closely associated with the degree of liver insufficiency [7,24].

As AMA in the serum include all three major immunoglobulin isotypes (IgG, IgA, and IgM), it is advisable to use antihuman polyvalent immunoglobulin (IgG, IgA, IgM) as secondary antibody in this assay [2]. In this context, Kuroda et al. reported that some patients negative for IgG-class anti-PDC-E2 antibody are both positive for IgA- or IgM-class antibody [25]. Of note, Rigopoulou et al. [26] have investigated AMA IgG isotypes and correlated the results with indices of disease severity and predictors of disease progression. This study demonstrated that while the expression of AMA is not restricted to a specific IgG subclass, IgG3 AMA positivity was associated with a more severe disease course, possibly reflecting the suitability of this isotype to recruit effector mechanisms of damage, regarding activation of complement system and recruitment of cells bearing the Fc receptor.

2.2. Enzyme-linked immunosorbent assays

For a long time, the IIF method on tissue sections has been considered 'the gold standard' for AMA detection. However,

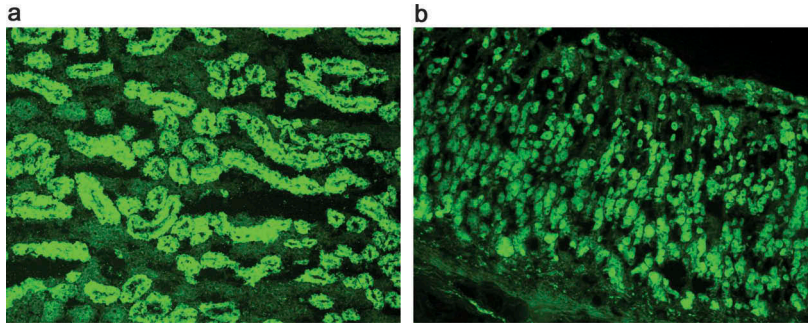

Figure 1. (a) AMA displays a fine granular cytoplasmic staining of both distal and proximal renal tubules. (b) AMA also stain the gastric parietal cells with a bright granular pattern.

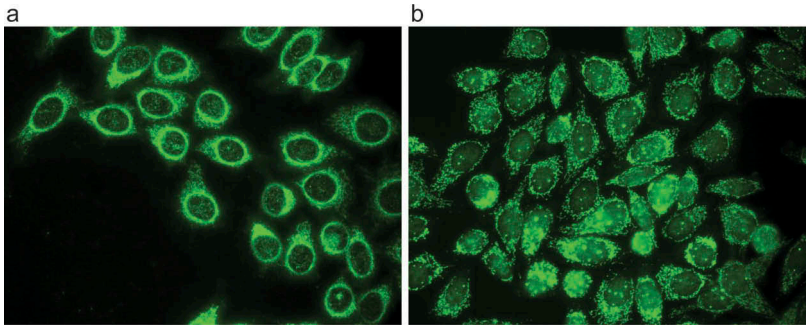

Figure 2. AMA on HEp2 cells provide a granular cytoplasmic staining. (a) RLM pattern gives a staining of nuclear membrane resulting from the reaction with nuclear pore complex antigens. (b) Multiple nuclear dots patterns are shown by 3–20 dots of variable size through the nucleus but sparing the nucleoli and the mitoses.

the limitations of IIF cannot be ignored. This method is labor-intensive, time-consuming, cannot be fully automated, and depends on a subjective interpretation of the observer. On the other hand, the identification of the molecular targets of AMA has allowed the establishment of molecular-based assays (enzyme-linked immunosorbent assays [ELISAs] and immunoblot) using recombinant or purified antigens. Therefore, when AMA are detected by IIF, further analysis is usually needed by using molecularly defined antigen preparations. First-generation ELISA tests utilized only PDC-E2 as the primary substrate for the detection of AMA. However, about 10% of histologically proven PBC patients are only positive for anti-BCOADC-E2 and/or anti-OGDC-E2 [27] and this may result in low sensitivity of the test.

ELISAs using recombinant proteins to the three known autoantigens are widely available and most frequently employed by commercial laboratories. Gershwin and colleagues achieved a higher diagnostic sensitivity than conventional ELISAs for AMA by creating a recombinant fusion protein (MIT3), which includes the immunodominant portions of the three primary targets of AMA (PDC-E2, BCOADC-E2, OGDC-E2) [28]. The new MIT3-based ELISA was shown to have enhanced performance over IIF or conventional PDC-E2-based ELISA tests as it was capable to unmask AMA positivity in up to half of AMA-negative samples by IIF [29–31]. Subsequently, a new ELISA (anti-M2-3E) utilizing a mixture of both purified PDC and MIT3 hybrid as antigenic targets has been developed, reaching a higher diagnostic sensitivity and a similar specificity when compared to anti-MIT3 alone, conventional anti-PDC, and IIF assays [32]. These findings may suggest the use of MIT3-based ELISAs as first-line investigation for AMA detection, particularly, when the laboratories are unfamiliar with the use and interpretation of the IIF patterns of AMA [31].

The group in Davis, USA developed a novel bead-based immunoassay, utilizing the three recombinant mitochondrial autoantigens (PDC-E2, BCOADC-E2, and OGDC-E2) [33]. This assay improved the sensitivity by unmasking 20% of 'AMA-negative' PBC cases by IIF. Interestingly, they showed that 100% of these newly detected AMA-positive patients were ANA-positive [33]. This method could also be customized to include variable sets of autoantibodies but also other markers of immunity or inflammation. However, this method requires significantly higher cost than traditional ELISAs.

2.3. Immunoblotting

Western blot is a sensitive and specific tool to identify and mainly to characterize the mitochondrial target antigens in PBC, by enabling the visualization of the indicative 74-kDa (PDC-E2), 52-kDa (BCOADC-E2), and 48-kDa (OGDC-E2) bands [2,27,30] (Figure 3). Preparations of mitochondrial fractions of human and/or rat liver or bovine heart have been used as sources of antigens for AMA detection by immunoblot testing [30,34]. Muratori et al. [30] achieved a better diagnostic performance for the detection of AMA, by using an antihuman polyvalent immunoglobulin (IgA, IgG, IgM) as secondary antibody in immunoblotting in comparison with IIF.

Moreover, in a study from our group [35], a computer-assisted imaging technology was used for a relative quantification of protein bands by calculating their intensity in pixels. In this study, it was demonstrated that AMA titers by IIF correlate to the number and magnitude of immunofixed 2-OADC bands in PBC sera, although the PDC-E2 was not always responsible for most of the reactivity seen by IIF [35].

Conflicting results exist regarding the clinical significance of specific immunoblotting patterns. Masuda et al. [36] have shown an association of IgA anti-2-OADC with advanced histopathological stages, IgA anti-PDC-E2 and IgA anti-E3BP

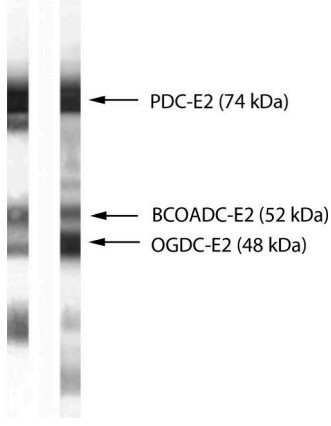

Figure 3. The major mitochondrial antigens, E2 subunits of pyruvate dehydrogenase complex (PDC-E2, 74 kDa), branched chain 2-oxo-acid dehydrogenase complex (BCOADC-E2, 52 kDa) and oxoglutarate dehydrogenase complex (OGDC-E2, 48 kDa) are demonstrated by immunoblotting on mitochondrial fractions of rat liver.

with bile duct loss as well as IgA anti-PDC-E2 with interface hepatitis and atypical ductular proliferation. Secretory IgA is the predominant immunoglobulin of the mucosal immune system as well as the major protein in bile. Therefore, a hypothesis of IgA-driven pathogenesis in PBC could be very attractive. However, neither our group [31] nor Muratori et al. [37] found any association between AMA-specific isotype patterns and clinical expression of liver disease.

3. ANA

In addition to AMAs, ANA are frequently detected in PBC in approximately 50–70% of patients [2,4,6]. The 'gold standard' method for the determination of ANA in human sera is IIF, using as a substrate the HEp-2 cells, a well-known human larynx epithelioma cancer cell line. Large nuclei and high rate of mitosis of HEp-2 cells allow for discrimination of different staining pattern [2,19].

3.1. PBC-specific antinuclear autoantibodies

PBC-specific ANA are found in up to 50% of patients [2,6], with two patterns of nuclear fluorescence described, a perinuclear rim-like membrane (RLM) and a pattern of multiple nuclear dots (MND) (Figure 2). In a previous study from our group, we have demonstrated a seropositivity of 65% for PBC-specific ANA by the utilization of specific antisera to each of the four IgG isotypes, compared to 15% seropositivity when using an anti-IgG (total) antiserum [8]. The prevailing IgG-specific isotype was IgG3 for MND and IgG1 for RLM patterns. In that study, PBC patients with specific ANA, in particular of the IgG3 isotype, had significantly more severe disease, possibly reflecting the peculiar ability of this isotype to engage mediators of damage [8]. However, most PBC sera are heterogeneous and have autoantibodies with different ANA specificities, resulting in several and frequently overlapping staining patterns in HEp-2 cells. In such cases, IIF assays are not able to distinguish between various kinds of circulating autoantibodies.

The RLM pattern gives a staining of nuclear membrane and results from autoantibody reaction with nuclear pore complex antigens (Figure 2(a)): 210-kDa glycoprotein (gp210), 62-kDa nucleoporin (NUP62), and the lamin B receptor (LBR) [38]. The prevalence of anti-gp210 antibodies reported in PBC patients ranges from 9% to 26% [8,39–41] and this variation is probably due to ethnical/geographical distinctions and to differences between assays used for their determination (in-house vs. commercial). Anti-gp210 antibodies are highly specific for PBC diagnosis and persist after liver transplantation, although no association with disease recurrence has been reported [42]. Recently, Nakamura et al. [43] showed for the first time that anti-gp210 positivity is significantly associated with worse response to treatment, and their findings suggested that anti-gp210 positivity may contribute to the progression of the clinical stage of the disease. However, similar studies from our group including also a considerable number of patients from Barcelona failed to reveal this autoantibody of major clinical significance regarding the disease progression, outcome, and response to treatment [7,41] suggesting that at least in Western European patients with PBC, this marker can be used only as a valuable laboratory indicator for PBC diagnosis. Anti-NUP62 prevalence varies from 15% to 55% of patients depending on the method used, while the association with severity and progression of the disease is under evaluation [44,45]. Anti-LBR positivity varies from 1.2% to 9% with high specificity for PBC [46].

The MND pattern is characterized by a staining of multiple dots of variable size distributed through the cell nucleus, but sparing the nucleoli and the mitoses, differentiating them from anti-centromere antibodies (ACA) (Figure 2(b)). It results from autoantibody reaction with components of the nuclear envelope; nuclear body speckled 100 kDa (sp100), 140 kDa (sp140), small ubiquitin-related modifiers, and promyelocytic leukemia proteins (PML) [2,6,45,47–50]. Anti-sp100 antibody can be detected in 20–40% of patients with PBC [51–53]. The occurrence of anti-MND antibodies against sp100 has been associated with an unfavorable disease course in terms of more severe biochemical and histological disease, faster disease progression, and worse outcome in seropositive Greek PBC patients from our group [7,8] as well as in other studies [54]. Moreover, regarding the serial changes of the titers of these autoantibodies, we have shown in a set of 512 sera obtained from 110 PBC patients that the decrease of anti-sp100 titers was associated with response to ursodeoxycholic acid (UDCA) treatment and improvement of the Mayo risk score [7]. These findings indicate that close serological monitoring of anti-sp100 titers may assist to discriminate patients with worse outcome. In this context, patients with stable or increased anti-sp100 titers during follow-up may be candidates for alternative treatment protocols [7]. Anti-PML-positive patients also appear to have a worse prognosis, but this may happen due to the fact that anti-PML mainly (>70%) coexist with anti-sp100 antibodies [54,55]. Similarly, anti-sp140 antibodies were found together with anti-sp100 antibodies in 90% and with anti-PML antibodies in 60% of PBC cases, although they were not associated with a specific clinical feature of PBC [50]. The findings that anti-sp140 and anti-PML antibodies almost exclusively occur in anti-sp100-positive patients, but not *vice versa*, led to the hypothesis that the nuclear body acts as a multiantigenic complex in which the immune response might involve first sp100, and only later spread to sp140 and PML that share the same subnuclear localization [50].

3.2. ANA not specific for PBC

Other ANA not specific for PBC, especially those already known in autoimmune rheumatic diseases like ACA, anti-SSA/Ro-52 kDa, and anti-dsDNA antibodies, can be frequently found in PBC [2,6,56]. Several methods have been applied for the determination of these autoantibodies such as IIF on HEp-2 cells (especially for the ACA antibodies with a fluorescence pattern of MND in interphase and mitotic cells, corresponding to the number of chromosomes in the cells), counter-immunoelectrophoresis, and immunoblot, while ELISAs have become the method of choice for routine screening in laboratories [56,57]. ACA antibodies have been reported in 10–30% of PBC patients [40,41,56,58], but their specificity for PBC is limited, as ACA positivity has also been reported in

miscellaneous rheumatologic diseases, as well as in chronic hepatitis C [59] and autoimmune hepatitis (AIH) [60]. Findings by Nakamura et al. [40,61] indicated that patients positive for ACA antibodies are more prone to develop portal hypertension and hepatocellular carcinoma, while histologically are characterized by more severe ductular reaction. In line with this study, Gao et al. [62] showed that PBC patients positive for ACA antibodies develop portal hypertension 10 times more frequently compared to ACA-negative PBC patients. However, other studies including studies from our group have failed to show such an association [41,58].

4. Multiplexed assays

Analytical systems for detecting and measuring autoantibodies have progressed significantly during the last 20 years and multiplex platforms have contributed to overcome some drawbacks of the conventional assays such as time spending, costs, universal standardization, volume of reagents and samples, turnaround time, and lack of harmonization, in order to ensure finally that different testing procedures used by different laboratories give equivalent results [63]. In the case of PBC, the overlap of PBC-specific antibodies is currently easier to be recorded due to the variety of available assays and multiplexed methods. These methods allow the simultaneous detection of other PBC-specific autoantibodies in addition to AMA and increase the diagnostic sensitivity, especially in AMA-negative cases.

Under this context, a new multi-analyte, dual isotype ELISA, which simultaneously screens for IgG and IgA antibodies to the three immunodominant epitopes of AMA, gp210, and sp100 (PBC Screen) in each test serum, has been developed [64]. Indeed, Liu et al. [64] evaluated the performance of PBC Screen in 1175 patients with PBC and 1232 subjects without PBC. The sensitivity was 83.8% and specificity 94.7%, similar to that obtained (96.1%) by the combined results of individual MIT3, sp100, and gp210 IgG ELISAs [64]. Another important point in this study was that almost half of IIF AMA-negative patients were positive for PBC-specific autoantibodies using the PBC Screen [64]. Similar findings revealed by another study by Bizzaro et al. where among IIF AMA-negative sera, 43% manifested reactivity using the PBC screening test [65]. These results indicate that the PBC Screen performs as well as ELISAs based on individual antigens and may therefore be considered as a first screening in excluding disease diagnosis (specificity 95%) particularly in settings with increased patients load.

Another method used to detect multiple PBC-related antibodies is the line immunoassay. Using this technique, several antigens are immobilized on strips and are incubated with serum samples obtained from patients with suspected autoimmune liver disease. Villalta et al. [66] reported recently their results on the multiplexed line-blot Autoimmune Liver Disease Profile 2 (ALD2) (Euroimmun, Lübeck, Germany), an assay which contains PBC-associated antigens (AMA-M2 natively purified from bovine heart; MIT3 enriched with native PDC antigen; sp100, PML, gp210 recombinant proteins), some non-PBC-specific nuclear autoantigens (Ro52, SSA, CENP-A, CENP-B, Scl70) and some AIH-specific autoantigens (LKM1, LC1, and SLA/LP). The ALD2 line-blot showed an excellent diagnostic accuracy for PBC (overall sensitivity and specificity were 98.3% and 93.7%) and a higher sensitivity than the IIF method to detect sp100 and gp210 autoantibodies.

5. New biomarkers

Despite the improvement achieved in the diagnosis of PBC by using assays to detect AMA and PBC-specific ANA, some patients are still serologically negative and may remain undiagnosed. Interestingly, novel serum markers have been recently proposed for PBC, although their clinical significance and diagnostic impact remain elusive (Table 2).

5.1. Novel autoantibodies

Recently, two novels PBC autoantigens have been identified by the use of proteomics, kelch-like 12 (KLHL12) [10] and hexokinase 1 (HK1) [10,67]. KLHL12 is a nuclear protein implicated in collagen export and ubiquitination of dopamine D4 receptor and Disheveled protein. HK1 is an enzyme localized to the outer membrane and plays an essential role in glucose metabolism, maintains the homeostasis of mitochondria, and modulates cellular susceptibility to apoptosis. The combination of the two new biomarkers resulted in improvement in overall sensitivity in AMA-negative PBC from 48.3% to 68.5% in ELISA and from 55% to 75% in immunoblot, suggesting that anti-KLHL12 and anti-HK1 autoantibodies can be used as important supplementary tools in the diagnosis of PBC. Of note, the specificity of both anti-KLHL12 and anti-HK1 autoantibodies in PBC was very high (≥95%) [10].

Table 2. New biomarkers in PBC.

Biomarkers	Findings
Novel autoantibodies	Anti-kelch-like 12 and anti-hexokinase 1 autoantibodies showed high specificity for PBC (≥95%) and improved sensitivity in AMA-negative PBC cases (68.5% in ELISA, 75% in immunoblot) [10]
Genetic markers	*IL12RB2, IL12A, IRF5, ORMDL3, STAT4, DENND1B, CD80, IL7R, CXR5, TNFRSF1A, CLEC16A, NFKB1, MMEL1, TYK2, PLCL2, ELMO1, TNFSF15, RPS6KA4, POU2AF1, SH2B3, TNFSF11, RAD51B, TNFAIP2, IRF8, IKZF3, CRHR1, SBIP, MAP3K7IP1* are PBC susceptible non-HLA genes [11,69–71]
Metabolomics	Differences in serum metabolites between PBC, PSC, and control groups [12]; upregulation of bile acids, free fatty acids, phosphatidylcholines, lysolecithins, and sphingomyelin in PBC compared to AIH patients [73]; increase of bile acids and decrease of carnitine levels as PBC progresses [74]
MicroRNAs	11 upregulated (e.g. miR-145, miR-328, miR-299-5p) and 24 downregulated (e.g. miR-122a, miR-26a) microRNAs in PBC patients [13]
Epigenetics	Involvement of telomere shortening in biliary senescence in PBC [75], increased frequency of monosomy X in PBC female patients [76], increased frequency of Y chromosomy loss in PBC male patients [78], histone modification [79], decreased DNA methylation of the CD40L promoter in PBC patients [80], dysregulation of long noncoding RNA expression [81]

PBC: Primary biliary cholangitis; AMA: antimitochondrial antibodies; ELISA: enzyme-linked immunosorbent assays; HLA: human leukocyte antigen; PSC: primary sclerosing cholangitis; AIH: autoimmune hepatitis; miR: micro RNA.

5.2. Genetic markers

First evidence for genetic susceptibility of the disease was provided by clinical observations revealing familial PBC aggregation, high concordance rates among monozygotic twins, and clustering of autoimmune diseases in PBC individuals and their siblings [68,77]. In the last decade, genome-wide association studies (GWAS) in large populations have significantly increased understanding of the genetic risk signature of this disease, providing associations between human leukocyte antigen (HLA) and non-HLA regions and PBC risk [11,69–71]. These risk loci highlight several immune pathways involved in antigen presentation, T-cell polarization, B-cell function, and myeloid cell differentiation. Indeed, future work will focus not only on the biologic implications of these discoveries but also on defining a genetic risk related to the selected sub-phenotypes of disease, such as the characteristics of presentation, treatment response, and outcome. For example, knowledge obtained regarding the role of interleukin-12 (IL-12) and IL-23 in the pathogenesis of PBC has already led to clinical studies investigating the effects of ustekinumab, a humanized monoclonal antibody that binds with specificity to the p40 protein subunit used by both the IL-12 and IL-23, in patients with PBC unresponsive to UDCA [72].

5.3. Metabolomic profiling

Metabolomics is the study of chemical processes involving metabolites and has arisen as a potent tool for discovering novel biomarkers for diagnosing and understanding pathogenesis of several diseases, including PBC. In a recent study, Bell et al. [12] performed a thorough investigation of global serum metabolome of patients with autoimmune cholestatic liver disease including PBC. They identified important differences in the serum metabolic profiles of patients with PBC or primary sclerosing cholangitis (PSC) as compared to healthy controls. Besides, alterations were found between PBC and PSC patients, regarding lipid metabolism, oxidative stress/lipid peroxidation, stress hormones, and protein/amino acid metabolism suggesting that these differences can be used as potential biomarkers for differentiating PBC from PSC [12]. Moreover, Lian et al. [73] identify different metabolic profiles that could be used to discriminate between PBC and AIH, while Tang et al. [74] suggested that the circulating levels of bile acids and carnitine are differentially altered in PBC patients. These variances could also provide insight into metabolic pathways that contribute to the pathogenesis of autoimmune liver diseases.

5.4. miRNAs

miRNAs are short, noncoding RNAs (20–22 nucleotides) that posttranscriptionally regulate gene expression. Therefore, it is not surprising that altered miRNA profiles underlie the deregulation of several proteins involved in the pathogenesis of PBC, as well as showing promise as diagnostic and prognostic tools. A recent miRNA microarray identified 35 differentially expressed miRNAs (11 upregulated and 24 downregulated in PBC patients compared with normal tissue) [13], but their true value as diagnostic tools requires further studies in larger cohort of patients, as well as standardization of methods for their determination.

5.5. Epigenetics

In recent years, accumulating evidence has demonstrated that, in addition to genetics, other complementary mechanisms are involved in the pathogenesis of autoimmunity, in particular, epigenetics. Epigenetic marks can be affected by age and other environmental triggers, providing a plausible link between environmental factors and the onset and development of various human diseases. Several mechanisms have been proposed to get involved in PBC pathogenesis, such as alterations in chromosome architecture like shortening of telomeres, X monosomy in females and Y chromosomy loss in males [75,76,78], histone modification [79], DNA methylation [80], and long noncoding RNA transcripts [81]. The identification of cell-specific targets of epigenetic deregulation may serve as clinical markers for diagnosis, disease progression, and therapy approaches.

6. Expert commentary

IIF assay on fresh frozen cryostat sections of rat liver, kidney, and stomach substrate should be used as the 'gold standard' for the screening for the detection of AMA. It must be emphasized that the influence of the tissue substrate and conjugate should be taken into account, in order to minimize discrepancies between different laboratories. Ideally, it could be better for each local laboratory to develop its own rodent tissue substrate sections because substrates that are commercially available are of variable quality, resulting sometimes in enhanced background staining, which usually limits interpretation of fluorescence patterns. In addition, the use of an anti-total human Ig-fluorescein isothiocyanate conjugate (IgG, IgA, IgM) as the revealing reagent is preferred compared with the use of single monospecific IgG antiserum.

HEp-2 cells should have only an auxiliary role in the investigation of patients with elevated cholestatic enzymes as this substrate is characterized by low sensitivity for the detection of AMA. However, HEp-2 cells' substrate could be helpful in identifying the PBC-specific ANA in particular in AMA-negative PBC cases by showing the typical RLM or MND patterns.

PBC diagnosis by the detection of AMA using the IIF assay continues to demand a great deal of knowledge and experience and both the laboratory personnel and physicians need to get more familiar with the interpretation of the liver autoimmune serology. This challenge is surely also the reason why most people working in this field exhibit great enthusiasm and intensity in facing this challenge, and why advanced training in this field is very important. An important consensus report published in 2004 [19] included guidelines and recommendations on this issue, making the first steps toward standardization. This report provides valuable information regarding the preparation of the substrate (optimal size, histological composition, and orientation of the tissues), application of test sera, dilution of sera and fluorochrome-labeled reagents, incubation times, controls, and immunofluorescence patterns.

However, IIF assay has many limitations as this method is labor-intensive, time-consuming, cannot be fully automated, and is observer dependent. Therefore, when AMA and/or PBC-specific ANA are observed by IIF, further analysis should include subclassification using molecularly defined antigen preparations by ELISA tests or blot assays. For AMA subclassification, at least the three immunodominant epitopes, namely PDC-E2, BCOADC-E2, and OGDC-E2, should be taken into account, in an attempt to achieve the maximum performance for AMA detection. Besides, the investigation should include all three main isotypes of AMA (i.e. IgG, IgM, IgA). The use of recombinant fusion protein (MIT3 or M2-E3) comprising the immunogenic domains of the E2 subunits of the PDC-E2, BCOADC-E2, and OGDC-E2 complexes provides higher sensitivity and specificity than the old M2-based assays. Similar investigation using molecularly defined antigens should be followed in suspected PBC cases (particularly in those with negative AMA results by IIF), utilizing assays including recombinant nuclear antigens, especially sp100 and gp210, when the diagnostic suspicion is high, but the results from first-level traditional assays are negative [2].

Multi-analyte assays, such as the PBC Screen, which combines mitochondrial (PDC-E2, BCOADC-E2, and OGDC-E2) and nuclear antigens (sp100, gp210), can be used as first-line method for diagnosing PBC in particular in laboratories which are not so familiar with PBC testing algorithm. Indeed, it could be beneficial for routine clinical laboratory testing, because of its semi-automated and nonsubjective readout, and may also help in the diagnosis of PBC patients, especially those negative for serological markers when using conventional methods such as IIF.

However, as we have shown previously with 17% (in 103 PBC sera) discrepancies in results between IIF and MIT3-based assays, there is no 'gold standard assay' with 100% sensitivity and 100% specificity for the detection of AMA [31], and therefore, efforts should be done in an attempt to remain IIF the standard first screening procedure.

Finally, development of clinical governance is of great importance in order to ensure that clinical standards are met, and that processes are in place to ensure continuing improvement and harmonization between laboratories [82]. The guidance should include the whole testing process, beginning from the formulation of a reasonable clinical suspicion and the request of the most appropriate autoantibody test and continuing with handling of biological samples. In addition, the laboratory autoimmunologists should take care to remain up-to-date about current technologies and methods for detecting and measuring autoantibodies [83]. In this regard, when a laboratory reports a result, it must accurately define the diagnostic performances of the assays used and provide explanatory comments in the laboratory report, in order to aid the clinician in the correct interpretation of the results and the appropriate clinical decision [84]. It is needless to underscore of course that internal and external quality assessment programs are necessary to ensure the quality of autoimmune serological diagnostic tests.

7. Five-year view

Laboratory diagnostics in the field of autoimmune liver diseases have made a huge improvement, and undoubtedly, the perspectives remain widely open. One of the issues that should be addressed in the near future is the standardization of the methodology employed, as there is awareness that variations in test results, like reference ranges and units, cause confusion in the clinicians but might be also dangerous for the patients. International quality controls and external validation of the assays should take place between laboratories, through the exchange of sera with known and calibrated reactivities [82].

The proportion of AMA-negative PBC patients will be significantly minimized by the use of new laboratory methods and recombinant antigens. Development of multiplex completely automated platforms incorporating a full panel of PBC-related autoantibodies, including a vast array of nuclear antigens, will finally lead to the optimization of the diagnostic algorithms.

Indeed, before the use of new diagnostic tools for routine screening, the assays should be validated in large prospective multicenter studies by incorporating newly diagnosed PBC patients of different race and stage of the disease. Furthermore, studies should be extended from established series of patients to the general population in order to assess the specificity of the assays.

Last but not the least, although GWAS have been critically informative, genetic markers could not be considered as an alternative diagnostic marker at present. Functional studies will be needed to identify precise causal variants and define their biologic effects. On the other side, epigenetics may determine not only a portion of genetic predisposition but may also govern the natural history of the disease.

Key issues

- PBC demands an accurate and early diagnosis, in order to halt progression of the disease by prompt initiation of treatment.
- Continual improvement in diagnostic assays has resulted in the detection of most PBC patients suffering from mild or apparently asymptomatic stages of the disease.
- AMA are detected in almost 95% of patients with PBC if an internationally accepted diagnostic road map is followed by the laboratories and therefore, they are considered as the diagnostic hallmark of the disease.
- PBC-specific ANA are also important tools for PBC diagnosis and probably for its prognosis.
- Screening for PBC is performed by IIF, but the specification of antigens has allowed the development of modern techniques, which could help to unmask the supposed 'PBC AMA-negative cases'.
- Standardization of the methodology through large prospective multi-center studies will lead to optimization of the diagnostic approach to patients with clinical suspicion of PBC.
- Clinical governance is very important to guarantee that clinical standards are met, and that processes are in place to ensure continuing improvement.
- New biomarkers such as novel autoantibodies, genetic polymorphisms, metabolomic profiling, micro-RNA and epigenetics may assist to the understanding, diagnosis and management of the disease.

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