ORIGINAL ARTICLE

Anti-kelch-like 12 and anti-hexokinase 1: novel autoantibodies in primary biliary cirrhosis

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Abstract

Background & Aims: Using high-density human recombinant protein microarrays, we identified two potential biomarkers, kelch-like 12 (KLHL12) and hexokinase-1 (HK1), in primary biliary cirrhosis (PBC). The objective of this study was to determine the diagnostic value of anti-KLHL12/HK1 autoantibodies in PBC. Initial discovery used sera from 22 patients with PBC and 62 non-PBC controls. KLHL12 and HK1 proteins were then analysed for immunoglobulin reactivity by immunoblot and enzyme-linked immunosorbent assay (ELISA) in two independent cohorts of PBC and disease/healthy control patients. *Methods:* Serum samples from 100 patients with PBC and 165 non-PBC disease controls were analysed by immunoblot and samples from 366 patients with PBC, 174 disease controls, and 80 healthy donors were tested by ELISA. *Results:* Anti-KLHL12 and anti-HK1 antibodies were each detected more frequently in PBC compared with non-PBC disease controls ($P < 0.001$). Not only are both markers highly specific for PBC ($\geq$95%) but they also yielded higher sensitivity than anti-gp210 and anti-sp100 antibodies. Combining anti-HK1 and anti-KLHL12 with available markers (MIT3, gp210 and sp100), increased the diagnostic sensitivity for PBC. Most importantly, anti-KLHL12 and anti-HK1 antibodies were present in 10–35% of anti-mitochondrial antibody (AMA)-negative PBC patients and adding these two biomarkers to conventional PBC assays dramatically improved the serological sensitivity in AMA-negative PBC from 55% to 75% in immunoblot and 48.3% to 68.5% in ELISA. *Conclusions:* The addition of tests for highly specific anti-KLHL12 and anti-HK1 antibodies to AMA and ANA serological assays significantly improves efficacy in the clinical detection and diagnosis of PBC, especially for AMA-negative subjects.

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Key Points

● Using high-density human recombinant protein microarrays, two new biomarkers in primary biliary cirrhosis coined kelch-like 12 and hexokinase-1 have been identified.
● Antibodies to KLHL12 and HK1 are more frequently identified ($P < 0.001$) in patients with PBC compared to controls.
● Antibodies to KLHL12 and anti-HK1 have higher sensitivity than anti-gp210 and anti-sp100.
● The use of these two new highly specific autoantibodies significantly improves efficacy in the diagnosis of PBC, especially in AMA-negative patients.

Advances in serodiagnostics for primary biliary cirrhosis (PBC) have resulted in detection of PBC-specific autoantibodies in 90–95% of PBC patients. While the majority (~90%) of patients have antibodies to the mitochondrial E2 subunit of the pyruvate dehydrogenase complex (PDC-E2), the branched chain 2-oxo-acid dehydrogenase complex (BCOADC-E2), and the 2-oxoglutarate dehydrogenase complex (OGDC-E2) (1), antibodies to the nuclear pore glycoprotein gp210 and the nuclear body-associated protein sp100 are found in some patients (~40%) and may be the only antibodies detected (2). Although utilization of assays to detect antibodies to these five proteins has increased detection of PBC patients, some patients are still serologically negative and may remain undiagnosed. Accurate diagnosis of PBC at early stages is important because early treatment can slow progression, delay liver failure and improve the survival rate of PBC. Moreover, treatment with ursodeoxycholic acid is most effective when employed at early stages of the disease (3–5). As detection of PBC-specific autoantibodies may predict the development of PBC in asymptomatic patients, sensitive markers to identify these individuals are needed (6).

To identify additional PBC autoantigens and improve the diagnostic rate, we utilized a proteomics strategy using high-density human recombinant protein microarrays for autoantigen discovery (7, 8), coupled with subsequent enzyme-linked immunosorbent assay (ELISA)-based validation. This approach resulted in our discovery of two novel PBC autoantigens, kelch-like 12 (KLHL12) and hexokinase 1 (HK1). Another study using an independent proteomics approach has recently confirmed our discovery (9). KLHL12 is a nuclear protein that regulates COPII assembly, which is crucial for collagen export (10). It also specifically binds to and directs ubiquitination of both the dopamine D4 receptor and the Dishevelled protein (11, 12). While KLHL12 is located inside the nucleus, HK1 is an enzyme that localizes to the outer membrane of mitochondria and phosphorylates glucose to yield glucose-6-phosphate (13). In addition to its essential role in glucose metabolism, HK also maintains the homoeostasis of

mitochondria and modulates cellular susceptibility to apoptosis (14). The possible association between these two proteins and the pathogenesis of PBC or other autoimmune diseases remains unknown.

We report herein the methodology used to verify the diagnostic value of these two novel biomarkers in PBC by immunoblot and ELISA in 466 subjects with PBC [anti-mitochondrial antibody (AMA) positive and AMA negative] and 419 control subjects (disease and healthy controls). We demonstrate that anti-KLHL12 and anti-HK1 antibodies were each detected more frequently in patients with PBC compared with non-PBC disease controls ($P < 0.001$). Both autoantibodies are highly specific to PBC (specificity $\geq 95\%$). Utilization of assays for the detection of both anti-KLHL12 and anti-HK1 antibodies can reduce the number of seronegative PBC patients and improve the overall sensitivity of PBC serological assays. Therefore, anti-KLHL12 and anti-HK1 antibodies can be considered new noninvasive biomarkers of PBC.

Materials and methods

This study involved three phases: (i) Biomarker discovery at AmberGen, Inc (Watertown, MA, USA); (ii) immunoblot analysis at the University of California, Davis and (iii) conventional ELISA development, validation and clinical evaluation at Inova Diagnostics (San Diego, CA, USA).

Patients

Each phase of the study used an independent cohort of patients. For the initial autoantigen discovery phase, sera from 18 subjects with PBC, 22 subjects with systemic lupus erythematosus (SLE), 2 with Sjogren's syndrome (SjS), 25 with colorectal cancer (CRC) and 13 normal controls were analysed using proteome microarrays. Ten SLE sera were from Bioreclamation Inc. (Hicksville, NY, USA). Normal sera were from ProMedDx, LLC (Norton, MA, USA) and CRC sera were from Asterand Inc. (Detroit, MI, USA). All remaining sera were from a biobank at Massachusetts General Hospital (Boston, MA, USA) of de-identified samples from patients with PBC and other autoimmune diseases. This study was approved by the Institutional Review Board at Partners Health Care; all subjects in this study signed informed consent. For immunoblot, serum samples from patients with liver disorders, including 100 subjects with PBC (50 early and 50 advanced stage), 38 subjects with primary sclerosing cholangitis (PSC), 55 subjects with acute liver failure (ALF) and 5 healthy controls were studied. The serum AMA and ANA status in PBC was predetermined by indirect immunofluorescence assay (IFA). In addition, serum samples from 72 non-liver disease control patients, including 43 subjects with scleroderma and 29 subjects with systemic lupus erythematosus (SLE) were studied in parallel. The protocol was approved by the Institutional Review Board

of the University of California, Davis. In all cases, the diagnosis of patients was made using international criteria and, in particular, in the case of PBC, based on elevation of alkaline phosphatase, a compatible liver biopsy, and the presence of AMAs (15). AMA-negative patients were defined using the same criteria of elevated alkaline phosphatase and a compatible liver biopsy. In all cases, the presence or absence of AMAs was based upon both immunofluorescence and immunoblotting with MIT3 (16, 17).

For ELISA, specimens from 366 patients with PBC (277 AMA positive and 89 AMA negative as predetermined by IFA), 174 patients with non-PBC disease, including 58 PSC, 7 autoimmune hepatitis (AIH)/PSC, 39 AIH, 16 SjS, 15 ulcerative colitis (UC), 10 Crohn's disease (CD), 10 hepatitis B virus (HBV), 10 hepatitis C virus (HCV), 7 hepatocellular carcinoma (HCC), 1 vanishing bile duct syndrome (VBDS), 1 liver sarcoidosis and 80 healthy controls were studied. All patients with autoimmune liver disease were from Toronto Western Hospital, University of Toronto, Canada and the protocol was approved by the local ethics board.

Serum screening and candidate biomarker selection on microarrays

Patient sera were screened on commercial human proteome microarrays comprised of ~8000 unique human recombinant (eukaryotically expressed) proteins printed in duplicate at high density to a 'chip' size of a standard microscope slide (Human ProtoArray v4.0, Invitrogen, Carlsbad, CA, USA). Microarray processing, imaging and data acquisition were performed according to the manufacturer's instructions. Candidate autoantigen biomarkers were selected from the microarray data with the ProtoArray Prospector v4.0 software package (Invitrogen, Carlsbad, CA, USA) using the Immune Response Profiling (IRP) add-on. Further selection and narrowing of candidate markers utilized M-statistics algorithms and Z-score analysis.

Antigens and immunoblotting

Recombinant proteins, KLHL12 and HK1, were purchased from Novus Biologicals (Littleton, CO, USA). Mammalian mitochondria were prepared as previously described (18). Reactivity against KLHL12 and HK1 was determined by immunoblotting as previously described (19). Positive and negative controls were analysed in parallel. In addition, the AMA status in disease controls was validated by immunoblot against a mammalian mitochondrial preparation.

ELISA

Microtitre plates were coated with human HK1 and KLHL12 full-length recombinant proteins. Diluted serum samples (1:101) were added to coated plates for 30 min. Plates were washed and HRP-conjugated goat-anti-human IgG antibody was added for 30 min. After washing plates, colour was developed by adding 3,3′5,5′-tetramethylbenzidine substrate for 30 min. The reaction was terminated by adding 0.1 N H_2SO_4 and read on a spectrophotometer at 450/620 nm. Results were calculated in reference to a low-positive calibrator. Samples were interpreted as negative ($\leq$20), equivocal (20.1–24.9) and positive ($\geq$25 units). In addition, sera were tested for PBC-specific antibodies using QUANTA Lite$^{\circledR}$ M2 EP (MIT3), sp100 and gp210 ELISA assays (Inova Diagnostics). Details of these ELISAs have been described previously (20).

Statistical analysis

Categorical data of antibody reactivity were compared between PBC and disease controls using Fisher's exact test. *P*-values less than 0.05 were considered statistically significant. Statistical analysis was performed using Prism software (Graphpad Software, La Jolla, CA, USA).

Results

Protein microarray identification of novel PBC autoantigens

After screening serum samples from 18 PBC patients and 62 non-PBC controls using microarrays comprised of approximately 8000 unique human recombinant proteins, HK1 and KLHL12 were both found to be valid PBC autoantigens (Fig. 1). HK1 and KLHL12 yielded M-Statistics *P*-values of 1×10^{-10} and 8×10^{-5} respectively. In this microarray sample set, HK1 had a diagnostic sensitivity of 85–89% and specificity of 84–90%, whereas KLHL12 had a lower sensitivity of 33–40% and higher specificity of 97–98%.

Immunoblot analysis and ELISA validation of anti-KLHL12 and anti-HK1 autoantibodies in PBC

By immunoblot, anti-KLHL12 and anti-HK1 antibodies were both detected among the 100 PBC patients, but not in healthy and HRP-conjugated anti-human secondary antibody-only controls (Fig. 2). Overall, anti-KLHL12 antibodies were detected in 16% (16/100) of the PBC patients (Table 1), including 14% (11/80) of AMA-positive and 25% (5/20) of AMA-negative PBC cases, whereas anti-HK1 antibodies were detected in 16% (16/100) of the PBC patients, including 18% (14/80) of AMA-positive and 10% (2/20) of AMA-negative PBC cases. The frequency of anti-KLHL12 and anti-HK1 antibodies in PBC was higher compared with all non-PBC disease controls (*P* < 0.001) (Table 1) with a specificity of 96.4% and 94.5% for anti-KLHL12 and anti-HK1 respectively. Although the sensitivity of anti-KLHL12 and anti-HK1 antibodies was relatively modest (16%, 16/100) as detected by immunoblot, both these autoantibodies were significantly more prevalent in 100
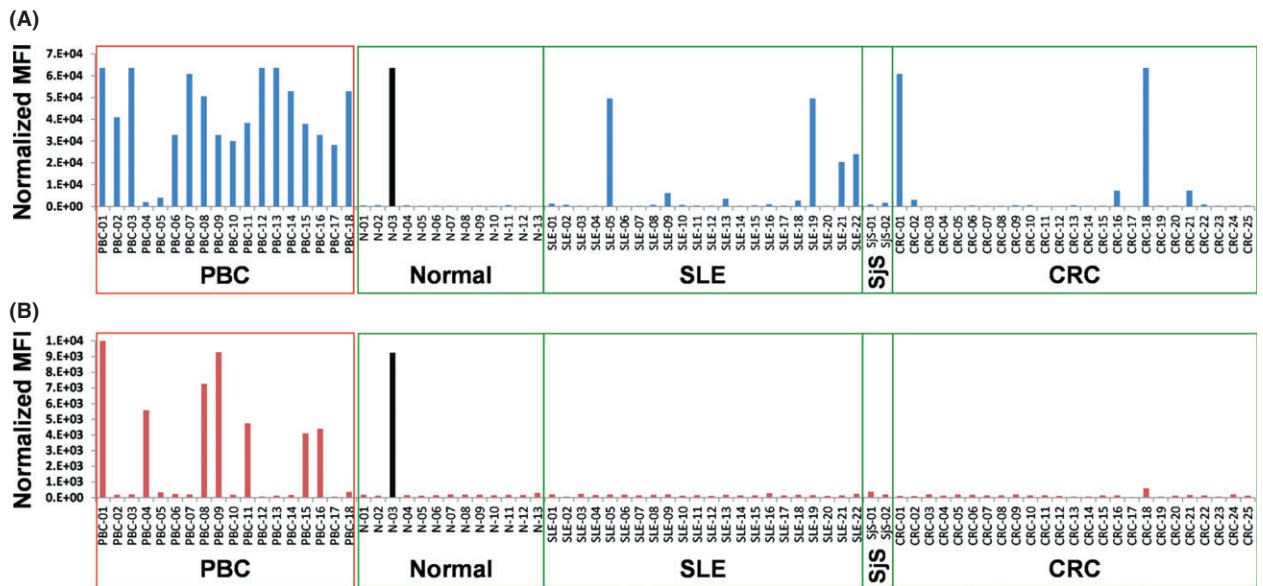
Fig. 1. Quantile normalized protein microarray autoantibody data for (A) HK1 and (B) KLHL12 for 80 distinct serum samples.
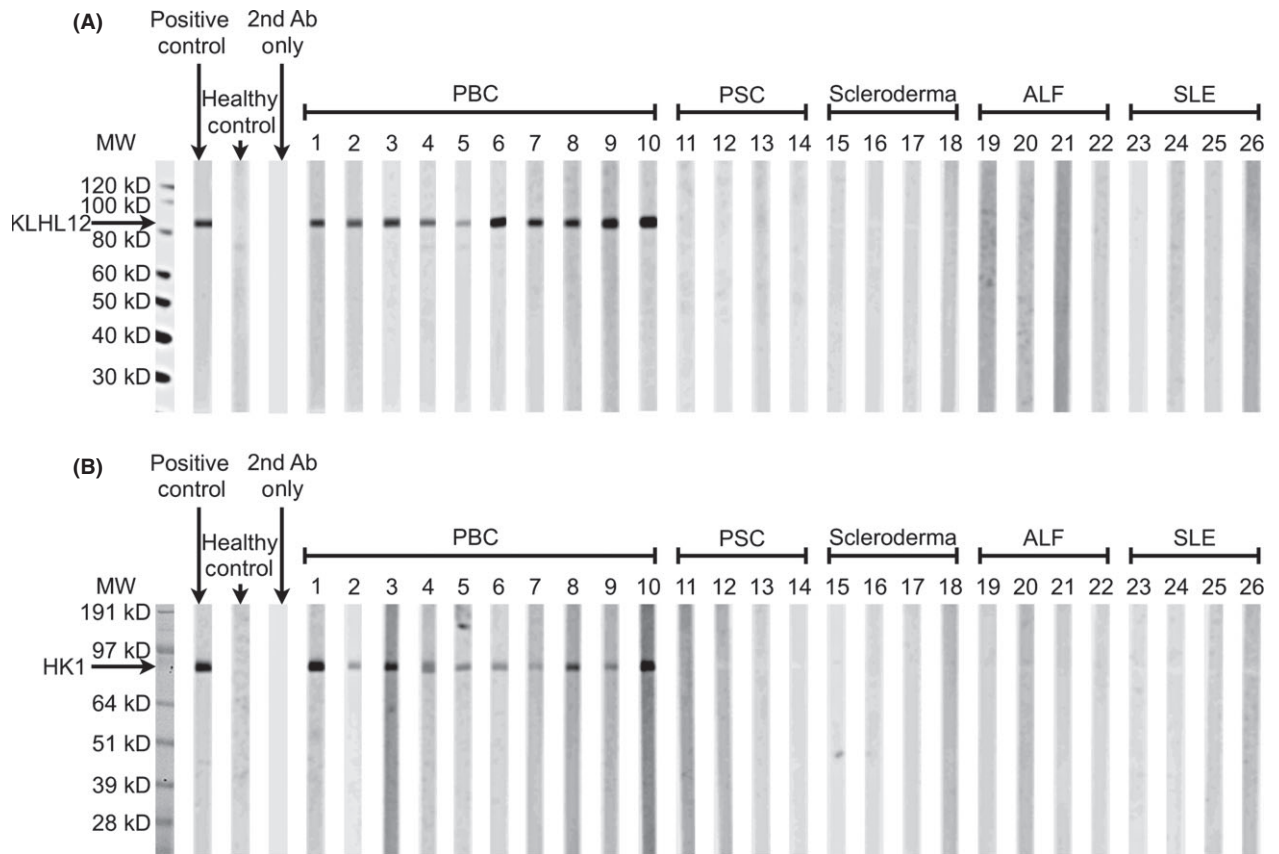

Fig. 2. Serological immunoglobulin reactivity of patients with PBC against (A) KLHL12 and (B) HK1 by immunoblot. Representative results from 10 anti-KLHL12/HK1-positive patients in PBC (lane 1–10) and 16 anti-KLHL12/HK1-negative patients in disease controls (lane 11–26) are shown. Healthy and HRP-conjugated anti-human secondary antibody-only (2nd Ab Only) controls were analysed in parallel.

PBC subjects than in 165 control patients with non-PBC disease.

By ELISA, 366 PBC patients were tested for anti-KLHL12 antibodies and anti-HK1 antibodies. Anti-KLHL12 antibodies were detected in 40% (147/366) of the total cohort of PBC patients, including 42% (116/277) of AMA-positive and 35% (31/89) of AMA-negative PBC (Fig. 3 and Table 1). In comparison, anti-HK1 antibodies were detected in 45% (166/366) of the total PBC patients, including 53% (146/277) of AMA-positive and 22% (20/89) of AMA-negative PBC. The frequency of anti-KLHL12 and anti-HK1 antibodies in PBC were each higher compared with all non-PBC disease controls ($P < 0.001$) (Table 1) with a specificity for PBC of 96.1% and 96.9% respectively. Of significance, the receiver operating curve (ROC) analysis showed the sensitivity of both anti-KLHL12 and anti-HK1 antibodies were higher compared with anti-gp210 and anti-sp100 in ELISA under the same false-positive rate (Fig. 4), indicating that anti-KLHL12 and anti-HK1 antibodies are better supplementary biomarkers. Interestingly, two patients with scleroderma, one patient with ALF and two patients with PSC, who were AMA positive by immunoblot or ELISA, also had anti-KLHL12 and/or anti-HK1 antibodies. The overlap of the three autoantibodies could be a result of concurrent autoimmune diseases in these disease control subjects; however, follow-up for possible PBC diagnosis was not possible in this retrospective study.

Increased cumulative sensitivity in AMA-negative PBC by testing for anti-KLHL12 and anti-HK1 antibodies in addition to conventional AMA and ANA

The distribution of the major autoantibodies in patients with PBC in our study is summarized in Fig. 5. By IFA, 80% and 52% of the 100 PBC patients were serologically positive for AMA or ANA, respectively, while 77.8% and 30.7% of the 366 PBC patients were seropositive for AMA or ANA (anti-gp210 and/or anti-sp100) by ELISA respectively. These results were consistent with previous studies using IFA (21, 22). Although the sensitivity of anti-KLHL12/HK1 antibodies was not high, combining detection of the two autoantibodies by immunoblot increased the overall sensitivity of AMA and ANA detected by IFA, from 91% (91/100) to 95% (95/100) (Fig. 6A). Notably, the sensitivity of serological markers in AMA-negative PBC was substantially improved from 55% to 75% using the combination of ANA, anti-KLHL12 and anti-HK1 antibodies compared with ANA alone (Fig. 6B). When tested on ELISA, the combined use of KLHL12 and HK1 ELISA on an independent PBC cohort increased the sensitivity of MIT3 and gp210/sp100 ELISAs from 82.1% to 88.7% (Fig. 6C). By adding

Table 1. The frequency and specificity of anti-KLHL12 and anti-HK1 autoantibodies by immunoblot and ELISA

	α-KLHL12+				α-HK1+			
	Immunoblot		ELISA		Immunoblot		ELISA	
PBC	16/100	(16%)*	147/366	(40%)*	16/100	(16%)*	166/366	(45%)*
AMA-IFA-positive PBC	11/80	(14%)†	116/277	(42%)†	14/80	(18%)*	146/277	(53%)*
AMA-IFA-negative PBC	5/20	(25%)†	31/89	(35%)*	2/20	(10%)	20/89	(22%)*
Scleroderma‡	2/43	(5%)	–	–	5/43	(12%)	–	–
ALF§	3/55	(5%)	–	–	4/55	(7%)	–	–
SLE	1/29	(3%)	–	–	0/29	(0%)	–	–
PSC¶	0/38	(0%)	1/58	(2%)	0/38	(0%)	5/58	(9%)
AIH/PSC	–	–	1/7	(14%)	–	–	0/7	(0%)
AIH	–	–	5/39	(13%)	–	–	2/39	(5%)
SjS	–	–	2/16	(13%)	–	–	0/16	(0%)
UC	–	–	0/15	(0%)	–	–	0/15	(0%)
CD	–	–	0/10	(0%)	–	–	0/10	(0%)
HBV	–	–	0/10	(0%)	–	–	0/10	(0%)
HCV	–	–	0/10	(0%)	–	–	0/10	(0%)
HCC	–	–	0/7	(0%)	–	–	0/7	(0%)
VBDS	–	–	0/1	(0%)	–	–	0/1	(0%)
Liver sarcoidosis	–	–	0/1	(0%)	–	–	0/1	(0%)
Healthy controls**	–	–	1/80	(1%)	–	–	1/80	(1%)
Total positive in non-PBC controls	6/165	(4%)	10/254	(4%)	9/165	(5%)	8/254	(3%)
Specificity	159/165	(96%)	244/254	(96%)	156/165	(95%)	246/254	(97%)

*The frequency of α-KLHL12+/HK1+ in PBC is higher compared with all non-PBC controls ($P < 0.001$).

†The frequency of α-KLHL12+/HK1+ in PBC is higher compared with all non-PBC controls ($P < 0.01$).

‡Two AMA+ disease control patients detected by immunoblot with scleroderma are also α-KLHL12 or α-HK1 positive.

§One AMA+ disease control patient detected by immunoblot with ALF is also α-HK1 positive.

¶Two AMA+ disease control patients detected by MIT3 ELISA with PSC are also α-KLHL12 and/or α-HK1 positive.

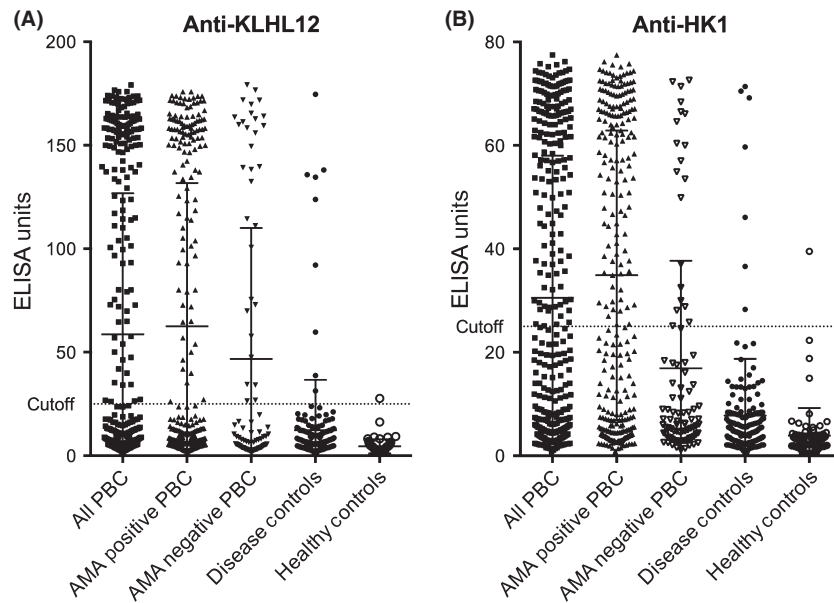**The one positive healthy control was weak positive (27 units, cut-off 25 units) on MIT3 ELISA.

Fig. 3. Distribution of (A) anti-KLHL12 and (B) anti-HK1 antibodies in different clinical groups by ELISA. Total of 366 PBC, including 277 AMA-IFA-positive and 89 AMA-IFA-negative as well as 174 non-PBC disease controls, and 80 healthy controls are shown.
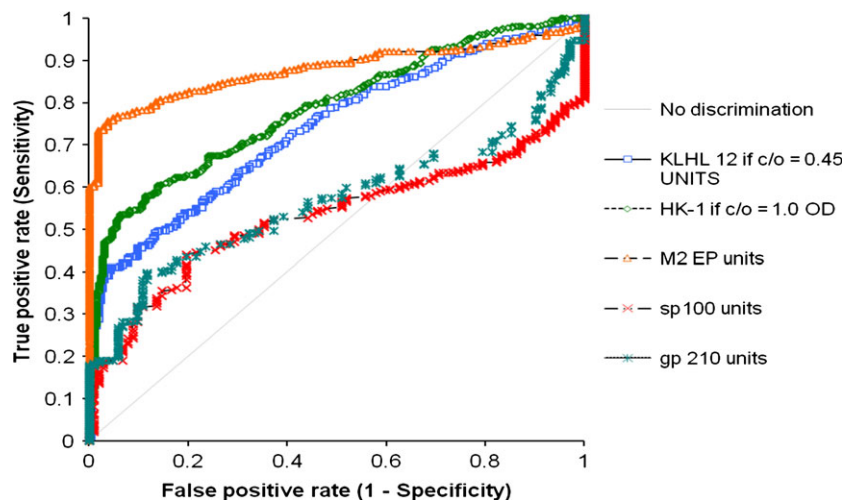

Fig. 4. Receiver operating characteristics (ROC) analysis for serological detection of PBC biomarkers by ELISA.

the two new biomarkers, the overall sensitivity of serological markers in AMA-negative PBC was improved from 48.3% to 68.5% (Fig. 6D), suggesting that anti-KLHL12 and anti-HK1 antibodies are important supplementary tools in the clinical diagnosis of PBC.

Discussion

While discovery of new biomarkers is an area of intense research, out of the thousands of biomarkers described in the literature, few have been translated into practical assays of value in clinical practice. The methodology used for discovery of KLHL12 and HK1 used an unbiased proteomics approach to identify potential autoantigens, statistical analysis of biochip hits, and multiple levels of testing to identify and validate candidate autoantigens. In clinical diagnosis, disease specificity is one of the most crucial criteria of a serological biomarker. AMA is the key diagnostic marker for PBC because of its high specificity (23). PBC is serologically characterized by a high titre of AMA, of which the major targets are PDC-E2, OGDC-E2 and BCOADC-E2 (24).
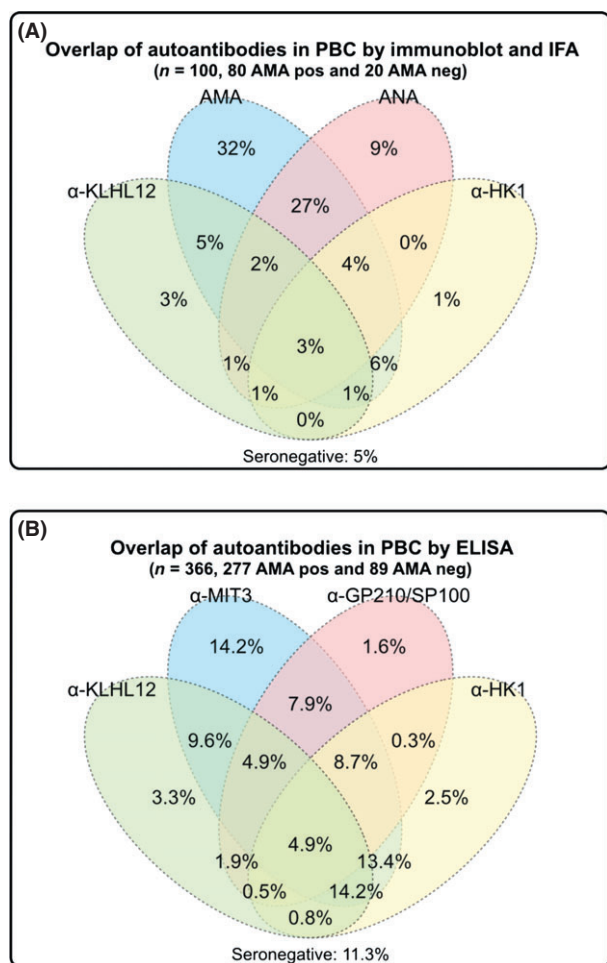
Fig. 5. Overlap of autoantibodies in PBC. Anti-KLHL12, anti-HK1, AMA and ANA were determined by (A) the combination of immunoblot and IFA (*n* = 100, including 80 AMA IFA positive and 20 AMA IFA negative) or by (B) ELISA (*n* = 366, including 277 AMA IFA positive and 89 AMA IFA negative). Majority of patients with PBC had AMA and/or ANA. Interestingly, 5–20.4% of the aforementioned PBC patients had concurrent anti-KLHL12 and anti-HK1 antibodies. The cohort tested by immunoblot and IFA was distinct from that tested by ELISA.

However, AMA has also been detected in sera from patients with other diseases, including SjS (25), scleroderma (26) and ALF (27). Consistent with these reports, we also observed seropositivity of AMA in scleroderma, ALF and PSC (Table 1) in our study. Therefore, newly identified autoantibodies, such as anti-KLHL12 and anti-HK1, could be important biomarkers for differentiating PBC from other autoimmune diseases. Despite the reported presence of anti-KLHL12 antibody in Sjögren's syndrome (28) and anti-HK1 antibody in the sera of children with autism (29), the specificity of both anti-KLHL12 and anti-HK1 antibodies in PBC was very high (≥95%).

A small percentage of clinically proven PBC are AMA negative (30). ANA has been considered a surrogate

screening tool for PBC, especially in AMA-negative patients (31), because of its high sensitivity, although lower specificity. The two major nuclear autoantigens in PBC are gp210 (IFA nuclear rim pattern) (32) and sp100 (IFA multiple nuclear dot pattern) (33). By combining gp210 and sp100 with the additional detection of anti-KLHL12 and anti-HK1 antibodies, the overall diagnostic sensitivity in AMA-negative PBC is dramatically improved. Therefore, anti-KLHL12 and anti-HK1 antibodies can be very effective diagnostic tools in PBC patients without the classic AMA reactivity.

AMA and ANA detected by IFA allow visualization of distinctive staining patterns, but is time-consuming, technically demanding and subjective. Given the molecular identification of the autoantigens over the past few years, molecular-based immunoassays, such as immunoblot, ELISA, and multiplex fluorescent bead-based assays, have emerged as popular screening tools with equivalent or higher sensitivity and specificity compared with traditional IFA (34–37). We used both immunoblot and ELISA analysis to detect autoantibodies directed against KLHL12 and HK1 in PBC patients and controls. Immunoblot is labour-intensive, but also highly specific, allowing visualization of seroreactivity against specific individual antigens and is less prone to false-positive results. On the other hand, ELISA provides advantages including automation, high throughput, the ability to normalize autoantigen concentration in the assay, objective/quantitative readout and facile interlaboratory standardization. We verified the KLHL12 and HK1 seropositivity in PBC using both immunoblot and ELISA on two independent cohorts and demonstrated that the frequency of these two antibodies in PBC was significantly higher compared with non-PBC disease controls. Most importantly, the combination of anti-KLHL12 and anti-HK1 antibodies remarkably increased the sensitivity of serological markers in AMA-negative PBC. Our results suggest the necessity to incorporate KLHL12 and HK1 into the serological assessment of PBC. The availability of new biomarkers to identify PBC patients who are not detected by the current repertoire of PBC markers will help remove diagnostic uncertainty and allow earlier institution of clinical management and treatment.

Like other PBC autoantibodies, the mechanism of anti-KLHL12 and anti-HK1 antibody generation remains unclear. It has been suggested that increased expression of gp210 in small bile ducts may contribute to the increased immunoreactivity of gp210 on the nuclear envelope of biliary epithelial cells (BECs) (38). Increased expression of mitochondrial antigens has also been observed in damaged BECs of small bile ducts in PBC (39). The abnormal expression of mitochondrial antigens was attributed to dysregulated autophagy under stressed conditions ultimately leading to autoimmune-mediated cytotoxic responses specifically against small bile ducts. It would be intriguing to investigate if the expression of KLHL12 and HK1 is elevated
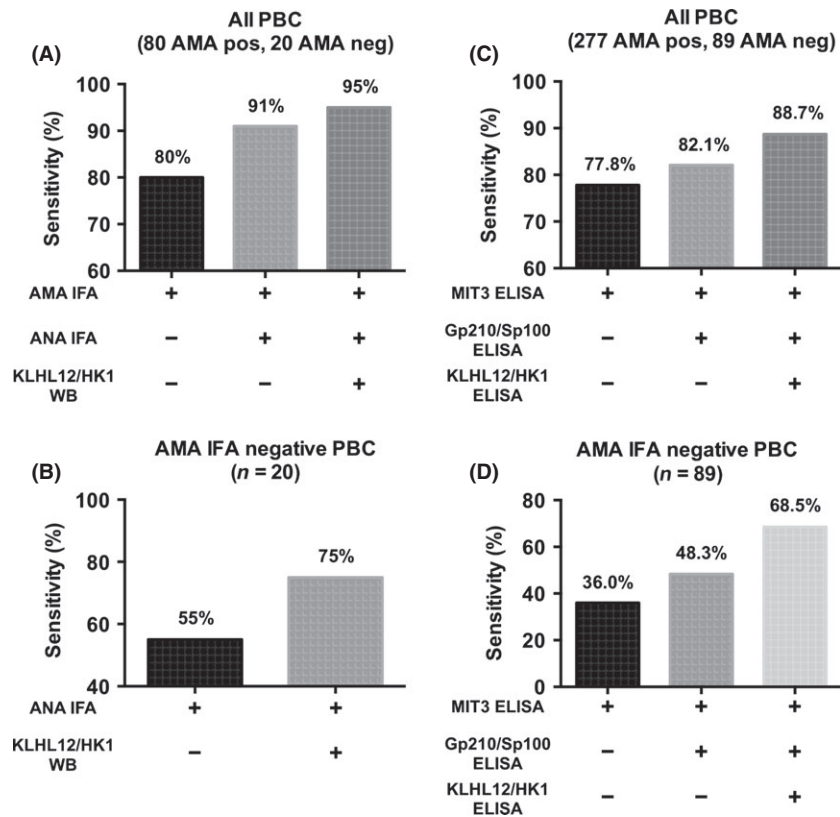
Fig. 6. Increasing sensitivity of serological biomarkers in (A) all PBC (*n* = 100, including 80 AMA-IFA-positive and 20 AMA-IFA-negative) and (B) AMA-IFA-negative PBC (*n* = 20) patients by the combination of immunoblot and IFA, and in (C) all PBC (*n* = 366, including 277 AMA-IFA-positive and 89 AMA-IFA-negative) and (D) AMA-IFA-negative PBC (*n* = 89) patients by ELISA. The 'all PBC' cohorts were selectively enriched in AMA-negative PBC patients and the cohorts in (A) and (B) were distinct from those in (C) and (D).

in BECs, and if anti-KLHL12/HK1 antibody production is cytokine-dependent.

We have previously demonstrated that covalent modification of lipoylated PDC-E2 by electrophilic drugs could inhibit the acyl transfer in the normal citric acid cycle, disrupt the ATP metabolism and induce cell death (40). The subsequent release of electrophile-modified PDC-E2 presented to the immune system of genetically susceptible individuals might eventually lead to the loss of self-tolerance by molecular mimicry and epitope spreading (41). Of note, HK1 is also a mitochondrial enzyme that regulates crucial cellular processes, namely, ATP synthesis and apoptosis. Studies have suggested the interaction between HK1 and the voltage-dependent anion channel in the outer membrane of mitochondria could inhibit cytochrome c release and prevent apoptosis of the cells (42–44). Therefore, the anti-HK1 production may be caused by the functional disruption of HK1, leading to cell death, release of mitochondrial HK1, and presentation to the immune system. The methodology used herein will have further application to identifying the specific underlying mechanisms and we suggest taking advantage of biliary cell lines to address the underlying pathways.

Continual improvement in diagnostic assays has resulted in the detection of individuals with mild or apparently asymptomatic disease. Clearly, there are multiple effector pathways in the pathogenesis of PBC and, further, it is likely that the mediators of immunopathology may change during different stages of disease based not only upon genetic predisposition but also upon the qualitative and quantitative changes in the inflammatory response (45–50). We should note that several independent panels of sera were used in this study and the number of PBC patients studied herein is a large series of over 350 patients. Additional work, particularly on stratifying based on patient genotype and phenotype, will be important in the future. While experienced clinicians may identify these individuals, the absence of classic markers of PBC, such as AMA and the more esoteric markers anti-gp210 and anti-sp100, may lead to delay in diagnosis and treatment. Our results have demonstrated that anti-KLHL12 and anti-HK1 antibodies are highly specific new markers of PBC and most importantly, are present in AMA-negative PBC patients, indicating that they are promising new candidates in the clinical diagnosis of PBC.

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