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Anti-Kelch-like 12 and Anti-Hexokinase 1: Novel Autoantibodies in Primary Biliary Cirrhosis

Short Title: Two novel biomarkers in PBC

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Conflict of interest: Gary L. Norman and Zakera Shums are employees of INOVA Diagnostics, Inc. Heather P. Ostendorff, Mark J. Lim, Amany Awad, and Kenneth J. Rothschild are employees of AmberGen Inc. All other authors declare no conflict of interest.

Abbreviations:

KLHL12, kelch-like 12; HK1, hexokinase-1; PBC, primary biliary cirrhosis; ELISA, enzyme linked immunosorbent assay; PDC-E2, pyruvate dehydrogenase complex; BCOADC-E2, branched chain 2-oxo-acid dehydrogenase complex; OGDC-E2, 2-oxo-glutarate dehydrogenase complex; SLE; systemic lupus erythematosus; SjS, Sjogren's syndrome, CRC, colorectal cancer; PSC, primary sclerosing cholangitis; ALF, acute liver failure; IFA, indirect immunofluorescence assay; UC, ulcerative colitis, CD, Crohn's disease; HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; VBDS, vanishing bile duct syndrome; IRP, Immune Response Profiling; BEC, biliary epithelial cells

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 KLH-12 and HK1 are more frequently identified ($p < 0.001$) in patients with PBC compared to controls.
- Antibodies to KLH-12 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.

Abstract

Background: 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.

Aims: Initial discovery used sera from 22 patients with PBC and 62 non-PBC controls. KLHL12 and HK1 proteins were then analyzed 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 analyzed 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 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.

Keywords: Liver, Serology, Diagnostics

Introduction

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-oxo-glutarate 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 5 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). Since 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 homeostasis 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 is 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 (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 ≥ 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: (A) Biomarker discovery at AmberGen laboratories, (B) immunoblot analysis at the University of California, Davis, and (C) conventional ELISA development, validation, and clinical evaluation at INOVA Diagnostics.

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 analyzed using proteome microarrays. Ten SLE sera were from Bioreclamation Inc. (Hicksville, NY). Normal sera were from ProMedDx, LLC (Norton, MA) and CRC sera were from Asterand Inc. (Detroit, MI). All remaining sera were from a biobank at Massachusetts General Hospital (Boston, MA) of de-identified samples from patients with PBC and other autoimmune diseases. The 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 ~8,000 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). 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) using the Immune Response Profiling (IRP) add-on. Further selection and narrowing of candidate markers utilized M-statistics algorithms and Z-score analysis.

Antigens & Immunoblotting

Recombinant proteins, KLHL12 and HK1, were purchased from Novus Biologicals (Littleton, CO). 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 analyzed in parallel. In addition, the AMA status in disease controls was validated by immunoblot against a mammalian mitochondrial preparation.

ELISA

Microtiter 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 added for 30 min. After washing plates, color was developed by adding 3,3'5,5'-tetramethylbenzidine substrate for 30 min. The reaction was terminated by adding 0.1N 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 (≤20), equivocal (20.1-24.9), and positive (≥25 units). Additionally, sera were tested for PBC-specific antibodies using QUANTA Lite® 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).

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 8,000 unique human recombinant proteins, HK1 and KLHL12 were both found to be valid PBC autoantigens (Figure 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 (Figure 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 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 (Figure 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 (Figure 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 Figure 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) (Figure 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 (Figure 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% (Figure 6C). By adding the two new biomarkers, the overall sensitivity of serological markers in AMA-negative PBC was improved from 48.3% to 68.5% (Figure 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 due to its high specificity (23). PBC is serologically characterized by a high titer of AMA, of which the major targets are PDC-E2, OGDC-E2, and BCOADC-E2 (24). 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), due to 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 labor-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 inter-laboratory 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 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.

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%)[a]	147/366	(40%)[a]	16/100	(16%)[a]	166/366	(45%)[a]
AMA-IFA-positive PBC	11/80	(14%)[b]	116/277	(42%)[a]	14/80	(18%)[a]	146/277	(53%)[a]
AMA-IFA-negative PBC	5/20	(25%)[b]	31/89	(35%)[a]	2/20	(10%)	20/89	(22%)[a]
Scleroderma[c]	2/43	(5%)	--	--	5/43	(12%)	--	--
ALF[d]	3/55	(5%)	--	--	4/55	(7%)	--	--
SLE	1/29	(3%)	--	--	0/29	(0%)	--	--
PSC[e]	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[f]	--	--	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%)

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

[b] The frequency of α-KLHL12+/HK1+ in PBC is higher compared with all non-PBC controls *(p* < 0.01).

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

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

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

[f] The one positive healthy control was weak positive (27 units, cutoff 25 units) on MIT3 ELISA.

Figure Legends

Figure 1. Quantile normalized protein microarray autoantibody data for (A) HK1 and (B) KLHL12 for 80 distinct serum samples.

Figure 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 (2^{nd} Ab Only) controls were analyzed in parallel.

Figure 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.

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

Figure 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.

Figure 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).

References

1. Hirschfield G M, Gershwin M E. The immunobiology and pathophysiology of primary biliary cirrhosis. Annu Rev Pathol 2013; 8: 303-30.

2. Bogdanos D P, Gershwin M E. What is new in primary biliary cirrhosis? Dig Dis 2012; 30 Suppl 1: 20-31.

3. Heathcote E J. Management of primary biliary cirrhosis. The American Association for the Study of Liver Diseases practice guidelines. Hepatology 2000; 31(4): 1005-13.

4. Poupon R E, Lindor K D, Pares A, et al. Combined analysis of the effect of treatment with ursodeoxycholic acid on histologic progression in primary biliary cirrhosis. J Hepatol 2003; 39(1): 12-6.

5. Kaplan M M, Gershwin M E. Primary biliary cirrhosis. N Engl J Med 2005; 353(12): 1261-73.

6. Metcalf J V, Mitchison H C, Palmer J M, et al. Natural history of early primary biliary cirrhosis. Lancet 1996; 348(9039): 1399-402.

7. Zhu H, Bilgin M, Bangham R, et al. Global analysis of protein activities using proteome chips. Science 2001; 293(5537): 2101-5.

8. Hudson M E, Pozdnyakova I, Haines K, Mor G, Snyder M. Identification of differentially expressed proteins in ovarian cancer using high-density protein microarrays. Proceedings of the National Academy of Sciences of the United States of America 2007; 104(44): 17494-9.

9. Hu C J, Song G, Huang W, et al. Identification of new autoantigens for primary biliary cirrhosis using human proteome microarrays. Molecular & cellular proteomics : MCP 2012; 11(9): 669-80.

10. Jin L, Pahuja K B, Wickliffe K E, et al. Ubiquitin-dependent regulation of COPII coat size and function. Nature 2012; 482(7386): 495-500.

11. Rondou P, Haegeman G, Vanhoenacker P, Van Craenenbroeck K. BTB Protein KLHL12 targets the dopamine D4 receptor for ubiquitination by a Cul3-based E3 ligase. The Journal of biological chemistry 2008; 283(17): 11083-96.

12. Funato Y, Terabayashi T, Sakamoto R, et al. Nucleoredoxin sustains Wnt/beta-catenin signaling by retaining a pool of inactive dishevelled protein. Current biology : CB 2010; 20(21): 1945-52.

13. Saraiva L M, Seixas Da Silva G S, Galina A, et al. Amyloid-beta triggers the release of neuronal hexokinase 1 from mitochondria. PloS one 2010; 5(12): e15230.

14. Robey R B, Hay N. Mitochondrial hexokinases: guardians of the mitochondria. Cell cycle 2005; 4(5): 654-8.

15. Lindor K D, Gershwin M E, Poupon R, et al. Primary biliary cirrhosis. Hepatology 2009; 50(1): 291-308.

16. Leung P S, Wang J, Naiyanetr P, et al. Environment and primary biliary cirrhosis: electrophilic drugs and the induction of AMA. J Autoimmun 2013; 41: 79-86.

17. Moteki S, Leung P S, Coppel R L, et al. Use of a designer triple expression hybrid clone for three different lipoyl domain for the detection of antimitochondrial autoantibodies. Hepatology 1996; 24(1): 97-103.

18. Leung P S, Manns M P, Coppel R L, Gershwin M E. Detection of antimitochondrial Autoantibodies in primary biliary cirrhosis and LKM antibodies in autoimmune hepatitis. 6 ed. Washington D.C.: ASM Press; 2002.

19. Leung P S, Yang G X, Dhirapong A, et al. Animal models of primary biliary cirrhosis: materials and methods. Methods in molecular biology 2012; 900: 291-316.

20. Milkiewicz P, Buwaneswaran H, Coltescu C, et al. Value of autoantibody analysis in the differential diagnosis of chronic cholestatic liver disease. Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association 2009; 7(12): 1355-60.

21. Gabeta S, Norman G L, Liaskos C, et al. Diagnostic relevance and clinical significance of the new enhanced performance M2 (MIT3) ELISA for the detection of IgA and IgG antimitochondrial antibodies in primary biliary cirrhosis. J Clin Immunol 2007; 27(4): 378-87.

22. Granito A, Yang W H, Muratori L, et al. PML nuclear body component Sp140 is a novel autoantigen in primary biliary cirrhosis. Am J Gastroenterol 2010; 105(1): 125-31.

23. Agmon-Levin N, Shapira Y, Selmi C, et al. A comprehensive evaluation of serum autoantibodies in primary biliary cirrhosis. J Autoimmun 2010; 34(1): 55-8.

24. Selmi C, Bowlus C L, Gershwin M E, Coppel R L. Primary biliary cirrhosis. Lancet 2011; 377(9777): 1600-9.

25. Nardi N, Brito-Zeron P, Ramos-Casals M, et al. Circulating auto-antibodies against nuclear and non-nuclear antigens in primary Sjogren's syndrome: prevalence and clinical significance in 335 patients. Clinical rheumatology 2006; 25(3): 341-6.

26. Skare T L, Nisihara R M, Haider O, Azevedo P M, Utiyama S R. Liver autoantibodies in patients with scleroderma. Clinical rheumatology 2011; 30(1): 129-32.

27. Leung P S, Rossaro L, Davis P A, et al. Antimitochondrial antibodies in acute liver failure: implications for primary biliary cirrhosis. Hepatology 2007; 46(5): 1436-42.

28. Uchida K, Akita Y, Matsuo K, et al. Identification of specific autoantigens in Sjogren's syndrome by SEREX. Immunology 2005; 116(1): 53-63.

29. Gonzalez-Gronow M, Cuchacovich M, Francos R, et al. Antibodies against the voltage-dependent anion channel (VDAC) and its protective ligand hexokinase-I in children with autism. Journal of neuroimmunology 2010; 227(1-2): 153-61.

30. Miyakawa H, Tanaka A, Kikuchi K, et al. Detection of antimitochondrial autoantibodies in immunofluorescent AMA-negative patients with primary biliary cirrhosis using recombinant autoantigens. Hepatology 2001; 34(2): 243-8.

31. Bizzaro N, Covini G, Rosina F, et al. Overcoming a "probable" diagnosis in antimitochondrial antibody negative primary biliary cirrhosis: study of 100 sera and review of the literature. Clinical reviews in allergy & immunology 2012; 42(3): 288-97.

32. Ruffatti A, Arslan P, Floreani A, et al. Nuclear membrane-staining antinuclear antibody in patients with primary biliary cirrhosis. J Clin Immunol 1985; 5(5): 357-61.

33. Szostecki C, Krippner H, Penner E, Bautz F A. Autoimmune sera recognize a 100 kD nuclear protein antigen (sp-100). Clin Exp Immunol 1987; 68(1): 108-16.

34. Copple S S, Martins T B, Masterson C, Joly E, Hill H R. Comparison of three multiplex immunoassays for detection of antibodies to extractable nuclear antibodies using clinically defined sera. Ann N Y Acad Sci 2007; 1109: 464-72.

35. Rigopoulou E I, Dalekos G N. Molecular diagnostics of primary biliary cirrhosis. Expert opinion on medical diagnostics 2008; 2(6): 621-34.

36. Dahnrich C, Pares A, Caballeria L, et al. New ELISA for detecting primary biliary cirrhosis-specific antimitochondrial antibodies. Clinical chemistry 2009; 55(5): 978-85.

37. Copple S S, Sawitzke A D, Wilson A M, Tebo A E, Hill H R. Enzyme-linked immunosorbent assay screening then indirect immunofluorescence confirmation of antinuclear antibodies: a statistical analysis. Am J Clin Pathol 2011; 135(5): 678-84.

38. Nakamura M, Takii Y, Ito M, et al. Increased expression of nuclear envelope gp210 antigen in small bile ducts in primary biliary cirrhosis. J Autoimmun 2006; 26(2): 138-45.

39. Sasaki M, Miyakoshi M, Sato Y, Nakanuma Y. Increased expression of mitochondrial proteins associated with autophagy in biliary epithelial lesions in primary biliary cirrhosis. Liver Int 2013; 33(2): 312-20.

40. Naiyanetr P, Butler J D, Meng L, et al. Electrophile-modified lipoic derivatives of PDC-E2 elicits anti-mitochondrial antibody reactivity. J Autoimmun 2011; 37(3): 209-16.

41. Leung P S, Lam K, Kurth M J, Coppel R L, Gershwin M E. Xenobiotics and autoimmunity: does acetaminophen cause primary biliary cirrhosis? Trends Mol Med 2012; 18(10): 577-82.

42. Azoulay-Zohar H, Israelson A, Abu-Hamad S, Shoshan-Barmatz V. In self-defence: hexokinase promotes voltage-dependent anion channel closure and prevents mitochondria-mediated apoptotic cell death. The Biochemical journal 2004; 377(Pt 2): 347-55.

43. Abu-Hamad S, Zaid H, Israelson A, Nahon E, Shoshan-Barmatz V. Hexokinase-I protection against apoptotic cell death is mediated via interaction with the voltage-dependent anion channel-1: mapping the site of binding. The Journal of biological chemistry 2008; 283(19): 13482-90.

44. Villinger S, Briones R, Giller K, et al. Functional dynamics in the voltage-dependent anion channel. Proceedings of the National Academy of Sciences of the United States of America 2010; 107(52): 22546-51.

45. Ando Y, Yang G X, Kenny T P, et al. Overexpression of microRNA-21 is associated with elevated pro-inflammatory cytokines in dominant-negative TGF-beta receptor type II mouse. J Autoimmun 2013; 41: 111-9.

46. Hudspeth K, Pontarini E, Tentorio P, et al. The role of natural killer cells in autoimmune liver disease: a comprehensive review. J Autoimmun 2013; 46: 55-65.

47. Kawata K, Yang G X, Ando Y, et al. Clonality, activated antigen-specific CD8(+) T cells, and development of autoimmune cholangitis in dnTGFbetaRII mice. Hepatology 2013; 58(3): 1094-104.

48. Wang L, Sun Y, Zhang Z, et al. CXCR5 CD4 T follicular helper cells participate in the pathogenesis of primary biliary cirrhosis. Hepatology 2014.

49. Zhang J, Zhang W, Leung P S, et al. Ongoing Activation of Autoantigen-Specific B cells in Primary Biliary Cirrhosis. Hepatology 2014.

50. Yang C Y, Ma X, Tsuneyama K, et al. IL-12/Th1 and IL-23/Th17 biliary microenvironment in primary biliary cirrhosis: implications for therapy. Hepatology 2014; 59(5): 1944-53.