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Detection of Anti-kelch-like 12 and Anti-hexokinase 1 Antibodies in Primary Biliary Cholangitis Patients in China

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ABSTRACT

Objectives
Primary Biliary Cholangitis (PBC) is a chronic cholestatic disease, characterized by positive anti-mitochondrial autoantibodies (AMA) in 90%-95% patients. Anti-kelch-like 12 (anti-KLHL12) and anti-hexokinase1 (anti-HK-1) antibodies have been identified as the two latest serum markers in recent years, which employed in diagnosing AMA negative PBC patients. The objective of the study is to examine the performance of these two new biomarkers in China.

Methods
A total of 192 patients were enrolled and screened for anti-KLHL12, anti-HK-1 antibodies and AMA by ELISA. Receiver Operating Characteristic (ROC curve) was applied to examine the diagnostic importance of AMA, anti-KLHL12 and anti-HK-1 antibodies. Furthermore, correlation analysis between some important biochemical indexes (ALT, AST, ALP, Bilirubin, γ-GT ), the staging of pathological changes of liver, and expression of novel antibodies in PBC patients were also examined.

Results
The positivity of anti-HK1 antibody in AMA-positive PBC patients and AMA-negative patients was found to be 44.7% and 33.3%, respectively. Specificity, PPV, and NPV were observed as 93%, 89%, 53%, respectively. In contrast, the positivity of anti-
KLHL12 antibody in AMA-positive and negative PBC patients was 41.2 % and 22.2 %, respectively. Specificity, PPV, and NPV were found as 98 %, 95 %, 52 %, respectively. The area under the curve (AUC) with anti-HK1 and anti-KLHL12 antibodies were 0.720 and 0.703. Through the combination with anti-HK1 and anti-KLHL12 antibodies, the AUC of AMA was increased from 0.889 to 0.891, raising the sensitivity from 0.764 to 0.836. Anti-KLHL12 and anti-HK1-positive patients had higher serum levels of ALP, γ-GT and bilirubin, with statistical differences (P<0.01) compared with anti-KLHL12 or anti-HK1-negative patients. Notably, correlation analysis showed a significantly positive correlation between antibody expression and ALP, γ-GT and bilirubin serum levels (r=0.735, 0.491, 0.466; P < 0.01).

**Conclusions**

Anti-HK1 and anti-KLHL12 antibodies can be identified as two significant biomarkers in PBC patients. Furthermore, the presence of these antibodies is likely to be correlated with the severity of PBC.

**Keywords**

Anti-kelch-like 12 antibody, anti-hexokinase 1 antibody, diagnosis, Primary Biliary Cholangitis

**INTRODUCTION**

Primary biliary cholangitis (PBC) is known to be an autoimmune liver disease, characterized by chronic cholestatic and destruction of the small intrahepatic bile ducts[1,2]. Over the last few decades, the annual incidence rates of PBC shows an upward trend in China. According to recent reports, the prevalence of PBC is up to 49/1000000 adults[3], contributing to the profound understanding of the disease and progress in detecting methods[4-6]. Furthermore, the pathophysiological mechanism of PBC is not very clear, with the onset of occult. Symptoms often appear in the middle and late stage, resulting in poor prognosis[3]. Thus, early diagnosis of PBC is of great clinical significance. Anti-mitochondrial autoantibodies (AMA) is a key serological
marker of PBC for its high specificity and sensitivity[7]. However, AMA was found to be negative among 10-40% of PBC patients[8], and AMA also exist in other diseases[9-12]. Specific antinuclear antibodies to PBC such as anti-gp210 and anti-sp100, have higher specificity, but lower sensitivity[13-14]. Therefore, the only way to confirm the diagnosis of PBC is to carry out a liver biopsy for highly suspected persons who are clinically AMA negative[15].

Autoantibodies to kelch-like12 protein(KLHL12) and to hexokinase1(HK-1) have recently been identified as novel biomarkers in patients with PBC recently[16-17]. KLHL12, a nuclear protein linked with collagen export, which directs the ubiquitination of dopamine D4 receptor and Dishevelled protein[18-20]. However, HK-1, as a member of the hexokinase family, is found in the outer mitochondrial membrane in tissues, allowing phosphorylating glucose to produce glucose-6-phosphate. Norman et al. showed that the combination of anti-KLHL12 and anti-HK1 antibodies in serological detection could significantly improve the efficiency of the clinical detection and diagnosis, particularly for AMA-negative individuals[16]. However, these two new antibodies have not been widely employed in clinical practice, and few relevant reports can be found in China. Therefore, there is an urgent need to verify the significance of anti-KLHL12 and anti-HK1 autoantibodies for the diagnosis of PBC.

MATERIALS AND METHODS

Study population
A total of 192 subjects, aged from 23 to 80 years old were enrolled at the Affiliated Hospital of Qingdao University between April and December of 2019. In this study, 112 patients had PBC, 40 individuals with AIH as disease control group and compared with 40 healthy individuals for the analysis. The clinical features of patients are presented in Table 1. The PBC patients were enrolled following the recommended criteria by the European Association for the study of the Liver (EASL)[21]. The diagnosis of PBC should meet any two of the following three criteria a) biochemical indicators of cholestasis, such as the elevated ALP, b) positive for AMA, and c) serum AMA was negative, while the liver biopsy was consistent with PBC. Exclusion criteria were followed as; a)
patients with positive serum hepatitis virus markers; b) patients with alcohol history (≥20g/d); c) medications that are toxic to the liver, bile ducts or that cause biliary obstruction d) patients with autoimmune hepatitis, and e) patients after liver transplantation. Our studies were approved by the ethics committe of Qingdao University and and were carried out following the ethical standards laid down in the 1964 Declaration of Helsinki and its subsequent amendments. All the enrolled participants had given informed consent.

**Detection of AMA, anti-KLHL12 and anti-HK1 antibodies by ELISA**

The level of AMA were detected by ELISA kits (Inova Diagnostics, San Diego, CA). 50 µl of control and sample was filled individually into the appropriate wells. Then added 100 µl of enzyme conjugate to control wells and sample wells except for the blank wells, covered with an adhesive strip, and incubated for 60 minutes at 37°C. Remove incubation mixture by aspirating contents of the plate into a sink or proper waste container. Each well was filled with completely with Wash Solution (1X) using a squirt bottle, then aspirated contents of the plate into a sink or proper waste container. This procedure was repeated four times. After the final wash, invert plate, and blot dry by hitting plate onto absorbent paper or paper towels until no moisture appears. Then, added Substrate A of 50 µl and Substrate B of 50 µl to each well. Gently mixed and incubated for 15 minutes at 37°C. 50 µl of stop solution was added to each well. The Optical Density (O.D.) at 450 nm was noted using a microtiter plate reader within 15 minutes. The level of anti-KLHL12 and anti-HK1 antibodies were measured by ELISA kits (Inova Diagnostics, San Diego, CA). All data were measured by the same operator using the same equipment. All the operations were carried out following the instructions provided in the kit.

**Evaluation of liver histopathological features of PBC**

The patient’s histopathologic examanation was evaluated based on Scheuer staging criterea[22]. PBC were divided into 4 phases. 1)Stage I, Cholangitis stage, the inflammation is confined to the portal area, 2)Stage II, bile duct proliferation,
inflammation in the portal area has been extended to the surrounding parenchyma, and the number of normal bile ducts has decreased, 3) Stage III, fibrosis stage, the adjacent manifolds were connected by fibrous septum 4) Stage IV, cirrhosis stage, obvious cirrhosis of regenerative nodules. Stage I and stage II are known to be the early stage of histological changes of PBC, while stages III and IV are considered to be the period of histological development of PBC.

**Statistical analysis**

SPSS 19.0 statistical software (IBM, Armonk, NY, USA) was used for statistical analysis of the data. The measurement data conforming to normal distribution are described through mean±SD, an independent sample t-test was used to compare the two groups. The measurement data for non-normal distribution are described by median(P50) and interquartile spacing(p25-p75). The comparison between the two groups was performed by the Mann Whitney test. Spearman rank correlation analysis performed a comparison between the two groups. Spearman rank correlation analysis was used to analyze the correlation between the serum level of liver function indexes, the staging of pathological changes in the liver, and the antibody expression. P < 0.05 was statistically significant.

**RESULTS**

**Frequency and diagnostic value of anti-KLHL12 and anti-HK1 auto-antibodies in PBC patients**

Among the enrolled participants, AMA was found in 75.9 % (85/112) PBC patients, whereas in 12.5 % (5/40) AIH patients, but not in health controls. The positivity of anti-KLHL12 and anti-HK1 autoantibodies in AMA-positive individuals was 41.2 % (35/85) and 44.7 % (38/85) respectively, as depicted in Table 2. Detection of AMA-negative PBC patients indicated that the prevalence of anti-KLHL12 and anti-HK1 autoantibodies was 22.2 % (6/27) and 33.3 % (9/27) respectively, as shown in Table 2. The sensitivity, specitivity, the proportion of positive patients (PPV) and the proportion of negative patients who were accurately diagnosed (NPV) of anti-HK-1 antibody were
42 %, 93 %, 89 %, 53 %, respectively. Sensitivity, specificity, PPV and NPV of anti-KLHL12 antibody were found to be 37 %, 98 %, 95 %, 52 % respectively. Table 3 summarizes a comparison of the diagnostic value of anti-KLHL12 and anti-HK1 autoantibodies in enrolled patients with specific antinuclear antibodies (anti-gp210 and anti-Sp100) to PBC. The positivity of gp210 and sp100 in AMA negative PBC patients was 37 %(10/27) and 7.4 %(2/27), respectively. Specitivity, PPV and NPV of anti-gp210 and anti-Sp100 were depicted in Table 3.

The ROC curve for AMA, anti-KLHL12, and anti-HK1 autoantibodies detected by ELISA kits is shown in Table 4. ROC curve analysis indicated that the areas under the curve(AUC) of anti-KLHL12 and anti-HK1 autoantibodies were calculated as 0.703 and 0.720, respectively. Besides, the addition of anti-HK-1 and anti-KLHL12 antibodies to AMA increased the AUC from 0.889 to 0.891. The sensitivity of AMA increased from 0.764 to 0.836 combined with anti-HK-1 and anti-KLHL12 antibodies; however, the specificity was decreased from 0.93 to 0.88, which was statistically significant(p<0.001).

**Correlation analysis between liver function index, the staging of pathological changes of liver and antibody expression**

As shown in Table 5, the levels of AST, ALP, γ-GT and bilirubin in sera of anti-HK-1 or anti-KLHL12 antibodies positive group were higher than those in anti-HK-1 and anti-KLH12 antibodies negative group, showing statistical significance(P < 0.05). A negligible difference in the level of ALP and albumin between the two groups was achieved during the analysis. According to Table 6, there was a positive correlation between antibody expression and serum levels of AST, ALP, γ-GT and bilirubin(r=0.332, 0.735, 0.491, 0.466; p<0.05), indicating that antibody positivity may suggest a higher serological level of AST, ALP, γ-GT and bilirubin. However, no significant correlation between antibody expression and ALT, albumin serum levels were noticed (P>0.05).

Among the enrolled 112 PBC patients, 41 patients performed liver biopsies. In these cases, 4 patients were in Stage I, 5 patients in stage II, 12 patients in stage III and 20 patients in stage IV. Overall, 32 cases (78 %) were in stage III and IV, only 9 cases (22 %)
were in stage I and II. Among the patients with stage III and stage IV, 34.4 %(11/32) were antibody positive, while 22.2 % (2/9) were in stage I and II. Based on the results of correlation analysis, there was no significant correlation between antibody expression and the staging of pathological changes of liver (r=0.108, P=0.501).

DISCUSSION

PBC is an autoimmune chronic cholestatic liver disease that can eventually lead to liver fibrosis or even cirrhosis and liver dysfunction without clinical intervention[23]. Ursodeoxycholic acid (UDCA) therapy is considered to be the first-line treatment for PBC patients[24-26]. Studies have shown that up to 40% of patients diagnosed with early PBC and rapidly initiated UDCA are still in the early stage at least 20 years after diagnosis[27]. The emergence of anti-KLHL12 and anti-HK1 auto-antibodies can be useful in improving the diagnostic efficiency and identifying asymptomatic PBC patients[16], thus laying a foundation for early application of UDCA.

In the diagnosis of PBC, liver biopsy is still the gold standard for assessing the degree of pathological damage in the liver[23]. However, liver biopsy is an invasive procedure with a mortality rate of 1:1000-1:10000[28,29], which is not easily accepted by patients. Although PBC has unique specific serological characteristics[8], as noninvasive diagnostic methods, serological analysis developed rapidly with a high diagnostic value and easy acceptance by patients in recent years. Our current findings have shown that anti-KLHL12 and anti-HK1 autoantibodies are present in PBC patients residing in China, especially in AMA positive patients. In this study, the positive rate of anti-KLHL12 antibody was 36.6 %, which was higher than 22 %-33.3 % in Europe and the United States[30]. We speculate regional and ethnic differences between the Asian population and the Western population, whereas the number of cases in the present study is limited. By comparing with anti-gp210 and anti-sp100 antibodies, we found that the specificity for novel antibodies was about 90 %. Notably, the sensitivity of novel antibodies was higher than that of anti-gp210 and anti-sp100, especially anti-sp100, suggesting that novel antibodies can be used as supplementary biomarkers. However, anti-HK-1 antibody detection rate was higher than the anti-KLHL12 antibody,
which could be attributed to the presence of HK-1 on the mitochondrial membranes[31]. It was observed that in small bile ducts, the expression of mitochondrial antigens was found to be elevated in PBC patients, resulting in cytotoxic responses against intrahepatic bile ducts[32]. While KLHL12 is located inside the nucleus, the potential relationship between these two proteins and the pathogenesis of PBC remains unknown[33-35]. The ROC analysis showed that the combination of AMA, anti-KLHL12 and anti-HK1 autoantibodies increased the sensitivity of AMA. In addition, the combination of two new antibodies could help in the diagnosis of PBC by improving the detection rate of AMA negative PBC patients, thus reducing the risk of liver biopsy and save costs.

The slow progress of PBC has significant differences among individuals, whereas the activity of serum ALP, γ-GT and bilirubin is an indirect indicator of cholestasis[36-40], using as a substitute indicator to evaluate the severity of the disease. The levels of ALP, γ-GT and bilirubin in sera of anti-HK-1 or anti-KLHL12 antibodies positive group were found to be higher than those of anti-HK-1 and anti-KLH12 antibodies negative group. Notably, the expression of anti-HK-1 and anti-KLHL12 was positively correlated with serum levels of ALP, γ-GT and bilirubin, suggesting that anti-HK-1 and anti-KLHL12 antibodies could be closely related to the severity of the disease. Therefore, anti-HK-1 and anti-KLHL12 antibodies may be used as supplements to reveal the severity of PBC, which is of considerable significance for the implementation of clinical plans.

This is the first study to examine the incidence of anti-HK-1 and anti-KLHL12 antibodies and to analyze the correlation between novel antibodies and liver functions, based on which the diagnostic value of anti-HK-1 and anti-KLHL12 antibodies was verified. Nevertheless, there are some limitations in our study, as we couldn’t examine the clinical phenotype of our PBC patients. Primary biliary cholangitis is known as a relatively heterogeneous disease, and the clinical phenotype of different patients may vary significantly from patient to patient. Thus, further study needs to be performed, in particular, based on the classification of patients by their genotype and phenotype.

In conclusion, anti-HK-1 and anti-KLHL12 antibodies are beneficial for diagnosing PBC patients, especially those not detected by current PBC markers. In addition, anti-
KLHL12 and anti-HK1 antibodies are possibly related to the severity of PBC, which had certain clinical significance for the diagnosis and treatment of diseases.

ACKNOWLEDGEMENTS
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Table 1: Clinical features of enrolled subjects

<table>
<thead>
<tr>
<th></th>
<th>PBC</th>
<th>AIH</th>
<th>HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years</td>
<td>55.2±12.5</td>
<td>56.2±12.3</td>
<td>43.2±8.2</td>
</tr>
<tr>
<td>Sex (Female/male)</td>
<td>108/4</td>
<td>39/1</td>
<td>31/9</td>
</tr>
<tr>
<td>ALT (u/L)</td>
<td>111.5±38.9</td>
<td>81.2±31.1</td>
<td>20.9±8.2</td>
</tr>
<tr>
<td>AST (u/L)</td>
<td>88.9±23.1</td>
<td>76.5±28.5</td>
<td>20.7±6.4</td>
</tr>
<tr>
<td>ALP (u/L)</td>
<td>243.4±52.4</td>
<td>158.5±35.5</td>
<td>65.2±12.1</td>
</tr>
</tbody>
</table>
Abbreviations AMA, anti-mitochondrial autoantibodies; anti-HK1, anti-hexokinase1 antibodies; anti-KLHL12, anti-kelch-like12 antibodies; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γ-GT, gamma-glutamyl transpeptidase; n.v., normal value.

Table 2 Frequency of anti-KLHL12 and anti-HK1 auto-antibodies in PBC patients

<table>
<thead>
<tr>
<th></th>
<th>AntihK1⁺</th>
<th>Anti-KLHL12⁺</th>
<th>Anti-HK1⁺ and/or Anti-KLHL12⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMA⁺</td>
<td>85</td>
<td>38(44.7 %)</td>
<td>35(41.2 %)</td>
</tr>
<tr>
<td>AMA⁻</td>
<td>27</td>
<td>9(33.3 %)</td>
<td>6(22.2 %)</td>
</tr>
<tr>
<td>p</td>
<td>0.4143</td>
<td><strong>0.0194</strong></td>
<td>0.1037</td>
</tr>
</tbody>
</table>

a n.v.7-40 μ/L; b n.v.13-35 μ/L; c n.v.50-135 μ/L; d n.v.7-45 μ/L; e n.v.3-22 μmol/L; f n.v.35-55 g/L
### Table 3 Evaluation of antibody diagnostic ability

<table>
<thead>
<tr>
<th></th>
<th>AMA⁺</th>
<th>Anti-KLHL12⁺</th>
<th>Anti-HK1⁺</th>
<th>Anti-SP100⁺</th>
<th>Anti-gp210⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sensitivity</strong></td>
<td>0.76</td>
<td>0.37</td>
<td>0.42</td>
<td>0.13</td>
<td>0.33</td>
</tr>
<tr>
<td><strong>Specificity</strong></td>
<td>0.94</td>
<td>0.98</td>
<td>0.93</td>
<td>0.98</td>
<td>0.96</td>
</tr>
<tr>
<td><strong>PPV</strong></td>
<td>0.94</td>
<td>0.95</td>
<td>0.89</td>
<td>0.88</td>
<td>0.92</td>
</tr>
<tr>
<td><strong>NPV</strong></td>
<td>0.74</td>
<td>0.52</td>
<td>0.53</td>
<td>0.44</td>
<td>0.51</td>
</tr>
</tbody>
</table>

Abbreviations PPV, positive predictive value; NPV, negative predictive value

### Table 4 ROC curve for AMA, Anti-HK-1 and Anti-KLHL12 antibodies by ELISA

<table>
<thead>
<tr>
<th></th>
<th>AUC</th>
<th>95% CI</th>
<th>P-value</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMA</td>
<td>0.889</td>
<td>0.827-</td>
<td>&lt;0.001</td>
<td>0.764</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.955</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-HK-1</td>
<td>0.720</td>
<td>0.618-</td>
<td>&lt;0.001</td>
<td>0.509</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-HK-1 and Anti-KLHL12\textsuperscript{−} \quad (n=82)</td>
<td>Anti-HK-1 or Anti-KLHL12\textsuperscript{+}</td>
<td>Z / t</td>
<td>P-value</td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>---------------------------------------------------------</td>
<td>-------------------------------------------</td>
<td>-------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>Anti-HK-1 and Anti-KLHL12\textsuperscript{−}</td>
<td>0.703 \quad 0.593</td>
<td>0.001 \quad 0.491</td>
<td>0.89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-KLHL12</td>
<td>0.802 \quad 0.955</td>
<td>&lt;0.001 \quad 0.836</td>
<td>0.88</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 5** Biochemical parameters of PBC patients with positive or negative for anti-HK-1 and anti-KLHL12 antibodies
### Table 6 Correlation analysis between antibody level and the liver function index

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>r</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (u/L)</td>
<td>42</td>
<td>0.279</td>
<td>0.074</td>
</tr>
</tbody>
</table>

Abbreviations anti-HK1, anti-hexokinase1 antibodies; anti-KLHL12, anti-kelch-like12 antibodies; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γ-GT, gamma-glutamyl transpeptidase; n.v., normal value.

- n.v.7-40 µ/L; b n.v.13-35 µ/L; c n.v.50-135 µ/L; d n.v.7-45 µ/L; e n.v.3-22 µmol/L; f n.v.35-55 g/L
<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (u/L)</td>
<td>42</td>
<td>0.332</td>
<td>0.032</td>
</tr>
<tr>
<td>ALP (u/L)</td>
<td>39</td>
<td>0.735</td>
<td>0.000</td>
</tr>
<tr>
<td>γ-GT(u/L)</td>
<td>37</td>
<td>0.491</td>
<td>0.002</td>
</tr>
<tr>
<td>Bilirubin (umol/L)</td>
<td>38</td>
<td>0.466</td>
<td>0.003</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>41</td>
<td>-0.036</td>
<td>0.821</td>
</tr>
</tbody>
</table>

Note P<0.05 indicated there was a correlation between antibody level and liver function index. In contrast, P > 0.05 indicated there was no correlation between antibody level and liver function index. The r value represented the correlation coefficient between variables in the sample. A positive value indicates a positive correlation between two variables, that is, the change trend is the same. Negative values were the opposite. The closer the absolute value of r was to 1, the stronger the correlation was.