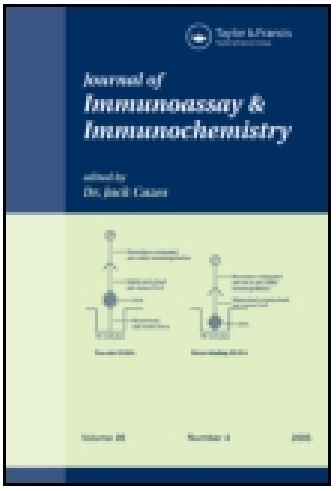

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Detection of Anti-Lactoferrin Antibodies and Anti-Myeloperoxidase Antibodies in Autoimmune Hepatitis: A Retrospective Study

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DETECTION OF ANTI-LACTOFERRIN ANTIBODIES AND ANTI-MYELOPEROXIDASE ANTIBODIES IN AUTOIMMUNE HEPATITIS: A RETROSPECTIVE STUDY

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□ *Anti-lactoferrin antibodies (ALA) and anti-myeloperoxidase antibodies (AMPA) are specific serological markers for autoimmune hepatitis (AIH). The project aimed to detect ALA and AMPA and explore their clinical significances in AIH patients. 59 AIH patients, 217 non AIH patients, and 50 healthy controls were enrolled in this study. ALA and AMPA were detected by ELISA. Antineutropil cytoplasmic antibodies (ANCA) and anti-smooth muscle antibodies (ASMA) were examined by indirect immunofluorescence. Antimitochondrial antibody M2 subtype (AMA-M2), anti-liver kidney microsomal antibody Type 1 (LKM1), anti-liver cytosol antibody Type 1 (LC1), and anti-soluble liver antigen/liver-pancreas antibodies (SLA/LP) were tested by immunoblot. The positivity for ALA was 18.6% in AIH group, only one patient in non-AIH group was positive for ALA; the positivity for AMPA was 59.3% in AIH group, with significant differences ($P < 0.01$) compared with other groups. The specificities for ALA and AMPA were 99.63% and 97.75%; the sensitivities were 18.64% and 59.32%; and the accuracy rates were 84.97% and 90.80%, respectively. A certain correlation was observed between ALA and SLA/LP, AMPA and ANCA, ASMA in AIH group. ALA and AMPA were associated with AIH, and had high clinical diagnostic value. Co-detection with other relative autoantibodies could play an important role in differential diagnosis of AIH.*

Keywords autoimmune hepatitis (AIH), anti-myeloperoxidase antibodies (AMPA), anti-lactoferrin antibodies (ALA)

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INTRODUCTION

Autoimmune hepatitis (AIH) is a chronic liver disease characterized with interface hepatitis, hypergammaglobulinaemia, extrahepatic syndromes, and a good response to immunosuppressive treatment.[1] However, the diagnosis is only confirmed by the exclusion of other liver diseases also featured by biochemical, histological, and clinical characteristics of chronic hepatitis.[2] Therefore, the precise diagnosis is necessary. The occurrence of AIH is more often followed by the development of clinical diagnostic technology and the prognosis will be severe if the disease could not be diagnosed early, even if many available treatments are applied.[3] There is no specific clinical symptom for AIH as compared with other liver diseases, therefore, detection of autoantibodies from sera becomes a helpful mean to diagnose AIH. As it is difficult to diagnose AIH accurately, identification of novel specific and sensitive autoantibodies for improving diagnosis of AIH is critical. In this study, we observed that the positivities for ALA and AMPA in AIH patients were higher than that in patients with other liver diseases. These two autoantibodies were useful in diagnosing AIH, and there was a certain correlation between the autoantibodies in AIH patients.

MATERIAL AND METHODS

Patients

59 AIH patients and 217 non AIH patients were enrolled in the study, including outpatient and inpatient cases from the Second Affiliated Hospital of Nanchang University. Data were assembled between October 2008 and December 2012. Among 59 AIH patients, 8 were male, aged 4–68 years (mean 42 years). In non AIH group, out of 35 patients suffered from primary biliary cirrhosis (PBC), 6 were male, aged 31–68 years (mean 47 years); out of 21 patients were diagnosed with primary sclerosing cholangitis (PSC), 16 were male, aged 21–62 years (mean 41 years); out of 78 patients were infected with hepatitis B (HB), 38 were male, aged 18–60 years (mean 39 years); out of 54 patients were infected with hepatitis C (HC), 28 were male, aged 20–59 years (mean 41 years); out of 29 patients were infected with hepatitis E (HE), 11 were male, aged 18–54 years (mean 44 years). In addition, sera obtained from 50 healthy blood donors (20 men) for physical examination in the same hospital were collected as negative controls, aged 23–58 years (mean 36 years). Informed consents were obtained from each participant included in this article.

Diagnostic Evidence

Autoimmune liver diseases were diagnosed according to the internationally agreed and recently revised criteria in 2008.[3] The diagnosis of

viral hepatitis was referred to the criteria recently revised by the Ministry of Health of the People's Republic of China in 2008.

Inclusion Criteria

The patients conformed to all of the following conditions were enrolled in this study: informed consent; accordance with the diagnostic criteria without any other liver disease; intact clinical and pathologic data; re-evaluation all the data of patients participated, without hereditary diseases, such as hemachromatosis, antitrypsin deficiency, or Wilson disease; no complication, including diabetes, arthrolithiasis, or phenylketonuria; no history of taking drug with hepatotoxicity, drinking a lot, suffering from cancer or any other liver disease, such as alcoholic liver disease before the occurrence of AIH.

Exclusion Criteria

The patients under any of the following conditions would be ruled out: no consistence with one or more bringing in standard rules; women in pregnancy or lactation; and decompensated cirrhosis.

Blood Samples

2–3 mL fasting blood samples from the vein were collected in a tube without any anticoagulant. After centrifugation at 1000 g for 15 min, sera were separated, divided into aliquots and frozen at −20°C.

Operating Instructions

In this article, all controls were designed, and the instructions were operated according to the protocols of commercial kits and standard operation procedure (SOP) of our laboratory.

Immunofluorescence

ASMA, ANCA were detected by indirect immunofluorescence (IF) with commercial kits from Euroimmun Company (Lübeck, Schleswig-Holstein, Germany). ASMA were tested on snap-frozen sections of rat stomach. Detection of ANCA was performed by IF on Granulocyte Mosaic (alcohol and methanal-fixed human neutrophils, HEp-2 cell,s and primate liver) from Euroimmun Company.

ELISA

ALA, AMPA were detected by enzyme linked immunosorbent assay (ELISA) kits from Euroimmun Company. Diluted sera and controls were added into the corresponding wells with 100 μL, incubated for 30 min at room temperature (RT). After washing with buffer for three times, 100 μL HRP-conjugated antibody was applied for 30 min at RT. After washing as described previously, the substrate buffer was added for 5 min incubation. The optical density (OD) was read at 450 nm and results were analyzed following stop buffer added.

Immunoblot Assay

LKM1, LC1, SLA/LP, and AMA-M2 were tested by immunoblot. Briefly, recombinant LKM1, LC1, SLA/LP and five subtypes of AMA-M2 (MM 36 kDa, 45 kDa, 51 kDa, 55 kDa, and 74 kDa) antigens were transblotted onto nitrocellulose membrane. 30μl serum samples initially diluted 1:101 were incubated with the membrane strips in 1.5 mL dilution buffer for 30 min at RT. After washing, AP-conjugated anti-human IgG antibody was added for 30 min incubation at RT on the rocking bed. After further washing, the strips were reacted with 1.5 mL NBT/BCIP substrate solution for 10 min. Then the reaction was stopped by distilled water. The result was referred as positive if the band was dyed strongly according to the standard control strip.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 software. Enumeration data were described in percentage. One-factor analysis of variance between groups were completed using chi-square test; advantage analysis between antibodies were performed using paired chi-square test. Differences were considered significant when $P < 0.05$. Differences were considered significant greatly when $P < 0.01$. Consistency analysis between antibodies were completed by calculating kappa value. When $P < 0.01$, if the kappa value was 0.4–0.6, the consistency will be regarded as moderate; if 0.6–0.8, the consistency will be regarded as high; if more than 0.8, the consistency will be regarded as great.

Evaluation Indicators

The clinical evaluation indicators of autoantibodies including sensitivity (SEN), specificity (SPE), accuracy (ACC), positive likelihood ratio (+LR), negative likelihood ratio (-LR), and Youden's index (YDI) were calculated according to the results of antibodies from patients diagnosed in golden

standard. It is valuable when accuracy > 0.75 and Youden's index > 0.5. In parallel tests, combined sensitivity = A sensitivity + [(1-A sensitivity) × B sensitivity]; combined specificity = A specificity × B specificity. In serial tests, combined sensitivity = A sensitivity × B sensitivity; combined specificity = A specificity + [(1-A specificity) × B specificity].

RESULTS

1. The detection of autoantibodies from patients with AIH or non AIH. The positivities of ALA and AMPA in AIH group were respectively 18.6% and 59.3%, while in non AIH group, only one patient was positive for ALA, as shown in Table 1.
2. The clinical evaluation indicators about the autoantibodies from AIH patients. In AIH group, the specificities of ALA and AMPA were respectively 99.63% and 97.75%, as listed in Table 2.
3. The advantage analysis (P) and consistency analysis [Kappa (κ)] between antibodies in AIH group was shown in Table 3.
4. The clinical evaluation indicators of autoantibodies in parallel and serial tests in AIH were listed in Table 4.

TABLE 1 The results of autoantibodies from patients with AIH or non AIH [cases(%)]

Group	N	ALA	SLA/LP	LKM-1	LC-1	AMPA	ANCA	ASMA	AMA-M2
non-AIH									
AIH	59	11 (18.6)[a]	9 (15.3)[a]	7 (11.9)[a]	2 (3.4)[a]	35 (59.3)[a]	38 (66.4)[a]	40 (67.8)[a]	3 (5.1)[a]
PBC	35	0	2 (5.7)[b]	0 [b]	0	3 (8.6)	3 (8.6)	2 (5.7)	32 (91.4)
PSC	21	1 (4.8)[b]	0	0	0	3 (14.3)	11 (52.4)[b]	0	0
HBV	78	0	0	0	1 (1.3)[b]	0	0	0	0
HCV	54	0	0	0	0	0	1 (1.9)	0	1 (1.9)[b]
HEV	29	0	0	0	0	0	0	0	3 (10.3)[b]
Control	50	0	0	0	0	0	0	0	0

[a]Versus non AIH and control, $P < 0.01$, but [b]versus AIH, $P > 0.05$.

TABLE 2 The clinical evaluation results of autoantibodies in AIH

Group	SEN%	SPE%	ACC%	+LR	−LR	YDI
ALA	18.64	99.63	84.97	49.78	0.82	0.18
SLA/LP	15.25	99.25	84.05	20.36	0.85	0.15
LKM-1	11.86	100	84.05	–	0.88	0.12
LC-1	3.39	99.63	82.21	9.05	0.97	0.03
AMPA	59.32	97.75	90.80	26.40	0.42	0.57
ANCA	66.10	94.38	88.96	11.46	0.38	0.61
ASMA	67.80	99.25	93.56	90.51	0.32	0.67
AMA-M2	5.08	86.51	71.78	0.38	1.10	−0.08

TABLE 3 Advantage analysis (P) and consistency analysis [Kappa(κ)] between antibodies in AIH group

Group	SLA/LP	LKM-1	LC-1	AMPA	ANCA	ASMA	AMA-M2
ALA							
p	0.687	0.388	0.004	0.000	0.000	0.000	0.039
κ	0.640*	0.220	0.226[a]	0.211[b]	0.110	0.085	0.068
AMPA							
p	0.000	0.000	0.000	–	0.453	0.180	0.000
κ	0.220	0.110	0.047	–	0.762[a]	0.673[a]	0.071

[a]$P < 0.01$, [b]$P < 0.05$.

TABLE 4 The clinical evaluation indicators of autoantibodies in parallel and serial test

Group	A	B	C	D	E	F	G	H	I
SEN%	41.55	26.58	95.56	39.71	97.40	81.18	76.22	80.19	17.81
SPE%	98.52	100	91.56	99.88	90.20	94.15	96.30	92.98	100
YDI	0.40	0.27	0.87	0.40	0.88	0.75	0.73	0.73	17.81

A: Parallel ALA, SLA/LP, LC-1, LKM-1; B: Series AMPA, ANCA, ASMA; C: Parallel AMPA, ANCA, ASMA; D: Series A, B; E: Parallel A, B; F: Series A, ASMA; G: Series A, AMPA; H: Series A, ANCA; I: Series C, ALA.

DISCUSSION

AIH is a chronic liver inflammatory disease that occurs when the body's immune system attacks liver cells. The characteristics of AIH are interface hepatitis in pathology, lymphocyte infiltration in portal area, high levels of aminotransferase and γ immunoglobin in serum, positive autoantibodies, and negative infection from virus[4] which leads to further complications, including cirrhosis.[5] AIH is also characterized by female preponderance. The etiology may be associated with the disturbance of immune microenvironment and the deficiency of immune tolerance caused by genetic predisposition and postnatal surroundings. AIH may be a result of autoimmune attack against the body's own hepatocytes as target antigen, but the particular mechanism is still unknown.[6,7] In AIH, some positive related autoantibodies are one of its serological parameters. So far, ASMA, SLA/LP, LKM, LC-1, and ANCA have played important roles in the differential diagnosis. The target antigens of ANCA were lysosomal enzymes in neutrophilic granulocytes, including lactoferrin and myeloperoxidase, which participated in the inflammation of autoimmune diseases resulting in pathologic injury of target organs such as liver. The target antigens may provide us a direction to explore novel autoantibodies with high specificity and sensitivity in serum to improve the diagnosis of AIH.

Lactoferrin (LF) is multifunctional globular glycoprotein which purified from milk and various secretory fluids, such as saliva and tears. LF is released from neutrophilic granulocytes after degranulation under the

second activation and is one of the components of innate immune system of the body; it also has specific natural immune regulation functions. Abundant data showed that LF played important role in regulating iron metabolism, antioxidation, killing or inhibiting microbial activity, antitumor, regulating cell growth and differentiation, anti-inflammation, and regulating immune responses.[8] The level of LF increased highly in infection and inflammation diseases. The underlying mechanism may be related to the type β secretion of bacteria.[9] Other data indicated that the production of tumor-necrosis factor-α (TNF-α), IL-6, IL-1B participated in the process of anti-inflammation.[10] The possible mechanism was that ALA produced by the body in immune disorder reacted with LF, which led to the inhibition of LF functions and increase of proinflammation factors, followed by the injury of liver after the exacerbation of inflammation. Myeloperoxidase (MPO) was released by the activated monocytes, macrophages, and neutrophilic granulocytes. MPO contained heme protein which had antiseptic effect and played a key role in innate immunity.

Increasing published data showed that MPO was closely related with acute and chronic inflammation. It can make use of hydrogen peroxide as oxidizing agent to produce hypochlorous acid which is cytotoxic, so as to cause tissue injury.[11,12] Antineutropil cytoplasmic antibodies (ANCA) can be classified to perinuclear ANCA (p-ANCA) and cytoplasmic ANCA (c-ANCA). MPO is the major target antigen of p-ANCA and the marker of activation of neutrophilic granulocytes, which could kill intracellular microorganism, destroy extracellular targets and regulate inflammation. p-ANCA stimulate the vascular endothelial cells to secrete E-selectin, cell adhesion molecule-1 and vascular cell adhesion molecule-1, and influence the inactivation of ceruloplasmin which inhibits the functions of MPO. Finally, the immune regulation is in disorder, followed by inflammatory injury of tissue cells, which may be the feature of AIH in pathology. In this article, among 59 AIH patients, the positivity of AMPA was 59.3% which was consistent with other similar studies; significant differences ($P < 0.01$) existed when compared with non AIH group; it had clinical importance as Youden's index was 0.57; and the accuracy was just next to ASMA. All the results indicated that AMPA had important value for differential diagnosis of AIH.

In the eight autoantibodies we detected, the positivity of ALA in AIH group was 18.6%. When compared with other groups (except PSC group), P value was less than 0.01 using Chi-square test with significant differences. It was worthy of being noted that only one PSC patient was positive for ALA in non AIH group. The specificity for ALA was as high as 99.7% in AIH, just next to 100% specificity for LKM-1. The advantage analysis between the two antibodies was performed using paired Chi-square test, in which P value was more than 0.05, showing no statistical significance. Because the positive for LKM-1 had been accepted universally as a serologic feature

of AIH Type 1, we may come to a conclusion that the diagnostic value of ALA for AIH is nearly equal to LKM-1. Furthermore, consistency analysis between ALA and LKM-1 was completed, and the kappa value was 0.220 ($P > 0.05$), showing no significance. It indicated that there was no consistency between ALA and LKM-1, supporting bad evidence for differential diagnosis of AIH. But it should be studied further that ALA may be important for the classification of AIH or specifically related with some other related diseases. Many studies observed that the target antigens of anti-soluble liver antigen/liver-pancreas (SLA/LP) were soluble proteins in liver cytoplasm which were highly specific for AIH, and can be acted as serologic diagnostic indicators for AIH. Although they may be useless for the classification of AIH, it would be greatly valuable for the evaluation of diseases. The advantage analysis between ALA and SLA/LP were performed using paired Chi-square test, in which P value was equal to 0.687, with no significant difference. In addition, consistency analysis between them was completed, and the kappa value was 0.640 ($P < 0.01$), showing significant differences. It indicated that there was high consistency between them. In other words, ALA and SLA/LP had certain correlation in AIH. In autoimmune liver disease group, only two AIH patients were positive for LC-1, while the results were negative in PBS or PSC group. The specificity of LC-1 for AIH was 99.63%, and the result showed that LC-1 were important for differential diagnosis of AIH. The advantage analysis between ALA and LC-1 was performed using paired Chi-square test, in which P value was less than 0.01, with significant differences. It showed that the total positivity of ALA for AIH was higher than LC-1. Moreover, consistency analysis between them was completed, and the kappa value was 0.226 ($P < 0.01$), with moderate significant differences in coincidence degree, but bad consistency.

In AIH group, the positivity of AMPA was 59.3%, and there were greatly significant differences compared with that in non AIH groups ($P < 0.01$). In addition, the specificity was as high as 97.75%, the sensitivity was 59.32%, and Youden's index was 0.57, showing clinical significances. The data indicated that AMPA had important value for differential diagnosis of AIH. When compared AMPA with ANCA or ASMA using paired Chi-square test, there was no significance for the detection of AIH ($P > 0.05$). The specificity of ASMA was 99.25%, and other indicators were the highest except negative likelihood ratio. Because ASMA were usually regarded as the specific marker of AIH Type 1, and ANCA were of clinical value in sensitivity, specificity, accuracy and Youden's index, consistency analysis, and correlation analysis were performed between AMPA and ANCA as well as ASMA. The kappa value was, respectively, 0.762 (AMPA vs. ANCA; $P < 0.01$) and 0.673 (AMPA vs. ASMA; $P < 0.01$). The results showed AMPA were of good

coincidence degree and great consistency with ANCA and ASMA, with statistical significances. AMPA may have equal significance in diagnosis like ANCA and ASMA, and be closely related with AIH so as to involve in the pathologic mechanism of AIH together.

We designed parallel test and serial test, which may be helpful to improve some indicators about AIH, such as sensitivity, specificity and Youden's index, and diagnosis of AIH. The positivities of ALA, SLA/LP, LC-1, LKM-1 in AIH group were respectively 18.6%, 15.3%, 3.4%, and 11.9%, which were similar to the data from other publishers. These antibodies were of high specificities for AIH. If any one of the above antibodies had been detected in the serum of a patient with liver diseases, he would be possibly suffered from AIH. When combined theses four antibodies together, the sensitivity increased up to 41.55%, and the rate of leave out diagnosis decreased. At the same time, more sensitive antibodies like AMPA, ANCA, or ASMA had been tested, if any one of which was positive, the patient might be more possiblely suffered from AIH, so as to reduce the rate of misdiagnosis and improve the rate of diagnosis comprehensively. Using the parallel test and serial test, the final Youden's index was larger than 0.7, indicating high clinical value and good validity, besides improving the rate of diagnosis of AIH. The sensitivities of AMPA, ANCA and ASMA for AIH were higher than other antibodies. If AMPA, ANCA, and ASMA were all positive in the same patient (in serial test), the final specificity for AIH would come up to 100%, indicating large possibility of AIH. When parallel test was done, the combination of AMPA, ANCA, and ASMA could further improve the sensitivity for AIH, and the Youden's index was as high as 0.87 with high validity for the diagnosis of AIH. When AMPA, ANCA and ASMA combined with more specific antibodies, such as ALA and SL/LP in serial test, the specificity for the diagnosis of AIH was 100%, supporting more sufficient evidences for AIH.

In summary, specific autoantibodies, such as ALA and AMPA, were AIH associated autoantibodies, and there was certain correlation between other related autoantibodies. Comprehensive analysis was of higher clinical diagnostic value for AIH. Therefore, joint detection of autoantibodies was very important for differential diagnosis of AIH.

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