Improved Diagnoses of Autoimmune Hepatitis Using an Anti-Actin ELISA

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The presence of antismooth muscle antibodies is one of the diagnostic criteria of autoimmune hepatitis. We evaluated a new anti-F-actin ELISA test and compared it with indirect immunofluorescence assay (IIFA) for antismooth muscle antibodies (ASMA). Two hundred and nine serum samples (35 autoimmune hepatitis, 174 other hepatopathies and control sera) were tested by IIFA on mouse stomach kidney sections for ASMA and by the Quanta Lite Actin ELISA for anti-F-actin antibodies. ASMA were detected in 26 of 35 sera from autoimmune hepatitis (74%) as compared with 25 (71%) with anti-actin antibodies, as well as in 25 of 49 (51%) samples from viral

hepatitis as compared with 7 (14%) with anti-actin antibodies. With regards to autoimmune hepatitis, though sensitivity (74.3 vs 71.4%) and negative predictive value (93.5 vs 93.9%) of ASMA and anti-actin ELISA were comparable, anti-actin ELISA was significantly better than ASMA IIFA in terms of specificity (89.7 vs 74.7%), and positive predictive value (58.1 vs 37.1%). Although frequently positive in HCV samples, a comparable sensitivity but better specificity makes the anti-actin ELISA a useful tool in combination with ASMA IIFA for the screening and diagnosis of autoimmune hepatitis. *J. Clin. Lab. Anal.* 22:340–345, 2008. © 2008 Wiley-Liss, Inc.

Key words: actin; autoimmune hepatitis; evaluation; immunofluorescence; antibodies

INTRODUCTION

Autoimmune hepatitis type I (AIH-I) is an inflammatory disease of the liver characterized by hypergammaglobulinemia, the presence of antinuclear autoantibodies (ANA) and antismooth muscle autoantibodies (ASMA) and a favorable response to steroids and immunosuppressive therapy (1). In 1993, Johnson and McFarlane published a scoring system for the diagnosis of AIH based on several clinical, serological and biological features, which included the presence of ANA and/or ASMA autoantibodies (2). During the last decade, it was shown that these autoantibodies were neither pathogenic nor disease specific (1,3). By indirect immunofluorescence (IIFA) on mouse stomach/kidney slides, ASMA may recognize different cytoskeleton proteins including actin, microtubules or intermediate filaments. ASMA are also detected in other liver diseases such as primary biliary cirrhosis and viral hepatitis, hence their low predictive value for AIH-I. (4,5). On the other hand, actin is the specific target of ASMA in AIH-I (6). Actin is a 42kd protein present in all eukaryotic cells in variable conditions of polymerization. The soluble form, or globular actin (G-actin),

associates in the presence of ATP to form double α -helix polymers and microfilaments (F-actin). The anti-actin autoantibodies only recognize the highly polymerized F-actin, which could hardly be detected in this form by ELISA or western blotting until recently (7).

Therefore, in order to improve the diagnosis of AIH-I, IIFA, which does not easily discriminate between the diverse ASMA targets, should logically be completed by an enzyme-linked immunosorbent assay (ELISA) using actin as solid phase. Although actin is the antigen recognized by ASMA in chronic active autoimmune hepatitis, no methods have been as of yet to be standardized used in the routine medical laboratory. We thus aimed to evaluate a new anti-F-actin ELISA test and to compare it with IIFA. To this purpose, a retrospective study on well-characterized series of

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patients expressing various autoantibodies and clinical conditions was performed.

MATERIALS AND METHODS

Sera

Serum samples used in this study ($n = 209$, 70 ASMA positive and 139 ASMA negative) consisted in-samples of different clinical or pathophysiological origins including autoimmune hepatitis ($n = 35$), sclerosing cholangitis ($n = 8$), primary biliary cirrhosis ($n = 5$) and undefined, nonalcoholic cirrhosis ($n = 11$), chronic viral hepatitis (HCV, $n = 33$ and HBV, $n = 16$) as well as 21 rheumatoid factor (RF) positive sera ($8 > 50$ IU/ml), 10 sera with hypergammaglobulinemia, 40 sera with positive autoantibodies (20 ANA and 20 ANCA without history of autoimmune hepatitis), 30 negative controls from healthy blood donors. All sera were stored at -80°C and thawed just before the analysis was performed.

Immunofluorescence Assay and Anti-Actin ELISA

IIFA was performed on commercial mouse stomach/kidney sections (KallestadTM Mouse Stomach/Kidney Slide 30441, Bio-Rad Laboratories, Redmond, WA), Hep-2 cells (ANA (Hep-2 actin) Slide 508095, Inova Diagnostics, Inc., San Diego, CA) and neutrophils (ANCA Slide 508290, Inova Diagnostics, Inc., San Diego, CA). Sera with a titer $< 1:80$ were considered negative; with a titer equal to $1:80$ equivocal and with a titer $> 1:80$ positive for ANA on Hep-2 cells as well as for ASMA on MSK slide. The cutoff value for ANCA was $1:20$. RF and gammaglobulins were quantified using

nephelometry on BNII (Dade Behring, Marburg GmbH, Germany). F-actin quantification was performed using the QUANTA LiteTM Actin ELISA (Inova Diagnostics, Inc., San Diego, CA). Prediluted "high" and "low" controls and diluted ($1:100$) patient's sera were measured in duplicates and incubated for 30 min at room temperature (RT), allowing serum anti-actin antibodies to bind to immobilized purified F-actin. After washing with Tris-buffered saline and Tween 20 solution, plates were incubated with horseradish peroxidase (HRP) labelled antihuman IgG antibody for 30 min at RT. After addition of TMB chromogenic substrate, optical density (OD) at 450 nm was measured using the absorbance module of the DSXTM automated ELISA System (ThermoLabsystems, Chantilly, VA). Results were expressed as the ratio of mean sample OD to mean low positive control as indicated by the manufacturer. Sera were classified as negative when < 20 Units, weakly positive when between 20–30 Units and moderately to strongly positive when > 30 Units.

RESULTS

An overall distribution of ASMA and anti-actin positivity or negativity among the 209 sera considered in this study is presented in Table 1. Seventy samples (33.5%) were ASMA positive by IIFA and 43 (20.6%) anti-actin positive by ELISA. Among the 70 ASMA positive samples, 34 (48.5%) were anti-actin positive by ELISA, whereas 9 (20.9%) out of the 43 anti-actin positive samples by ELISA were ASMA negative. Furthermore, four out of these nine anti-actin positive samples were considered weakly positive (20–30 units)

TABLE 1. Distribution of positive and negative ASMA (by IIFA) and anti-actin antibodies (by ELISA) in all sera tested

	ASMA positive			ASMA negative		
	<i>n</i>	Anti-actin positive (%)	Anti-actin negative (%)	<i>n</i>	Anti-actin positive (%)	Anti-actin negative (%)
Autoimmune hepatitis ($n = 35$)	26	21 (80)	5 (20)	9	4 (44)	5 (56)
HCV ($n = 33$)	19	6 (31)	13 (69)	14	1 (7)	13 (93)
HBV ($n = 16$)	6	0 (0)	6 (100)	10	0 (0)	10 (100)
Cirrhosis ($n = 16$) ^a	6	1 (17)	5 (83)	10	0 (0)	10 (100)
Cholangitis ($n = 8$)	1	1 (100)	0 (0)	7	0 (0)	7 (100)
Positive RF ($n = 21$) ^b	4	2 (50)	2 (50)	17	0 (0)	17 (100)
Hypergammaglobulinemia ($n = 10$) ^c	2	0 (0)	2 (100)	8	1 (12)	7 (88)
ANA pos ($n = 20$)	2	0 (0)	2 (100)	18	1 (6)	17 (94)
ANCA pos ($n = 20$)	3	3 (100)	0 (0)	17	2 (11)	15 (89)
Negative control ($n = 30$)	1	0 (0)	1 (100)	29	0 (0)	29 (100)
TOTAL	70	34 (49)	36 (51)	139	9 (7)	130 (93)

Undefined origin.

^a > 50 IU/ml.

^b > 20 g/l.

TABLE 2. Low vs moderately to high anti-actin positive but ASMA negative samples ($n = 9$): clinical diagnosis

Anti-actin (U)	Diagnosis
24	Chronic hepatitis C
25	Churg–Strauss syndrome
27	SLE with inflammatory syndrome
28	Autoimmune hepatitis
40	Autoimmune hepatitis
40	Waldenström's hyperglobulinemia with Sjögren's syndrome
47	Sjögren's syndrome with autoimmune hepatitis
66	SLE with autoimmune hepatitis
98	p-ANCA pos. serum with chronic bronchitis

SLE, systemic lupus erythematosus.

by ELISA and only one had a history compatible with AIH (Table 2).

Among the 35 AIH, 26 were ASMA positive and 9 ASMA negative compared with 25 anti-actin positive and 10 anti-actin negative (Table 1). Within the 26 ASMA positive AIH, 21 were also anti-actin positive (80%), indicating an anti-actin false-negative rate of 20%. Spearman's correlation coefficient (r) between the two assays (ASMA in dilution titers vs anti-actin antibodies in U) was -0.63 (95% CI -0.80 to -0.37) (Fig. 1).

With regards to the 33 HCV samples (Table 1), 19 were ASMA positive and 14 ASMA negative compared with 7 anti-actin positive and 26 anti-actin negative samples. All but one anti-actin positive HCV samples were also ASMA positive. This improve specificity of the anti-actin test as compared with the ASMA IIFA was confirmed in the non-AIH hepatopathies group including HBV, cirrhosis and cholangitis in which we found 13 ASMA positive and 27 ASMA negative samples compared with 2 anti-actin positive and 38 anti-actin negative samples. None of the 16 HBV positive sera were anti-actin positive whereas 6 were ASMA positive.

Data derived from RF positive sera, hypergammaglobulinemia sera, ANA or ANCA positive samples as well as healthy blood donor samples further supported the superior specificity of the anti-actin ELISA. Indeed, of the 21 RF positive sera, 4 were ASMA positive and 17 were ASMA negative, compared with 2 anti-actin positive and 19 anti-actin negative samples. Two out of the 10 hypergammaglobulinemic samples were ASMA positive and none anti-actin positive. Among the eight ASMA negative samples, one was anti-actin moderately positive (40 units) and corresponded to Waldenström's hyperglobulinemia with Sjögren's syndrome. Among the 20 ANA positive samples, 2 were

Fig. 1. Correlation between ASMA titers as measured by IIFA and anti-actin antibodies as measured by ELISA. R^2 determination coefficient of the logarithmic tendency curve, Spearman coefficient, $r = -0.63$, $P < 0.0001$.

ASMA positive and anti-actin negative, but among the 18 ASMA negative sera, 1 showed a low positivity for anti-actin (27 units) and corresponded to a systemic lupus erythematosus (SLE) with polyarthralgia and systemic inflammation. Among the 20 ANCA positive samples, 3 were ASMA positive, all confirmed by a positive anti-actin result. One of these patients developed an AIH 3 years later. However, among the 17 ANCA positive/ASMA negative samples, 2 only were anti-actin positive at 25 and 98 units, respectively. One case corresponded to Churg–Strauss syndrome and the other one to chronic bronchitis with a p-ANCA positive serum.

None of the 30 healthy blood donor samples reached the anti-actin positivity cutoff level whereas one had an ASMA equivocal titer of 1:80.

Taken together these data allow the calculation of a sensitivity and a specificity of 71.4 and 89.7% for the anti-actin test (Fisher's test $P < 0.0001$) vs 74.3 and 74.7% for ASMA IIFA (Fisher's test $P < 0.0001$) respectively. Accordingly the PPV and NPV was 58.1 and 93.9% for the anti/actin ELISA and 37.1 and 93.5% for the ASMA IIFA, respectively (Table 3).

DISCUSSION

We have shown in this study that a new anti-actin ELISA was comparable to ASMA IIFA in terms of sensitivity, however was superior in terms of specificity.

TABLE 3. Test characteristics of the anti-actin ELISA and the ASMA IIFA with regards to the diagnosis of autoimmune hepatitis

Test characteristics	Anti-actin	95% CI	ASMA	95% CI	Anti-actin or ASMA	Anti-actin and ASMA
Sensitivity (%)	71.4	53.7–85.3	74.3%	56.8–87.5	85.7%	60%
Specificity (%)	89.7	84.1–94.8	74.7%	67.6–81.0	71.8%	92.5%
PPV (%)	58.1	42.1–73.0	37.1%	25.9–49.5	37.9%	61.8%
NPV (%)	93.9	89.2–97.1	93.5%	88.1–97.0	96.1%	92%

PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval.

TABLE 4. Anti-actin ELISA positivity according to ASMA-IIFA titer

ASMA – IIFA titer	Number	Anti-actin pos. (>20 U)	% Positive
1:1280	4	3	75
1:640	6	4	66
1:320	17	9	53
1:160	10	5	50
1:80	33	13	39
Total	70	34	49

As shown in Table 1, half the total number of ASMA positive sera tested turned out to be anti-actin positivity with a trend for IIFA samples with the highest titers to be anti-actin ELISA positive (Table 4). This is consistent with the multiple antigenic cytoskeleton targets of ASMA, the anti-actin specificity representing only a fraction of them (1,8).

If we consider the 35 sera from AIH out of the 209 sera tested, the sensitivity of anti-actin antibodies was very close to that of ASMA (71.4 vs 74.3%). In these 35 samples, the concordance of positive or negative results for actin and smooth muscle antibodies was high (26/35) and discrepant in only nine cases (Table 1). These data are in agreement with the data previously published (1,9) indicating a proportionally comparable percentage of patients with anti-actin antibodies (74%) or ASMA (86%). This suggests that conventional determinations of ASMA may be sufficient for the routine screening of type I autoimmune hepatitis. However, the same study showed that end-point outcome such as death or liver transplantation occurred more frequently in anti-actin positive than in anti-actin negative patients. The reasons for this association are still unclear but may identify a subset of patients with a more aggressive pathogenic process. On the other hand, a screening limited to antibodies to actin would have failed in detecting five autoimmune hepatitis in our study. When both tests were combined, the sensitivity of the assay was enhanced to 85.7%, supporting the use of positivity in either one or the other assay for screening purposes and

ROC curve analysis **Fig. 2.** ROC curve analysis of anti-actin ELISA results using 60, 40, 20 or 10 Units as cutoff value (red line) compared with ASMA using 1:640, 1:320, 1:160 or 1:80 as positive titer (blue line).

both anti-actin and ASMA positivity for diagnostic purposes, which enhanced the specificity of the assay to 92.5% (Table 3).

The positive predictive value of ASMA in the diagnosis of type I autoimmune hepatitis in our study was low (37.1%) and close to the value already mentioned (8). The ROC curve analysis shows that ASMA determination alone is not the most accurate test for HAI diagnosis (Fig. 2). It is also well known that IIFA is largely operator-dependant and that the experience of the technician could modify the evaluation of pattern and titer. To avoid false-positive results, ASMA positive sera should be confirmed using anti-actin ELISA. Thus, using both ASMA and anti-actin tests gives an advantage in diagnosing autoimmune hepatopathies.

We observed with the anti-actin ELISA that test values between 20 and 40 units, considered as weakly or

moderately positive, could be reached in the absence of clinical history of autoimmune hepatitis (Table 2). If we modify the cutoff value of the anti-actin ELISA from 20 to 40 units, we will rather lose in sensitivity than gain in specificity (Fig. 2). Prospective studies will be useful to better understand the predictive value of a weakly positive anti-actin result in such cases. In our series, weakly positive anti-actin titers were found in three of four sera from patients with some kind of polyclonal immune activation (autoimmune manifestations, chronic HCV hepatitis) and may indeed have no clinical relevance as markers of autoimmune hepatitis. Evolution of ASMA and ANA titers has been studied over a period of 128 months without any significant correlation with therapy or disease prognosis (10). Currently, we still do not know how useful would be the follow-up of patients under therapy using the semiquantitative anti-actin ELISA.

Although the presence of anti-actin antibodies in patients with chronic hepatitis C infection was previously described (11–14), the prevalence of either ASMA or anti-actin positivity was particularly high in our study. Out of the 33 HCV samples, 19 (57%) were ASMA positive, 7 (21%) were anti-actin positive and 6 (18%) of them both ASMA and anti-actin positive. In contrast, in another study, if the prevalence of ASMA positive sera among 290 HCV infected samples was 20%, only 2% of them were anti-actin specific (15). The relatively high prevalence of ASMA and anti-actin positivity in our HCV samples can be explained by the bias introduced in our study in selecting a panel of ASMA positive patient first. This can also in part be explained by the well-known propensity of chronic hepatitis C to secrete high level of autoantibodies. In patients with hepatitis, the presence of multiple autoantibody specificities, partially explained by molecular mimicry with HCV epitopes, may suggest falsely the development of AIH (16). Nonetheless, the clinical message behind this observation is still a high prevalence of false-positive test results for autoimmune hepatitis among sera from HCV infected patients. This is not without significance with regards to the therapy, interferon- α being contraindicated in autoimmune hepatitis (17). In comparison to ASMA, anti-actin antibody false-positive rate was more than two-folds lower.

Three samples among the ANCA positive samples (20 nonviral hepatitis/nonautoimmune hepatitis samples) were positive for both ASMA and anti-actin antibodies. One case out of the three cases was classified as possibly autoimmune hepatitis, the two others being reported with muscle and arthritic pains and fever, compatible with systemic vasculitis. Interestingly, ANCA have been described in sera of patients with autoimmune hepatitis

(18). More recently, the cytoskeletal protein actin has been identified as an ANCA antigen (19). In the description of actin as target for ANCA, it was mentioned that ANCA were detected in 24 of 32 sera from AIH patients (75%). Targan et al. (18) demonstrated a p-ANCA pattern in 92% of 104 patients with AIH. These data suggest that the ANCA test may be another way to identify anti-actin antibodies in AIH. Retrospectively, we looked for the presence of ANCA in the 35 AIH samples. Because of the low volume of available sera, only 18 samples were analyzed. From these 18 sera, 14 were ANCA positive (77%). Positive ANCA samples were equally distributed among the ASMA positive and negative groups (seven ANCA positive out of nine ASMA positive or negative samples, respectively). Interestingly, when we looked at the ANCA prevalence in the AIH anti-actin positive sera, we observed 55% (10/18) of ANCA positive samples. We can thus confirm in our AIH panel of sera the association of anti-actin antibodies with the ANCA pattern by IIFA. None of the samples were positive for antimyeloperoxidase or antiproteinase-3 antibodies (data not shown). This strongly suggests that antineutrophil cytoplasmic antibodies were in most cases targeting neutrophil actin, although an ANCA specific epitope, different from actin, cannot be fully excluded. The question of a correlation between disease activity or prognosis and ANCA fluorescence positivity is open.

Used as a screening test, the anti-actin ELISA would have missed ten autoimmune hepatitis from which five would have been ASMA positive (Tables 1 and 5). In these cases, ASMA titers were never $>1:320$ and considering the clinical history of the samples, only a single serum (Table 5, second line) might be considered as a false-negative result for anti-actin. The other four samples were probable ASMA targeting other epitope(s) than actin such as vimentin or tubulin (7). If we consider the four anti-actin negative samples as true negative, this would boost the anti-actin assay sensitivity, specificity, PPV and NPV up to 80.6, 89.9, 58.1 and 96.4%, respectively. On the other hand, the use of ASMA alone would have missed nine autoimmune hepatitis from which four would have been anti-actin positive (Tables 1 and 5). Taken together, the use of ASMA or/and anti-actin ELISA as screening tests appears rational, joined to clinical and pathology parameters for diagnostic purposes. Positive predictive values (PPV) were in favor of the anti-actin ELISA, as well as in favor of an approach combining ASMA with anti-actin ELISA (Table 3). Accordingly, the use of both ASMA and anti-actin detection has recently been supported to improve diagnostic yield in the assessment of patients with raised transaminases (20).

TABLE 5. Clinical history or biochemical characteristics from autoimmune hepatitis samples with discrepancies between ASMA and anti-actin results

ASMA titer	Anti-actin (U)	History
<i>ASMA positive/anti-actin negative sera (n = 5)</i>		
1:320	4	Anti-vimentin antibody positive
1:320	12	Autoimmune hepatitis I and II
1:160	18	Drug induced autoimmune hepatitis
1:80	9	Nonconfirmed AIH
1:80	9	Nonconfirmed AIH
ANA titer and IIFA pattern	Anti-actin (U)	History
<i>ASMA negative/anti-actin positive sera (n = 4)</i>		
1:1,280, homogeneous	40	Autoimmune hepatitis with cirrhosis
Not done	28	Autoimmune hepatitis I and II
1:5,120, homogeneous	66	Autoimmune hepatitis with SLE
1:1,289, homogeneous	47	Sjögren, CBP, autoimmune hepatitis

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