

Serum anti-modified citrullinated vimentin antibody concentration is associated with liver fibrosis in patients with chronic hepatitis

Suad Abdeen
Samuel O Olusi
Sunila George

Department of Pathology,
Faculty of Medicine,
Kuwait University, Safat, Kuwait

Background and aims: The hepatic stellate cell, which plays a pivotal role in hepatic fibrosis, contains the filament vimentin which is known to undergo protein citrullination and become immunogenic. The aims of this study were to find out if anti-modified citrullinated vimentin (anti-MCV) antibodies are produced in patients with chronic hepatitis and if such production is associated with liver fibrosis.

Methods: Sera and liver biopsy specimens were collected from 100 patients with chronic hepatitis. Sera were also collected from 100 healthy controls. The liver biopsies were graded according to the Metavir fibrosis scores. The serum concentrations of anti-MCV antibody were measured in both patients and controls by ELISA using commercially available kits.

Results: The mean serum concentration of anti-MCV antibody in patients with chronic hepatitis (54.90 ± 6.09 U/mL) was significantly higher ($P = 0.001$) than that of controls (17.38 ± 0.56 U/mL). Furthermore, serum anti-MCV antibody titer was able to separate patients with no fibrosis from those with moderate or severe fibrosis or cirrhosis. Using receiver operating characteristic curves, a serum concentration of anti-MCV antibody of 8.82 U/mL was able to diagnose cirrhosis with 60% specificity and 60% sensitivity.

Conclusion: We concluded that serum anti-MCV antibody concentration may be a sensitive noninvasive marker for liver cirrhosis that needs to be investigated further.

Keywords: anti-MCV antibody, serum marker, liver fibrosis

Introduction

Protein citrullination or deimination is a recently described post-translational modification of arginine residues within a peptide sequence into citrulline residues. Deimination is catalyzed by a family of calcium-dependent enzymes, the peptidylarginine deiminases (PADs, EC 3.5.3.15). To date, 5 isoenzymes have been identified. Their genomic organization, subcellular localization, and tissue-specific expression have been determined (for a review, see ref¹). All PAD1 genes are localized in 1 cluster at 1p 36.13 within a 300 kb region.^{1,2} The product of citrullination is the nonstandard amino acid citrulline, which contributes to the backbone of certain proteins such as histones.³ By decreasing the net positive charge, citrullination can alter the primary, secondary, and tertiary structures of proteins with a potential influence on intermolecular interactions. Proteins known to be citrullinated in vivo include keratin,⁴ filaggrin,⁵ trichohyalin,⁶ vimentin,⁷ myelin basic proteins,⁸ histones,^{9,10} fibrinogen,^{11,12} fibrins,¹³ and collagen type I.¹⁴ Physiologically, protein citrullination plays an essential role in cell differentiation,⁴ nerve growth,^{8,15} embryonic development,¹⁶ cell death,^{17,18} and gene regulation.^{9,10}

Correspondence: SO Olusi
Department of Pathology, Faculty
of Medicine, Kuwait University,
PO Box 24923, Safat 13110, Kuwait
Tel +965 5319476
Fax +965 5338905
Email olusoji@hsc.edu.kw

Protein citrullination is involved in the pathogenesis of certain human diseases, the best example being rheumatoid arthritis (RA). The most specific family of RA antibodies is the antibodies directed against citrullinated proteins (reviewed in¹⁹). These antibodies can be detected in almost 80% of RA with a specificity of 99%.²⁰ Besides being very specific for RA, anti-citrullinated protein antibodies can be detected very early in the disease and appear to predict clinical outcome,^{21–23} making them a very useful diagnostic tool for rheumatologists. The anti-citrullinated protein antibodies are produced locally in the inflamed synovium.^{24–26} Since hepatic stellate cells, which play a pivotal role in hepatic fibrosis, contain vimentin, we hypothesized that protein citrullination of vimentin may also occur in chronic hepatitis and may partly explain the fibrosis seen in this disease. The objectives of this study therefore were to find out if production of anti-modified citrullinated vimentin (anti-MCV) antibody is increased in the blood of patients with chronic hepatitis and to find out if there is any relationship between the serum concentrations of these antibodies and the Metavir fibrosis scores.

Materials and methods

Patients and serum samples

One hundred patients with chronic liver disease of varying duration who reported for liver biopsy at the hepatology clinics of Al-Amiri and Mubarak Al-Kabeer University Teaching Hospitals, Kuwait between September 2006 and May 2007 were recruited for this study. They had reported for liver biopsy because of past history of jaundice and persistently elevated serum transaminases. None of these patients fulfilled any of the American Rheumatism Association (ACR) criteria for the diagnosis of rheumatoid arthritis.²⁷ All the patients gave informed consent for use of data and serum for research purposes, and this study was approved by the local research ethical committee.

A fasting blood sample was collected by venipuncture from each patient before liver biopsy. The blood samples were centrifuged and sera separated. The serum samples were kept deep frozen at -80°C until ready for assay.

Controls

One hundred age- and sex-matched healthy controls were recruited from the central blood bank and serum obtained from each person in a similar way. All the volunteers gave informed consent for use of their blood for research purposes.

Histological analysis of liver biopsies

Liver biopsies were formalin-fixed, paraffin-embedded, and stained with H&E stain as well as other special stains used for liver tissue diagnosis such as Masson's trichrome stain for collagen, reticulin stain, PAS stain with and without diastase digestion, Perl's iron stain, and orcein stain. Two histopathologists, completely unaware of patient characteristics, examined the biopsies under the microscope to assess the degree of fibrosis (stage) and the extent of inflammatory activity (grade) according to the Metavir scoring system.²⁸ Fibrosis was staged on a scale of 0 to 4; F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = few septa, F3 = numerous septa without fibrosis, F4 = fibrosis. Activity (based on the intensity of macroinflammatory activity, interface hepatitis and lobulitis) was graded as follows: A0 = no histological activity, A1 = mild activity, A2 = moderate activity, A3 = severe activity. To assess liver biopsy quality, Regev quality criteria²⁹ were used. A biopsy between 10 and 15 mm in length, with fewer than 5 portal tracts or fragmented, was considered as 'fair quality biopsy'. A 'poor quality' biopsy was less than 10 mm in length. All the 6 poor quality biopsies were excluded from the study.

Serum assay of antibodies to modified citrullinated vimentin (anti-MCV)

Concentrations of anti-MCV antibody were measured in the sera of patients and controls using a commercially available ELISA kit (ORGENTIC Diagnostica, GmbH, Mainz, Germany) according to the manufacturer's instructions. In brief, serum samples were diluted 1:100 and incubated on MCV-coated microtiter wells for 30 minutes at room temperature on a horizontal shaking platform (100/second). Plates were washed 3 times and incubated with peroxidase-labeled anti-human IgG conjugate for 15 minutes. 3',3',5,5'-tetramethylbenzidine substrate was added and incubated for 15 minutes after additional washing. Color development was stopped with 1M HCl solution and the optical density of each well was read at 450 nm on an ELISA reader. Results were expressed in U/mL using a sample point-point curve fitting method. The intra-assay and inter-assay coefficients of variation were 6% and 8.5%, respectively, in our laboratory.

Biochemical data

Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and albumin concentrations were measured in patients and control sera on a Beckmann Coulter LX-20 Chemistry Analyser using dedicated reagents.

Statistical analysis of data

Data entry and statistical analysis were performed by using SPSS version 15.0. We evaluated the associations between serum anti-MCV antibody concentrations and Metavir fibrosis and activity scores. We used error bar plots with 95% confidence intervals to display these associations. ROC (receiver operating characteristics) curves were used to identify the optimal serum anti-MCV antibody concentration that would give the maximum sensitivity and specificity for the diagnosis of liver fibrosis.

Results

Patient characteristics

The demographic, biochemical, and histological data, which have been described in previous publications,^{30,31} are summarized in Table 1.

Serum concentration of anti-MCV antibody

Serum concentrations of anti-MCV antibody in hepatitis C virus (HCV) patients (58.80 ± 6.7 U/mL) were significantly higher than those of healthy controls (17.38 ± 0.57 U/mL), patients with hepatitis B virus (HBV) (43.86 ± 7.75 U/mL), or patients with nonalcoholic steatohepatitis (NASH) (23.10 ± 2.88 U/mL) (Table 2). Concentrations in HBV patients were also significantly higher than those in NASH patients and healthy controls. Values in NASH patients did not differ significantly from those of healthy controls.

Mean serum anti-MCV antibody concentration in patients with chronic hepatitis (54.90 ± 6.09 U/mL) was significantly ($P < 0.01$) higher than that of healthy controls (17.38 ± 0.563 U/mL) (Figure 1). None of the healthy controls had a serum concentration greater than 20 U/mL (the upper limit of normal recommended by the manufacturers of the ELISA kit) while 86 of 94 patients with chronic hepatitis had serum anti-MCV antibody concentrations greater than 20 U/mL.

Association among serum concentration of anti-MCV antibody and serum concentrations of liver enzymes and serum albumin

In patients with chronic hepatitis, serum concentration of anti-MCV antibody was positively associated with serum ALT ($r = 0.423$, $P = 0.02$) and serum AST ($r = 0.44$, $P = 0.01$), and negatively associated with the serum albumin concentration ($r = -0.44$, $P = 0.01$).

Relationship between serum anti-MCV antibody concentrations and Metavir fibrosis scores

Mean anti-MCV antibody concentration at stage F0 (no fibrosis) was 25.26 ± 5.25 U/mL (Figure 2), not significantly ($P = 0.116$) different from the value at stage F1 (30.50 ± 5.82 U/mL) but significantly different from those at F2 (39.11 ± 5.31 U/mL), F3 (40.56 ± 3.40 U/mL), or F4 (78.62 ± 11.16 U/mL). Thus anti-MCV antibody concentration could differentiate patients with no liver fibrosis from those with moderate fibrosis, severe fibrosis, or cirrhosis (F2–F4). The association among serum anti-MCV antibody concentrations and Metavir fibrosis scores are shown in Table 3. Using ROC curves (Figure 3) a cut-off point of 8.82 U/mL of serum anti-MCV antibody concentration was 60% specific and 60% sensitive for predicting liver cirrhosis.

Discussion

Our study showed that significant protein citrullination of vimentin occurs in patients with chronic hepatitis and that the serum concentration of anti-MCV antibody could differentiate patients with no liver fibrosis from those with moderate to severe fibrosis. If this study can be confirmed in a larger sample size, it would seem that serum concentrations of anti-MCV antibody can be used as a sensitive noninvasive marker for staging liver fibrosis

Table 1 Demographic and biochemical characteristics of the patients

Fibrosis stage	n	Male/female	Mean age (years)	Serum ALT (U/L) mean \pm SEM	Serum AST (U/L) mean \pm SEM	HCV (n)	HBV (n)	NASH (n)
F0	14	11/3	41.89 \pm 4.16	69.2 \pm 5.77	57.75 \pm 12.52	4	2	10
F1	9	8/1	46.8 \pm 1.65	79.85 \pm 15.3	70.43 \pm 16.21	4	4	7
F2	13	9/4	44 \pm 1.31	84.25 \pm 25.23	75.71 \pm 13.07	8	4	5
F3	34	24/10	45.47 \pm 2.05	180.6 \pm 25.8	136.67 \pm 84.06	16	6	4
F4	24	17/7	42.8 \pm 1.54	191.63 \pm 78.39	145.18 \pm 59.23	14	4	2
Total	94	69/25	44.1 \pm 1.1	114.62 \pm 22.69	93.88 \pm 16.46	46	20	28

Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; HCV, hepatitis C virus; HBV, hepatitis B virus; NASH, nonalcoholic steatohepatitis; n, number; SEM, standard error of mean.

Table 2 Mean concentrations of anti-modified citrullinated vimentin (anti-MCV) antibody in patients with HCV, HBV, NASH, and healthy controls

Group of patients	Anti-MCV concentrations (U/mL) (mean ± SEM)	n
HCV	58.80 ± 6.70	46
HBV	43.86 ± 7.75	20
NASH	23.10 ± 2.88	28
Controls	17.38 ± 0.57	100

Notes: HCV vs controls: $P = 0.000$; HBV vs controls: $P = 0.01$; NASH vs controls: $P = 0.91$; HCV vs HBV: $P = 0.151$; HCV vs NASH: $P = 0.000$; HBV vs NASH: $P = 0.01$.

Abbreviations: HCV, hepatitis C virus; HBV, hepatitis B virus; NASH, nonalcoholic steatohepatitis; n, number; SEM, standard error of mean.

in patients with chronic hepatitis. Hitherto, a variety of indirect and direct noninvasive serum markers of liver fibrosis have been evaluated. The indirect markers include derived variables such as the AST/ALT ratio,³²⁻³⁵ platelet count,³⁶ and prothrombin index.³⁷ Other indirect tests include the PGA index which combines measurement of the prothrombin index, gamma glutamyl transferase, and apolipoprotein A1.³⁸⁻⁴⁰

More recently, Halfon and colleagues reported on a score derived from 6 markers found to be useful in discriminating between early and more advanced liver fibrosis.⁴¹ Other indirect tests include the FibroTest,⁴² ActiTest,⁴³ Forns test,⁴⁴ and APRI test.⁴⁵ The direct markers of liver fibrosis include collagen propeptides (PIIINP),⁴⁶ hyaluronic acid,⁴⁷ metalloproteinases and inhibitors of metalloproteinases,⁴⁸ and a host of others. Unfortunately most indirect and direct serum

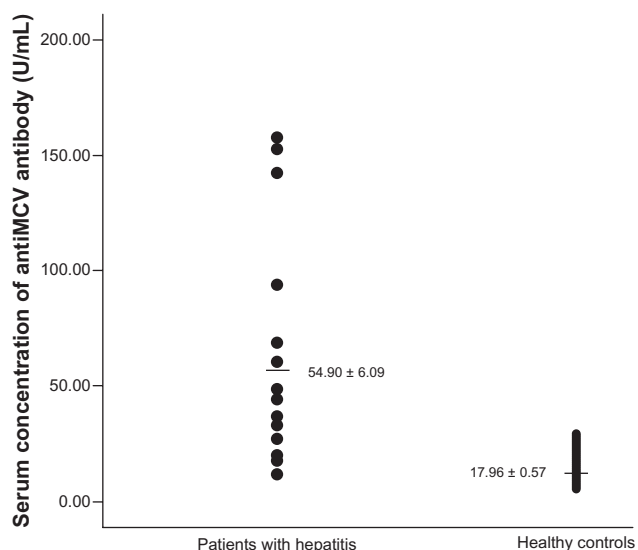


Figure 1 Scatterplot of the serum concentration of anti-modified citrullinated vimentin (anti-MCV) antibody in patients with chronic hepatitis and in healthy controls.

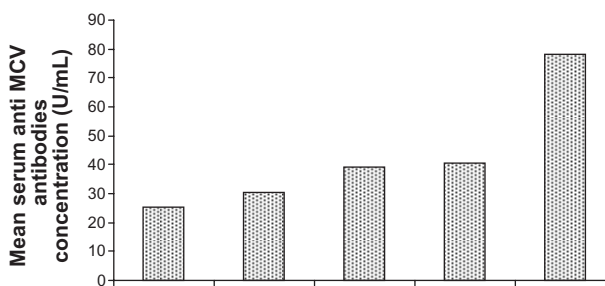


Figure 2 Distribution of serum concentration of anti-modified citrullinated vimentin (anti-MCV) antibody according to Metavir fibrosis score in patients with chronic hepatitis.

markers are complex and lack sensitivity. Moreover most studies on their diagnostic usefulness have been limited by their retrospective design and poor reporting on liver biopsy methods. Therefore, there has always been a need to develop new noninvasive markers for liver fibrosis in patients with chronic hepatitis.

This study will be the first to report the presence of anti-MCV antibody in patients with chronic hepatitis and has demonstrated that it can be used to diagnose liver cirrhosis with 60% sensitivity and specificity. It could not differentiate between F0 and F2 or F3 degrees of liver fibrosis. Because of our relatively small sample size, there is a need to confirm this observation in a larger sample size. We are still collecting samples to validate this result and to compare the diagnostic sensitivity and specificity of serum anti-MCV concentrations with other noninvasive markers of liver fibrosis.

The formation of anti-MCV antibody in patients with chronic hepatitis is not difficult to explain. Hepatic stellate cells contain vimentin.^{49,50} Oxidative stress due to liver injury can modify this vimentin so that it becomes immunogenic stimulating the production of anti-MCV antibody. Our finding that the serum concentration of anti-MCV antibody is associated with the degree of liver fibrosis in patients with chronic hepatitis supports the theory that hepatic stellate cells play a central role in liver fibrosis.

Table 3 Association among serum concentrations of anti-modified citrullinated vimentin (anti-MCV) antibody and degrees of hepatic fibrosis in patients with chronic liver disease

Metavir fibrosis core	Serum concentration of anti-MCV (U/mL)	Standard error	P value vs F ₀
F0	25.26	5.25	–
F1	30.50	5.82	0.12
F2	39.11	5.31	0.07
F3	40.56	3.40	0.02
F4	78.62	11.16	0.001

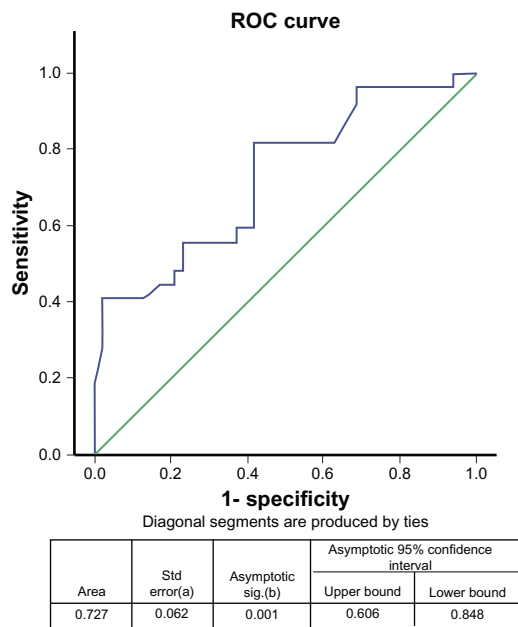


Figure 3 Area under the curve for serum concentration of anti-modified citrullinated vimentin (anti-MCV) antibody and stage of hepatic fibrosis.

Note: The test result variable(s): anti-MCV (U/mL) has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased

^aUnder the nonparametric assumption

^bNullhypothesis true area = 0.5

Abbreviation: ROC, receiver operating characteristics.

Furthermore, our finding that protein citrullination occurs in patients with chronic hepatitis supports previous reports that protein citrullination is not specific for RA since it has been found to occur in psoriasis,^{51,52} multiple sclerosis,^{15,53,54} and various tumors.⁵⁵ We are now investigating the diagnostic properties of serum anti-MCV antibody concentration for staging liver fibrosis in a large cohort of patients with chronic liver disease, especially viral hepatitis.

Disclosure

The authors declare no conflicts of interest.

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