Serum Autoantibodies in Chronic Hepatitis C: Comparison with Hepatitis C/Autoimmune Hepatitis Overlap Syndrome in Egypt

Khaled Metwally[1], Samia A. Abdo[2], Soheir Badr[3], Maryam A. Abdurrhman[4] and Nazek K. Saafan[5],Abear Mohamady Abdel- Bary[5] and Manal H. Abbas

[1]Rheumatology Unit, [2]Gastroenterology unit, [3]Oncology diagnostic unit, [4]Rheumatology department and [5]Clinical Pathology Department, Ain Shams University, Cairo-Egypt.
Soheir_badr@yahoo.com

Abstract: Background: Hepatitis C virus—autoimmune hepatitis (HCV/AIH) overlap syndrome had been described in the literature since the early 1990s with numerous case reports and proposed guidelines of management. However, definitive diagnosis for the syndrome remains controversial and there are still no formalized treatment strategies. The aim of this study was to test how hepatitis C virus (HCV)-associated autoantibodies differs from those of AIH in terms of titer and sub specificities and to find the best combination of antibodies in discrimination between the two conditions. **Methods:** Liver biopsy and blood samples were taken from clinically and serologically confirmed patients with chronic HCV infection (n=57) and patients suspected to have HCV/AIH overlap syndrome (n=21).HCV infection was determined by detection of anti-HCV antibodies using a third-generation enzyme immunoassay and active virus replication defined by quantitative measurement of HCV RNA. Antinuclear antibodies (ANA) and its pattern, anti-smooth muscle antibodies (ASMA), Anti-Actin antibody (AA), Antimitochondrial antibody (AMA), anti liver-Kidney microsome-1(anti-LKM1) and perinuclear staining of antineutrophil cytoplasmic antibodies (p-ANCA) were detected by immunofluorescence assay. Anti-soluble liver antigen (SLA) was measured by ELIZA. Serum protein electrophoresis was done for gamma globulin measurement**. Results:** Statically significant difference regarding serum autoantibodies positivity and subspecificities were evident between chronic hepatitis C and HCV/AIH overlap syndrome patients. Elevated γ-globulins was the best test for selecting HCV/AIH overlap syndrome patients out of chronic HCV patients, followed by anti-actin, atypical p-ANCA, the homogeneous ANA pattern, the worse was ASMA. Positivity for AMA, anti-LKM-1 and SLA antibodies were not observed in all patients sera. The best combination was homogeneous ANA, anti-actin SMA and p –ANCA with sensitivity 85.71%, specificity 73.68%, and accuracy 76.92%. **Conclusions:** Serum autoantibodies positivity and sub-specificities should be used in differentiation between chronic hepatitis C and HCV/AIH overlap syndrome. The best combination was homogeneous ANA, anti-actin SMA and p–ANCA.

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1. Introduction

Worldwide, 130–170 million persons are living with chronic hepatitis C virus infection [1] which, if left untreated, can result in cirrhosis and liver cancer. Egypt has the largest burden of HCV infection in the world, and incidence rates have been estimated at 2.4/1000 person-years (165,000 new infections annually) [2]. Hepatitis C is the main cause of liver-related morbidity and mortality and represents a worldwide public health problem [3]. Chronically infected HCV frequently leads to autoimmune response including the production of autoantibodies and the coincidence of autoimmune diseases [4]. The diversity of autoantibodies in particularly non-organ-specific autoantibodies has been widely established in sera of patients with HCV-related chronic liver disease. Anti smooth muscle (SMA) and anti- nuclear (ANA) antibodies have been detected in approximately one third of the cases [5,6], while antibodies to liver/- kidney microsomes type 1 (anti-LKM1) have been found rarely (from 0% to 5%) [6, 7]. In addition to these "conventional" autoantibodies, patients with AIH have a wide range of other autoantibodies. Those most frequently found include perinuclear staining antineutrophil cytoplasmic antibodies (p-ANCA), and soluble liver antigen (SLA) [8].

Hepatitis C virus—autoimmune hepatitis (HCV/AIH) overlap syndrome has been described since the early 1990s [9]. Following the identification of HCV/AIH overlap syndrome, reports began to appear that a high proportion of patients who otherwise fulfilled criteria for AIH had evidence of HCV infection, suggesting that this virus might be an aetiological factor in the development of AIH[10].

The diagnosis of AIH is based on the revised descriptive criteria for diagnosis of AIH reported by the International Autoimmune Hepatitis Group (IAHG) in 1999[11]. Whether the application of the AIH scoring system is useful in cases of AIH/overlap syndromes and furthermore, in cases of AIH with concurrent other liver disease has not been extensively validated so far[12]. However, some of the features that may support AIH diagnosis, such as elevated serum IgG, detection of autoantibodies and histologically evident interface hepatitis, can occur with variable frequency in a wide range of other liver disorders [13]. The problem arising in diagnosis makes the decision for clinical management difficult as corticosteroids, which constitute the treatment of choice in AIH are usually contraindicated in HCV as they can induce exacerbation of viral hepatitis, and the presence of AIH appears to predispose to severe adverse reactions during antiviral treatment with interferon-alpha. Such patients need to be treated with caution for the underlying diseases [12].

The aim of this study was to test how hepatitis C virus (HCV)-associated autoantibodies differs from those of AIH in terms of titer and sub specificities and to find the best combination of antibodies in discrimination between the two conditions.

2. Materials and Methods

This was a cross-sectional case control study that included 78 patients attending an HCV out-patient clinic at Ain Shams University Hospital. Studied patients included 57 with chronic HCV infection and mean age ± SD of 31.6±0.8 years and 21 suspected to have HCV/AIH overlap syndrome with a mean age ± SD of 32.2±0.9 years. Informed consent was obtained from all subjects with approval granted by Ain shams Research and Ethics Committee "Ain-shams faculty of medicine federal number IRB00006444, prior to sample collection. The diagnosis of chronic hepatitis C (HCV) was based on clinical and laboratory evaluation. Serologic evidence of chronic HCV infection as determined by detection of antibodies to HCV (anti-HCV) using a third-generation enzyme immunoassay(Abbott Diagnostics) and active virus replication as defined by the detection of HCV RNA using a quantitative HCV RNA serum detection using (Amplicor; Roche Diagnostic Systems). None of the patients had ever received treatment with pegylated interferon and/or ribavirin.

Complete data for calculation of the revised IAHG score were reviewed retrospectively [11].A pretreatment score of 10 points or higher, indicate ''probable'' AIH. A pretreatment score of 15 points, indicate ''definite" AIH. Exclusion criteria included patients with hepatitis B infection, excluded on the basis of immunoassay negativity for serological markers (HBsAg, HBeAg, anti HBs, anti HBc, anti HBe, and PCR DNA for HBV). Intake of alcohol or potentially hepatotoxic drugs was ruled out in all patients.

All patients were submitted to the following: full history taking, thorough clinical examinations with record of any sign of cirrhosis or autoimmunity, full blood chemistry including complete liver function tests, liver biopsy and abdominal ultrasonograghy to detect ascites and/or liver cirrhosis.

Immunoflourescence measurements:

The standard indirect immunofluorescence (IF) technique was used for the detection of AMA, ASMA, anti-actin and anti-LKM-1 using a composite substrate comprising liver , kidney and stomach from rodents (DiaSorin Inc , USA) with titer ≥ 40 considered positive results[14]. ANA test was performed by IF technique on HEp-2 cells (DiaSorin Inc, USA) with a starting dilution of 1/40 [15]. Atypical p-ANCA was determined by the method of Terjung *et al.,*[16] using fixed neutrophils (Immco. , USA) at a dilution of 1/20.

Anti- soluble liver antigen (SLA) measurement:

Anti- SLA was detected by enzyme linked immunosorbent assay (ELISA) kit (Inova diagnostics Inc, USA) We had investigated reactivity of SLA positive sera against α-enolase and tRNP(Ser) sec using rat and primate liver homogenate and the recombinant antigens).

Biochemical measurements:

Alkaline phosphatase, ALT, AST and total protein were measured by commercially available kits (Randox-UK).

Gamma globulin measurement:

Serum protein electrophoresis was performed according to the method of Alper [17]. Level of protein fractions can be estimated by measuring the total serum protein and then multiplying that by the relative percentage of each protein fraction.

Statistical analysis;

Data was analyzed using SPSS statistical package version 15. Numerical data were expressed as mean, standard deviation. Qualitative data were expressed as frequency and percentage. Correlation between different antibodies frequencies were evaluated by Pearson's $\chi 2$ test. Comparison of continuous variables was made by means of the Student's t test.

3. Results:

Comparing patients with probable AIH along HCV (HCV/AIH) overlap syndrome with the patients with only chronic viral infection, It was found that patients with AIH / HCV had significant higher levels of AST and ALT, and lower levels of ALP and low HCV load than the patients with only viral pattern (p<0.001). On the other hand, higher serum gamma globulin was observed among patients with HCV/AIH patients in

comparison to those with HCV infection (p <0.001). Non-significant difference was found regarding age and gender (Table 1).

Liver biopsy of chronic HCV patients showed, mild portal inflammation with lymphoid aggregates, mild periportal piecemeal necrosis, steatosis, bile duct damage, and apoptosis. Portal fibrosis was detected in all of our patients in both groups. Patients with HCV/AIH overlap syndrome had mixed histological findings of chronic HCV with one of the following: Interface hepatitis (5 patients 23.8%), lymphoplasmocytic infiltrate in the portal tracts (10 patients 47.6%), or hepatic rosette formation (6 patients 28.6%). (Table 2).

Compared with HCV/AIH overlap syndrome, HCV-associated ANA and SMA exhibited ANA-homogenous pattern and SMA-anti actin antibodies at a lower prevalence (0% with median titer 80(40-160) Vs. 47.61% with median titer 320 (160-640) and P<0.001) and (0% with median titer 40(40-80) Vs. 57.14%, with median titer 320(320-640) and P <0.001) respectively. While p- ANCA positivity was detected in 24.56% of HCV patients and 52.38% of HCV/AIH overlap syndrome (P =0.004). Positivity for AMA, anti-LKM-1 and SLA Abs were not observed in all patients sera (Table-3).

Using autoantibodies in different combinations revealed that the best results were obtained when combining Homogeneous ANA, anti-actin SMA and p –ANCA with sensitivity 85.71%, specificity 73.68%, and Accuracy 76.92% (Table 4).

Table 1: Comparison between patients with chronic HCV and patients with HCV/AIH overlap syndrome

Parameter	Chronic HCV (n=57)	HCV/AIH overlap syndrome (n=21)	p-value
Age	31.6±0.8	32.2±0.9	0.58
Sex (M/F)	39/18	15/6	0.983
AST (U/L)	158.29± 10.22	173.61± 6.85	<0.001
ALT (U/L)	142.29± 4.65	164.1± 6.69	<0.001
ALP (U/L)	89.8± 0.50	73.2±0.44	<0.001
PCR (IU/mL)	846587.75±3878.25	451786.46± 2877.71	<0.001
γ globulin ≥1.5 (g/dl)	0/57	21/21	<0.001

M/F: Male/female, AST: Aspartate Aminotransferase, ALT: Alanine Aminotransferase, ALP: Alkaline phosphatase, ANA:
Antinuclear antibodies

Table 2: Liver biopsy in patients with chronic HCV and patients with HCV/AIH overlap syndrome

Parameter	Chronic HCV (n=57)	HCV/AIH overlap syndrome (n=21)
Fibrosis	57(100%)	21(100%)
Portal-periportal necroinflammation	55(96.4%)	20(95.23%)
Lobular necroinflammation	50(87.71%)	18(85.71%)
Interface hepatitis	0(0%)	5(23.8%)
Intense plasma cell infiltrate	0(0%)	10(47.61%)
Rosettes	0(0%)	6(28.57%)

Table (3): Serum ANA, ASMA and p ANCA in Chronic HCV: A Comparison with HCV/AIH overlap syndrome

Parameter	Chronic HCV (n=57)	HCV/AIH overlap syndrome (n=21)	p-value
	ANA-Positive chronic HCV	ANA-Positive HCV/AIH overlap	
	(44/57 patients)	(20/21 patients)	
ANA titer*	80 (40-160)	320(160-640)	<0.001
Homogeneous ANA	0 (0%)	10(47.61%)	<0.001
	ASMA-Positive chronic HCV	ASMA-Positive HCV/AIH overlap	
	(34/57 patients)	(16/21 patients)	
ASMA titer*	40 (40-80)	320(160-640)	<0.001
Anti-actin ASMA	0 (0%)	12(57.14%)	<0.001
p-ANCA	14(24.56%)	11(52.38%)	<0.001

*Reciprocal of the dilution; median (range).
ANA: Antinuclear antibodies, ASMA: Anti-smooth muscle antibodies and p-ANCA: perinuclear anti-neutrophil cytoplasmic antibody

Table (4): Value of combined use of autoantibodies in discrimination between Chronic HCV and HCV/AIH overlap syndrome

Parameter	Sensitivity	specificity	PPV	NPV	Accuracy
ANA	95.24%	22.81%	37.04%	92.86%	42.31%
Homogenous pattern	47.62%	100%	100%	83.82%	85.89%
ASMA	76.19%	40.35%	32%	82.14%	50%
AA-ASMA	57.71%	100%	100%	86.36%	88.46%
p-ANCA	52.38%	75.44%	44%	81.13%	69.23%
ANA + ASMA	90.48%	38.80%	35.19%	91.67%	52.56%
Homogenous pattern +ASMA	90.48%	38.60%	35.19%	91.67%	52.56%
Homogenous pattern +AA-ASMA	76.19%	100%	100%	91.94%	93.59%
Homogenous pattern +p-ANCA	76.19%	73.68%	51.61%	89.36%	74.36%
AA-ASMA+p-ANCA	71.43%	75.44%	51.72%	87.76%	74.36%
Homogenous pattern +AA-ASMA +p-ANCA	85.71%	73.68%	54.55%	93.33%	76.92%

ANA: Antinuclear antibodies, ASMA: Anti-smooth muscle antibodies, AA: Anti-actin and p-ANCA: perinuclear anti-neutrophil cytoplasmic antibody.PPV: Positive predictive value. NPV: Negative predictive value.

4. Discussion:

The diagnosis of true HCV-AIH overlap syndrome is often challenging, as the concurrent presence of serologic markers typically found in AIH and serologic evidence of HCV infection is well documented [5,18]. Autoantibodies such as antinuclear antibody, anti-smooth muscle antibody, or anti—liver and kidney microsomal 1 antibody have been reported in 9-38%, 5-91%, and 0-10%, respectively, of patients with chronic HCV infection [19]. Therefore, in order to definitively diagnose HCV-AIH overlap syndrome as a distinct entity, the existence of both diseases must be confirmed independently.

In the current study, on comparing autoantibody profile of HCV/AIH overlap syndrome patients with those in chronic HCV infection patients. Associated ANA, ASMA, and AA-ASMA exhibited a higher prevalence (95.24% vs. 59.65%, 76.19% vs. 59.65%, and 57.71% vs. 0%) respectively while, the concomitant positivity of ANA-Homogenous pattern and AA-ASMA was evident in 76.19% of HCV/AIH overlap syndrome patients and never in the sera of chronic HCV patients. The prevalence of autoantibodies detected in the present study was substantially in accordance with previous reports [20,21] which used the same immunofluorescence antibody screening dilution. Also, Rigopoulou and Dalekos [22] reported that the homogeneous ANA pattern is one of the Features of AIH in patients with chronic HCV.

The American Association for the Study of Liver Diseases has recommended the use of atypical p-ANCA for diagnosis of AIH since, occasionally, it may be the only autoantibodies present [23]. Indeed, it must be noted that p-ANCA in patients with AIH (atypical pANCA) differ from the classical p-ANCA by retention of the perinuclear staining (produced primarily on ethanol fixed cells) on formaldehyde fixed cells. Zauli *et al.*[24] found atypical p-ANCA in 65% of patients with AIH and in 13 % of patients with chronic C with autoimmune features in accordance to our results which revealed positivity rates of 52.38% vs. 24.56% respectively. They found that all HCV patients with positive atypical p-ANCA showed ASMA with actin specificity, 10/12 (83%) of our anti-actin positive patients were also positive for atypical p-ANCA. Using autoantibodies in different combinations revealed that the best results were obtained when combining Homogeneous ANA, anti-actin SMA and p –ANCA with sensitivity 85.71%, specificity 73.68%, and Accuracy 76.92%.

Hypergammaglobulinemia was present in 100% of HCV/AIH overlap syndrome patients and in non of chronic HCV patients (p<0.001). Possible explanation of the above data was that since AIH is characterized by the presence of many kinds of autoantibodies the majority of them are of class IgG which result in elevation of γ -globulin concentration in patients sera while, HCV infection characterized by presence of one or 2 types of autoantibodies not enough to elevate IgG as high as in AIH. Hypergammaglobulinemia is well accepted to be a distinct feature of AIH, thus our result is in agreement with many investigators who collectively cited that serum level of γ -globulin rises in patients with AIH in comparison to HCV infection and healthy controls [25,26].

The mechanism by which, chronic HCV triggers autoantibodies (e.g. ANA and ASMA), is unclear. The virus may facilitate the expression of immunological abnormalities by stimulating the production of endogenous interferon [26]. Additionally, it may down regulate cell mediated immunity, exaggerating the humoral immunity with increased production of soluble CD23, which inhibits B cells apoptosis. CD23 has been found to be

elevated in; chronic HCV patients, systemic lupus erythematosus, Sjogren's syndrome, and rheumatoid arthritis patients [27, 28]. It is possible that this immune dysregulation seen in patients with chronic hepatitis C (CHC) may be compounded by the use of interferon α in treatment of hepatitis C patients [29].

With the availability of highly sensitive assay techniques, some chemical tests become standard laboratory procedures in clinical practice for diagnostic and prognostic purposes. Therefore, in the present study biochemical parameters including serum AST, ALT, and alkaline phosphates were selected, there was significant association of aminotransferase with AIH, since this study showed that the highest concentration of aminotransferase AST and ALT were observed among patients with HCV/AIH overlap syndrome in comparison to those with HCV infection, this is probably due to the fact that AIH are aggressive form of the disease since the level of serum aminotransferase reflect severity of disease.

Histologic diagnosis of the overlap syndrome is another important consideration. Although no single histologic feature is pathognomonic of either HCV or AIH, distinct composite histologic patterns have been described for each entity. In general, patients with AIH are more likely to have severe lobular necrosis and inflammation, piecemeal necrosis, multinucleated hepatocytes, and broad areas of parenchymal collapse, whereas patients with HCV are more likely to have bile duct damage, bile duct loss, steatosis, and lymphoid cell follicles within portal tracts [30]. The combination of portal lymphoid aggregates and steatosis was found to have 91% specificity for HCV, whereas the pattern of lymphoplasmacytic portal, interface, and acinar hepatitis had 81% specificity for AIH [31]. In accordance with these findings, our results revealed that moderate to severe plasma cell infiltration of the portal tracts, Interface hepatitis and Rosettes were more common in patients with HCV/AIH overlap syndrome while, Portal-periportal necroinflammation and Lobular necroinflammation were more common in patients with chronic HCV infection.

To date, there are no standard guidelines on how to approach patients with HCV/AIH overlap syndrome [23].One management strategy is to determine the predominant entity in order to select the appropriate type of therapy[19,32].Chronic HCV-AIH overlap syndrome can be divided into autoimmune- or viral-predominant disease. Patients with autoimmune-predominant disease have ASMA or ANA titers of equal to or more than 1:320 or have ASMA and ANA titers of equal to or more than 1:40, as well as histology findings that include piecemeal necrosis (interface hepatitis), lobular hepatitis, and portal plasma cell infiltrates. Patients with viral-predominant disease have ASMA or ANA titers of less than 1:320 or have antibodies to LKM type-1 and hepatitis C viremia, as well as histologic findings that include portal lymphoid aggregates, steatosis or bile duct injury. Tissue damage is more focal in HCV and more diffuse in AIH liver histology [33,34].

5. Conclusions:

In chronic hepatitis C, serum autoantibodies are common, but their sub specificities are distinct from those occurring in AIH. Elevated γ -globulins is the best single test for selecting HCV/AIH overlap syndrome patients out of chronic HCV patients, followed by anti-actin, atypical pANCA, ANA, and the homogeneous ANA pattern, the worse was ASMA. Using antibodies in different combinations revealed that the best results were obtained when combining 3 antibodies: Homogeneous ANA, anti-actin SMA and p–ANCA. The absence of elevated γ-globulin levels, the low autoantibodies titre, absence of both anti-actin and the homogeneous ANA pattern should favour diagnosis of chronic viral hepatitis. It is recommended that: if the diagnosis HCV/AIH overlap syndrome is suspected these auto-antibodies should be looked at before the type of treatment is determined. Corticosteroids and/or immunosuppressive treatment can be the frontline therapy for patients with the overlap syndrome with predominant immunological features of AIH. IFN can be used as frontline therapy for patients with the overlap syndrome when the immunological features are more consistent with chronic HCV.

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