Autoantibodies to a miRNA-binding protein Argonaute2 (Su antigen) in patients with hepatitis C virus infection

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Abstract

Objectives
Chronic liver diseases caused by hepatitis B (HBV) or C virus (HCV) are common worldwide. Despite reports on autoimmunity in viral hepatitis, studies on autoantibodies associated with systemic rheumatic diseases are inconsistent. Testing of a small number of selected autoantibody specificities using ELISA appears to be one reason for inconsistency. Sera from patients with viral hepatitis were tested by immunoprecipitation that will allow unbiased screening of autoantibodies found in systemic rheumatic diseases.

Methods
Ninety Mexican patients (37 male, 53 female, 26 HBV, 6 HBV+HCV, 58 HCV) with chronic viral hepatitis, confirmed by nested or RT-nested-PCR, HBsAg and anti-HCV antibodies, were studied. Autoantibodies were tested by immunofluorescence, immunoprecipitation and ELISA. Specificities were verified using reference sera.

Results
Antinuclear antibodies were found in 38% HBV, 17% HBV+HCV, and 28% in HCV. Autontibodies to Argonaute (Ago2, Su antigen), a microRNA binding protein that plays a key role in RNA-induced silencing complex (RISC), was found in 5% (4/64) of HCV or HBV+HCV coinfected patients but not in HBV (0/26). Anti-Ago2/Su was found in 1/2 of I-IFN-treated case vs. 3/62 in cases without I-IFN. HCV did not have other lupus autoantibodies whereas 19% (5/26) of HBV had anti-U1RNP+Ku, Ro+La, RNA polymerase II, or possible U5snRNPs.

Conclusion
Lupus autoantibodies were uncommon in HCV except anti-Ago2/Su. HCV and I-IFN have many ways to affect TLR signaling, miRNA and miRNA binding protein Ago2/Su. To understand the mechanism of specific targeting of Ago2 in HCV may provide a clue to understand the mechanism of specific autoantibody production.

Key words
hepatitis C, hepatitis B, viral hepatitis, autoantibodies, Argonaute2, microRNA

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Abbreviations
Ago2:   Argonaute2;
ANA: antinuclear antibodies;
β2GPI: β2 glycoprotein-I;
ENA: extractable nuclear antigen;
HBsAg: HBV surface antigen;
HBV: hepatitis B virus;
HCV: hepatitis C virus;
I-IFN: type I interferon;
MEIA: microparticle enzyme immuno-
              assay;
miRNA: microRNA;
RF: rheumatoid factor;
RISC: RNA-induced silencing complex;
RT: reverse-transcription;
SjS: Sjögren's syndrome;
SLE: systemic lupus erythematosus;
TLR: toll-like receptor.

Introduction

Liver diseases caused by hepatitis B (HBV) or hepatitis C virus (HCV) are one of the most common chronic infectious diseases affecting 2–5% of the population worldwide (1). A classic example of vasculitis caused by HBV immune complex is well known and other studies reported autoimmune features associated with HBV infection (2, 3). However, the main focus on the link between viral hepatitis and rheumatology has shifted to HCV since its discovery and development of screening tests for HCV infection in the late 80s. HCV infection is often accompanied by extrahepatic symptoms and characterised by the high prevalence of mixed cryoglobulinemia and associated immune complex-mediated disease including musculoskeletal and skin symptoms (4-7).

HCV patients are often positive for rheumatoid factor (RF) and other autoantibodies, and in some cases accompanied by autoimmune thyroiditis, Sjögren's syndrome (SjS), and other autoimmune diseases. Use of type I interferon (IFN-α, β, I-IFN) as standard treatment of chronic HCV infection further complicates the issue (8) because the overproduction of I-IFN is thought to play a critical role in the pathogenesis of systemic lupus erythematosus (SLE) in human as well as in animal models (9). Thus, I-IFN can theoretically affects autoimmune status as suggested in reports on induction of autoantibodies, exacerbation of pre-existing autoimmune conditions, and triggering *de novo* autoimmune diseases including SLE-like features (4, 5, 8). Stimulation of toll-like receptors (TLR) by viral products induces overproduction of I-IFN that may induce or accelerate autoimmune conditions. Viral DNA can bind to TLR9 whereas viral RNAs bind to TLR7, 8, or 3 (10). Thus, viral hepatitis could be a very interesting condition to understand the interaction of viral infection, TLR stimulation and exogenous I-IFN in development of autoimmunity.

The prevalence of non-organ specific autoantibodies, antinuclear antibodies (ANA), in HCV is similar to controls (~5%) in some studies, whereas it was as high as 54% in other reports (11-13). Autoantibodies found in SLE and other systemic autoimmune rheumatic diseases in HCV have been examined mainly by ELISA, and the results are inconsistent (14-19). In the present study, autoantibodies associated with systemic rheumatic diseases in sera from patients with HCV and HBV infection were examined by immunoprecipitation.

Materials and methods
Patients

A total of 90 Mexican patients (37 male, 53 female) from Hospital Civil "Fray Antonio Alcalde" (Guadalajara, Jalisco, México) with age ranging from 12 to 79 years were studied. Among them, 47 had at least 1 blood transfusion and four had a history of drug abuse. These patients were referred to the service from a blood bank or other sections in the hospital to confirm the HBV and/or HCV infection. Thus, the majority of cases (5/6 in HBV+HCV coinfection, 57/58 in HCV) were without I-IFN treatment. A standardised, structured questionnaire was used to collect data from the patient regarding demography, relevant antecedents, risk factors and treatment. This study was approved by the Ethics Board of the hospital, meets and is in compliance with all ethical standards in medicine, and written informed consent was obtained from all patients according to the Declaration of Helsinki.

Serologic and molecular diagnosis of HBV and HCV infection

Chronic HBV or HCV infection was confirmed by the serology (HBV surface antigen (HBsAg) and anti-HCV antibodies) and molecular diagnosis (viral DNA (HBV) or RNA (HCV) by nested-PCR and reverse-transcription (RT)-nested-PCR, respectively). HBsAg and anti-HCV antibodies were tested by microparticle enzyme immunoassay (MEIA), performed according to the manufacturer's instruction and analysed in the IMX equipment (ABBOTT Laboratories, North Chicago, IL). DNA extraction was carried out from 100μl of serum by the phenol-chloroform method (20). HBV-DNA

was detected by standardised first-round and nested PCR of S-gene fragment using the primers and conditions described previously (21). Total RNA was extracted from each serum, using QIAamp Viral RNA Mini Kit (QIAGEN) as indicated by the manufacturer. HCV-RNA detection was performed by qualitative nested RT-PCR with two pairs of primers that hybridise in a segment of the 5' non-coding region of the HCV genome as described (22).

Immunoprecipitation
Autoantibodies in sera were screened by immunoprecipitation using ^{35}S-methionine labelled K562 cell extract as described (23). Specificity of autoantibodies was determined using previously described reference sera (24).

Immunofluorescent antinuclear antibodies
Immunofluorescent antinuclear/cytoplasmic antibodies (HEp-2 ANA slides; INOVA Diagnostics, San Diego, CA) were tested using a 1:100-diluted human serum and Alexa Fluor 488 goat anti-human IgG (1:400 dilution; Molecular Probes, Eugene, OR) (25).

ELISA
Sera were tested for IgG anti-dsDNA and β2 glycoprotein-I (β2GPI, a gift from Dr J. Kaburaki, Tokyo Electric Power Company Hospital, Tokyo, Japan) antibodies by ELISA (25).

Statistical analysis
Prevalence of autoantibodies was compared by Fisher's exact test using Prism 5.0 for Macintosh (GraphPad Software, Inc., San Diego, CA, USA). $p<0.05$ was considered significant.

Results
Autoantibody analysis in patients with HBV, HBV and HCV co-infection, and HCV is summarised (Table I). Prevalence of antinuclear antibodies was similar between HBV and HCV or between male and female.
Anti-Argonaute2 (Ago2)/Su was found only in patients with HCV (3/58) or HCV+HBV co-infection (1/6) but not in HBV (0/26) (Table I, Fig.1A). In patients treated with I-IFN, anti-Ago2/Su

Table I. Autoantibodies in patients with hepatitis virus infection.

	Hepatitis B		Hepatitis B + C		Hepatitis C	
	IFN Tx No	IFN Tx Yes	IFN Tx No	IFN Tx Yes	IFN Tx No	IFN Tx Yes
N.	25[1]	1	5	1	57	1
M: F	13: 12	1: 0	3: 2	0: 1	19: 38	1: 0
ANA (IF) M: F	32% (8/25)[2] 31% (4/13): 33% (4/12)	(1/1)	20% (1/5)	0	26% (15/57) 21% (4/19): 29% (11/38)	0
Anti-Ago2/Su M: F	0%	0	0%	(1/1) 0:1	5% (3/57) 0:3	0
Anti-dsDNA M: F	4% (1/25) 0:1	0	0%	0	4% (2/57) 1:1	0
Anti-β2GPI M: F	4% (1/25) 1:0	0	0%	0	7% (4/57) 2:2	0

[1]include 10 cases with incomplete HCV tests, 2: include a case of a patient with incomplete HCV tests.
ANA: antinuclear antibodies; IF: immunofluorescence; anti-Ago2/Su by immunoprecipitation; anti-dsDNA and β2GPI by ELISA.

was found in 1/2 case *vs*. 3/62 in cases without I-IFN treatment in HCV positive patients. The number of patients with I-IFN treatment is too small to draw any conclusion on the effects of I-IFN on autoantibodies. Thus, we cannot rule out the possibility that I-IFN enhances the production of anti-Ago2/Su, however, the presence of anti-Ago2/Su in 3 cases without I-IFN treatment indicates that this specificity is not necessarily induced by I-IFN therapy. Low levels of anti-dsDNA or β2GPI antibodies were found in some patients in both HBV and HCV without I-IFN treatment (Table I).
Interestingly, while anti-Ago2/Su were found only in HCV or HCV+HBV co-infection, other specificities associated with systemic rheumatic diseases were found only in 5/26 (19%) of HBV single infected group; anti-U1RNP, Ro, La, Ku, RNA polymerase II and possible U5snRNPs (Fig.1BCD, E panel e). GW body (GWB) staining was verified in a case of HCV with anti-Ago2/Su (Fig.1E panel a), however, other 3 cases had granular cytoplasmic staining and GWB staining was not clear (Fig. 1E panel b). Cajal body staining (Fig. 1E panel c) was found in one case and verified by double staining using rabbit anti-serum. Autoantibodies to p80 coilin in Cajal body have been described in SjS, SLE, atopic dermatitis, and other diseases at low prevalence but without

known clinical significance (26, 27). A centromere pattern (Fig. 1E panel d) was found in one case in HCV.

Discussion
Association of HCV infection with mixed cryoglobulinemia, RF and other autoantibodies, and various musculoskeletal and other extrahepatic symptoms are well described (4, 5). Coexistence of autoimmune diseases such as Hashimoto's thyroiditis and SjS also has been reported. Since the role of I-IFN in the pathogenesis of SLE has been emphasised (9), several reports suggested an induction or exacerbation of autoimmune diseases such as autoimmune thyroiditis and SjS by I-IFN therapy used in HCV infection (8). High prevalence of ANA in HCV has been reported, however, not many studies systematically characterised specific autoantibodies seen in autoimmune rheumatic diseases (15-17, 19, 28) (Table II). Some studies reported high (11–23%) prevalence of certain specificities (16-18). D'Amico *et al*. reported a high prevalence (46%) of anti-ENA (extractable nuclear antigens) including anti-Ro (23%), -La (20%), and -U1RNP (12%) (17). Omagari *et al*. also reported a high prevalence (36%) of anti-ENA including anti-La (23%) and anti-Scl70 (11%) by ELISA (18). Tzang *et al*. focused on anti-PCNA and reported a high prevalence of this
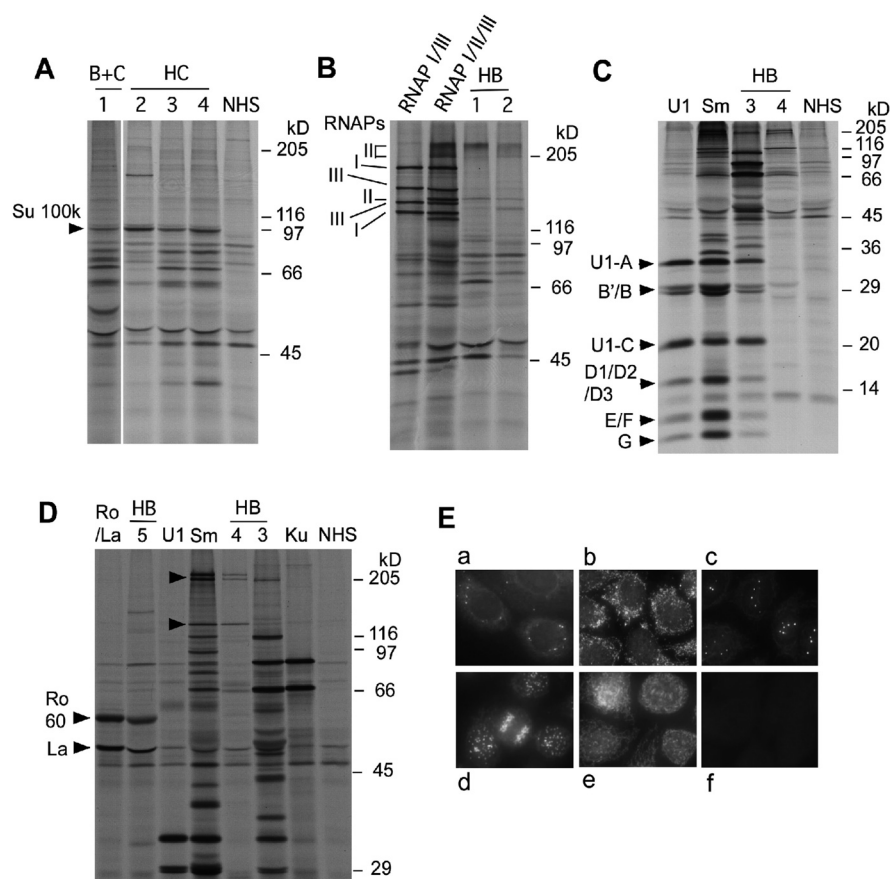
Fig. 1. Autoantibodies in sera from patients with chronic hepatitis B or hepatitis C infection.
A. Anti-Ago2/Su antibodies. A case of HCV+HBV (B+C) and 3 cases of HCV (HC) patients who had anti-Ago2/Su. NHS, normal human serum.
B. Anti-RNA polymerase II. Two cases of HBV infection (HB) that had anti-RNA polymerase II antibodies.
C. Anti-snRNPs antibodies. A case of HBV (lane 3) had anti-U1RNP and other case (lane 4) appeared to immunoprecipitate components of U5snRNPs (see panel D); however, Sm core particle was not clearly seen.
D. Anti-Ro and -La, U5snRNPs, and Ku. A HBV case had anti-Ro plus anti-La (lane HB 5). Immunoprecipitation of YRNAs was also verified by urea-PAGE and silver staining (not shown). A HBV serum (lane HB 4) immunoprecipitated proteins that comigrate with U5-200kD doublet and ~140kD protein immunoprecipitated by anti-Sm sera and considered anti-U5RNP (arrow heads); however, immunoprecipitation of U5RNA was not clear by silver staining. A case of HBV (lane HB 3) had anti-Ku in addition to anti-U1RNP.
E. Immunofluorescence: a) GWB staining by anti-Ago2/Su positive HCV serum, b) Granular cytoplasmic staining by anti-Ago2/Su positive serum, c) Cajal body by a serum from a HCV patient, Specificity was confirmed by double staining using a rabbit anti-p80-coilin serum (not shown), d) Anticentromere antibodies in a HCV patient, e) Immunofluorescence of anti-U1RNP and Ku positive HBV patient (lane 3 in panel C and D), f) normal human serum.

specificity in HCV patients; 18.7% by ELISA and 10.8% by Western blot (16). However, high cut-off (OD=0.842) with poor signal/noise ratios in ELISA and poor reactivity in Western blot in this study raise questions regarding the specificity of the assay. In striking contrast, anti-ENA antibodies were very low or absent in other studies (14, 15, 19). Cacoub *et al.* reported 3% prevalence of anti-ENA by immunodiffusion, but the specificities were not described (5). In the present study, anti-Ago2/Su was

the only specificity identified in HCV patients, and all other anti-ENA antibodies were absent. Thus, the results of specific autoantibodies associated with rheumatic diseases in HCV patients are quite inconsistent. There are several possible explanations for this. First, all these studies were from different countries; the difference in genetic and environmental factors in the study cohort may affect the prevalence of specific autoantibodies. Selection bias of the subjects also could be a factor;

e.g. HCV patients seen at rheumatology clinics and hepatology clinics may be different. The percentage of patients treated with I-IFN is different as the majority of patients in the present study were untreated. Nevertheless, little evidence of induction of anti-ENA by I-IFN is shown despite the role of I-IFN in pathogenesis of SLE. One study described cases of reduction of anti-ENA antibodies following I-IFN treatment (18). The more important factor for the inconsistent results appears to be reliability and selection of the assays since most studies used false positive-prone ELISA (29). A case of HCV with anti-replication protein A was reported (25), but this does not appear to be a common specificity either.

Anti-Ago2/Su antibodies were previously reported in 10–20% of patients with various systemic rheumatic diseases (30, 31). The 100kD protein recognised by anti-Su antibodies was recently identified as a miRNA binding protein Ago2, a key component of RNA-induced silencing complex (RISC) (32, 33). Although the prevalence is not high, specific presence of anti-Ago2/Su antibodies in patients with HCV suggests their specific association with HCV. This also indicates that anti-Ago2/Su can be produced in conditions other than systemic rheumatic diseases. Production of anti-Ago2/Su in a mouse model of pristane-induced lupus is I-IFN and TLR7 dependent (34, 35). Only one of anti-Ago2/Su positive cases had previously treated with I-IFN; three cases were without I-IFN, suggesting that anti-Ago2/Su is not necessarily induced by I-IFN therapy.

There are several links that can be made between HCV, I-IFN, and Ago2 to potentially explain why Ago2/Su is specifically targeted by autoantibodies in HCV infection (Fig. 2). First, Ago2 is a key component of RISC (32) and miRNAs that bind to Ago2 play a critical role in HCV replication (36, 37). miR-122, abundantly found in the hepatocytes, facilitates the HCV replication (38) whereas I-IFN treatment appears to reduce the expression of miR-122 and inhibits the HCV replication (38, 39). Another study showed that reduced miR-122 expression prior to I-IFN

Table II. Specific autoantibodies associated with rheumatic diseases in HCV infection.

Author	n. country	Method	U1/Sm %	Ro/La %	R-P %	PCNA %	Scl %	Jo-1 %	Ago2 (Su) %
Buskila D 1998 (14)	81 Israel	ELISA	0/0	1/0	NA	NA	NA	NA	NA
Verbaan H 1999 (15)	21 Sweden	ID	0/0	0/0	NA	0	0	0	NA
Tzang BS 1999 (16)	379 Taiwan	ELISA WB	NA	NA	NA	18.7	NA	NA	NA
D'Amico E 2002 (17)	69 Italy	ELISA WB	12/1.4	23.1/20.2	1.4	NA	NA	1.4	NA
Omagari K 2003 (18)	44 Japan	ELISA	4.5/NA	2.5/23	NA	NA	11	NA	NA
Kessen-Brock K 2007 (19)	68 Germany Canada Brazil	ELISA	1.4/NA	NA	2.9	NA	NA	NA	NA
Vazquez-Del Mercado M 2010 (the current study)	64 Mexico	IP	0/0	0/0	0	0	0	0	6

U1: U1RNP; R-P: ribosomal P; PCNA: proliferating cell nuclear antigen; Scl: Scl-70; ID: immunodiffusion; ELISA: enzyme-linked immunosorbent assay; WB: western blot; IP: immunoprecipitation.

treatment correlate with poor response to I-IFN therapy (40). I-IFN pathway-independent inhibition of HCV replication by miR-199a has also been shown recently (37). Accumulation of HCV replicon RNA to the RISC was observed when miR-199a was overexpressed *in vitro*. Both HCV and I-IFN therapy can affect miRNA expression (41) and modify RISC in which Ago2 plays a critical role. Ago2 is known to be phosphorylated and the phosphorylation status affects its localisation to GW bodies (also known as mammalian processing bodies or P bodies) in the cytoplasm (42). Both HCV and I-IFN may affect the expression, phosphorylation, localisation of Ago2 or interaction of Ago2 with miRNA and alter its antigenicity.

Stimulation of I-IFN production via binding of viral RNAs to TLR3, 7, and 8 is a key component of innate immunity against viruses. HCV RNAs are known to bind to TLR7 and induces I-IFN signature (43). Similar to the effects of exogenous viral RNAs, RNA components of the endogenous RNA-protein complex targeted by autoantibodies in systemic rheumatic diseases such as U1RNA (UsnRNPs, U1RNP, Sm) and Y RNAs (Ro-La complex) were shown to stimulate TLR7 and induce IFN signatures in human and mouse cells (10, 44). Certain siRNAs and other short single-stranded RNAs have been shown to stimulate TLR7 in a sequence dependent manner (45, 46). Thus, it is possible that certain miRNA, especially when aberrantly expressed, can stimulate I-IFN production via TLR7 in a similar manner. TLR7-dependent production of autoantibodies to UsnRNPs in mouse models of SLE was shown. Similarly, anti-Ago2/Su antibody production was recently shown to be TLR7 dependent in a mouse model of pristane-induced lupus (35). Thus, HCV RNA and some endogenous RNA can stimulate TLR7, induce I-IFN and help breaking tolerance and trigger autoimmune response. HCV E2 envelope protein is known to interact with Ago2 and suppresses RNAi pathway (47). This may be reminiscent to a mouse model of induction of p53 autoimmunity by self p53/viral SV40 large T (SVT) protein complex; immunization of p53 alone did not induce anti-p53 autoimmune response, however, immunization of self p53/viral SVT complex induced anti-p53 autoimmune response in addition to antibodies to SVT (48). Thus, interaction of viral proteins and endogenous protein can be an important step to trigger autoimmune response either
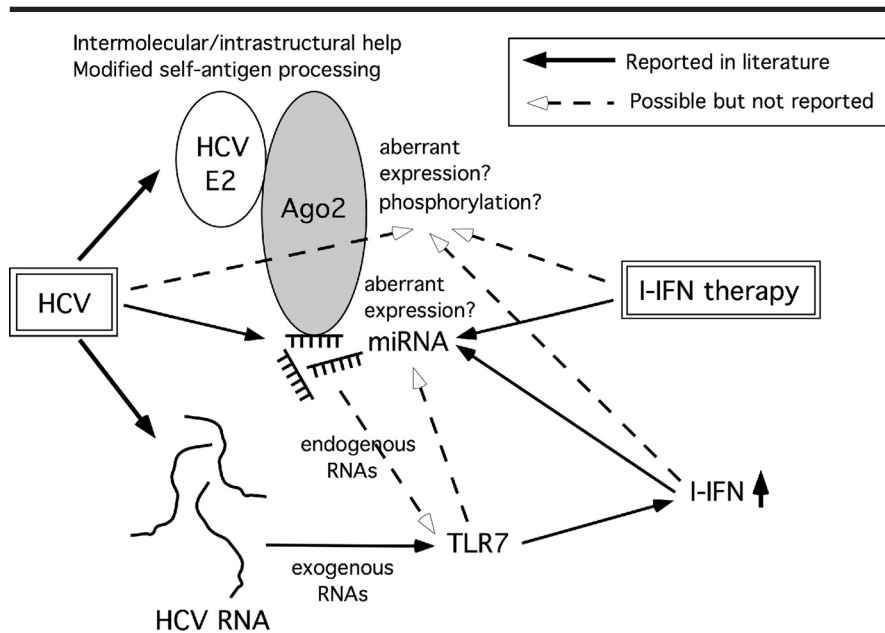

Fig. 2. Interaction of HCV, type I-IFN therapy, miRNA, and Ago2 as a potential mechanism of production of anti-Ago2/Su in HCV patients.
Solid line with a black arrowhead, reported in literature; dashed line with a white arrowhead, possible but not reported. See Discussion for description.

via intermolecular intrastructural help, exposure of cryptic epitopes by altering self-antigen processing, and other mechanisms. It is tempting to speculate that a combination of HCV RNA or miRNA stimulation of TLR7 and HCV E2-Ago2 interaction is involved in selection of the miRNA-binding protein Ago2 as a target of specific autoimmune response.

The role of TLRs and I-IFN in lupus and other autoimmune diseases has been recently under extensive investigations. HCV infection itself and I-IFN therapy have many ways of affecting TLR signalling pathways, miRNA expression, and RISC. To understand the mechanism of specific targeting of Ago2 in HCV autoimmune response may provide a clue to understand the mechanism of selection of a target in specific autoantibody production in rheumatic diseases.

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