Research paper

Detection of residual HCV-RNA in patients who have achieved sustained virological response is associated with persistent histological abnormality

Yijin Wang [a,1], Huiying Rao [b,1], Xiumei Chi [c,1], Boan Li [d,1], Hongyang Liu [a], Liyuan Wu [a], Hao Zhang [d], Shuhong Liu [a], Gaungde Zhou [a], Na Li [e], Junqi Niu [c,***], Lai Wei [f,**], Jingmin Zhao [a,*]

[a] Department of Pathology and Hepatology, the 5th Medical Center, Chinese PLA General Hospital, China
[b] Peking University Hepatology Institute, Peking University People's Hospital, Peking University Health Science Center, Beijing, China
[c] Department of Hepatology, the First Hospital of Jilin University, Changchun, China
[d] Center for Clinical Laboratory, the 5th Medical Center, Chinese PLA General Hospital, China
[e] Advanced Cell Diagnostics, 3960 Point Eden Way, Hayward, CA 94545, USA
[f] Beijing Tsinghua Changgung Hospital, Tsinghua University, China

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ABSTRACT

Background: Whether achieving sustained virological response (SVR) in patients with hepatitis C attains complete elimination of hepatitis C virus (HCV) is unknown, because occult HCV infection (OCI), defined as the detection of HCV-RNA in hepatocytes or peripheral blood mononuclear cells (PBMC) in absence of serum HCV-RNA, may occur. We thus investigated the prevalence and clinical relevance of OCI.

Methods: Subjects from three hospitals who had achieved serum HCV clearance, including 60 of Direct-acting antiviral agents (DAAs) induced SVR, 50 of pegylated interferon plus ribavirin (PR) induced SVR, and 30 of spontaneous resolution, were subjected to detect HCV-RNA in liver by robust RNAscope assay and PBMC by qPCR. Paired liver biopsies at baseline and at SVR24 were analyzed.

Results: OCI was detected in 16 of 140 subjects (11.4%), with 15.0% in DAA-based group, 10.0% in PR group and 6.7% in spontaneously resolved group. In DAA-based subgroups, the incidence of OCI was gradually increased in group of solely DAA(s) therapy, combining DAA and PR therapy and combining DAA and ribavirin therapy. OCI is more frequent in patients with genotype 3. No correlation between baseline viral load, interleukin-28B genotype, baseline transaminases, post-SVR transaminases and OCI were found. However, OCI was significantly linked with severity of fibrosis and active inflammation at post-SVR, even considering basal fibrosis status. In addition, both the magnitude and the frequency of fibrosis regression were lower in patients with OCI than in those without OCI. In the multivariate analysis, PR therapy was identified an independent negative prognostic factor for both hepatic inflammation ($P = .022$) and fibrosis regression ($P = .015$). Importantly, we found HCV relapse in one of the OCI patients at 48 weeks after the end of PR treatment.

Conclusions: HCV-RNA can persist in hepatocytes and/or PBMC in a certain of patients who achieved spontaneous or treatment-induced HCV RNA clearance from serum and associated with persistent histological abnormality. Our findings provide new insights into cure of HCV and could influence the following-up scenario after SVR.

Research in context

Evidence before this study

The rates of SVR have achieved extremely high curative ratio in DAA treated HCV patients, leading to the extensive use of these regimens worldwide. However, whether achieving SVR with DAA treatment means completely viral eradication is uncertain because occult HCV infection (OCI) in other compartments may occur despite HCV-RNA clearance from serum. We searched PubMed using the terms "occult HCV infection", and "direct-acting antiviral agents" to identify results of clinical studies published before June 1, 2019. We identified 10 relevant studies and additional relevant articles from reference lists were also identified. However, all of these studies were limited to patients of immunosuppressive who achieved spontaneous or treatment induced serum viral clearance or patients with long-standing abnormal tests of live function with unknown origin. We found no studies of the prevalence of OCI in immunocompetent patients of clinical HCV resolution, especially in context of DAA regimens. Additionally, there is no description of the concomitant clinical impact of OCI in previous studies. Furthermore, it is worth noting that most studies tested OCI based on utilization of PBMC, other that tissue samples, resulting in under-estimated incidence of OCI.

Added value of this study

To the best of our knowledge, this is the first study to assess the presence of residual HCV genome in both PBMC and liver tissue in the immunocompetent host following SVR24 with DAA treatment and compared the incidence of OCI in patients with DAA and PR regiments, as well as subjects of spontaneous HCV resolution in a multicenter cohort. Overall, 16 of 140 subjects (11.42%) were found to have OCI, with the highest (15.0%) rate in DAA treated subjects. The onset of OCI is more frequent in patients with genotype 3 HCV but less in patients with genotype 1. Importantly, OCI was significantly linked with severity of fibrosis and active inflammation at SVR24, even considering basal fibrosis status. In addition, both the magnitude and the frequency of fibrosis regression were lower in patients with OCI than in those without OCI. Finally, we found HCV relapse in one of the OCI patients at 48 weeks after the end of PR treatment.

Implications of all the available evidence

Our findings provide new insight into cure of HCV and could influence the following-up scenario after SVR. A complete elimination of virus after clinical diagnosis of SVR after DAA regimens seems unlikely despite of substantially improved anti-HCV efficacy. The chronic liver injury concomitant residual HCV-RNA could remain an issue after SVR. Longitudinal outcomes of OCI are required to be followed up by monitoring of virus reactivation, liver function tests and fibrosis evolution and diseases progression. Certainly, additional work on how to deal with such patients is in urgent.

1. Introduction

The approval of direct-acting antiviral agents (DAAs) has opened a new scenario for treating chronic hepatitis C virus (HCV) infection. Sustained virological response (SVR)24, defined as undetectable HCV-RNA in serum by standard clinical assay at 24 weeks after withdrawal of antiviral agents, is viewed the true cure and primary goal for hepatitis C. However, whether achieving SVR means completely viral elimination

is uncertain because occult HCV infection (OCI) in other compartments may occur despite HCV-RNA clearance from serum [1–5].

OCI is characterized by the presence of HCV-RNA in liver tissues or peripheral blood mononuclear cells (PBMC) in the patients whose serum is constantly negative for HCV-RNA, with or without presence of HCV antibodies [6]. It is evident by the persistence of HCV-RNA at low level in hepatic tissue or PBMC for many years after achievement of SVR [1,4,7–9]. Investigations of OCI have been mainly in patients who received organ transplantation and achieved spontaneous or treatment induced serum viral clearance, as the use of immunosuppressants may impair host immunity and result in high susceptibility to develop OCI [5,10–12]. Alternatively, OCI was also studied in patients with long-standing abnormal tests of liver function with unknown origin [13]. Nevertheless, the existence of OCI is disputed owing to the controversy on whether it has clinical impact. Although OCI in a subset of individuals was found to coincide with elevated liver function tests and/or various degree of fibrosis, some other studies argued that the persistent aminotransferase or histological abnormalities might be related to long-term inflammation or reactivation of tolerant immunologic microenvironment after antiviral therapy [14,15]. It is well-known that chronic HCV infection is associated with exhaustion of HCV-specific CD8+ T cells. During chronic HCV infection, CD8+ T cells sequentially loss their effector function, while the inhibitory receptorswere persistently high expressed. In this setting, the impaired function of circulating CD8+ T lymphocytes inactivates allospecific T cell clones and promotes development of a tolerogenic liver microenvironment. After HCV clearance, the recovery of CD8+ T-cell function might reverse this tolerogenic environment, facilitating subclinical rejection or other autoimmune phenomena that could explain abnormal liver tests and histologic inflammatory changes [16,17]. Thus the assessment of OCI in individuals without selection of hepatic function other than in individuals with abnormal liver function could be more favorable to provide extensive perspectives.

The incidences of OCI in previous studies differ substantially as the utilization of specimens for detecting HCV-RNA and the accuracy of detection methods varied [18]. Hepatic tissue would be a gold standard for diagnosis of OCI, but liver biopsies are rarely available due to lack of justification for that. Given that HCV are lymphotropic as well and capable of establishing persisting infection in circulating immune system, detection of HCV in PBMCs is an alternative method [19–21]. Nevertheless, the lower levels of HCV genome per cell in lymphocytes than in hepatocytes would result in under-estimated incidence of OCI [22]. OCI is not well substantiated and has typically been attributable to poor technique as well. As the detection of HCV protein by immunohistochemistry was limited, utilization of nested PCR or nucleic acid hybridization would apparently improve the identification of HCV-RNA. Therefore, precise diagnosis of OCI should be better to base on utilization of tissue samples and the assay with sufficient specificity and sensitivity.

Taking together, it remains to be determined the prevalence and clinical relevance of OCI in immunocompetent patients with serum HCV resolution. We thus tested HCV-RNA in liver tissues by in situ hybridization with advanced RNAscope assay, and in PBMC by PCR from HCV patients who achieved SVR24 with DAAs treatment, or pegylated interferon (peg-IFN) plus ribavirin (PR) treatment, as well as population with spontaneous resolved (SR).

2. Materials and methods

2.1. Patients

The participants were from previously established cohort study in three tertiary hospitals: Peking University People's Hospital, the first Hospital of Jilin University and Chinese PLA General Hospital. The study population consisted of three groups: (a) 60 chronic HCV patients who received DAA-based treatment with or without PR/ribavirin for 12 weeks or 24 weeks between March 2014 and December 2016;

(b) 50 chronic HCV patients who received standard dual therapy of sub-cutaneous injection of recombinant IFN-α2b (5 MU, three times/ week) and oral ribavirin (RBV) (800 mg/ day for 75 kg) for 48 weeks between 2011 and 2014; (c) 30 patients who achieved spontaneous HCV resolution between 2011 and 2014. All of the enrolled patients were eligible for the inclusion criteria: (a) Achieved SVR24 or spontaneous HCV resolution; (b) Age ≥ 18; (c) 18.5 ≤ BMI ≤ 23.9, and the exclusion criteria: (a) Co-infection with hepatitis B or human immunodeficiency virus; (b) Presence of other chronic liver diseases (autoimmune hepatitis, primary biliary cirrhosis, haemochromatosis); (c) Hepatic decompensation (ascites, variceal bleeding or hepatic encephalopathy); (d) Hepatocellular carcinoma or other malignant tumor at enrollment; (e) Excessive alcohol consumption (> 40 g/day) or intravenous drug abuse; (f) Any complication of severe heart, lung, kidney, brain, or blood diseases or other severe systematic diseases; (g) Pregnant or lactating women. Procedure of specimens' collection was shown in Fig. S1A.

2.2. Study design

Prior obtained paired liver biopsies at baseline and at SVR24 from 60 DAA treated and 50 PR treated patients were collected. Post-SVR PMBC from 50 PR treated patients and 30 SR individuals, as well as DAA treated subjects who had detectable HCV-RNA in liver biopsies were collected (Fig. S1B). Liver biopsies were used to detect intrahepatic HCV-RNA and for histological evaluation. PBMC specimens were used for detection and quantification of HCV-RNA, as well as viral sequencing in positive subjects (Fig. S1C).

2.3. Test of HCV-RNA in liver tissues by RNAscope assay

In situ hybridization assays on liver biopsies were performed using the RNAscope® 2·5 HD-Brown assay (Advanced Cell Diagnostics, Inc., Newark, CA, USA). The detailed information of each probe was: RNAscope® Probe- V-HCV-GT1 (Cat#423221, target region: 36–2399), RNAscope® Probe- V-HCV-GT2 (Cat#423231, target region: 19–2439), RNAscope® Probe- V-HCV-GT3 (Cat#423241, target region: 178–2454), RNAscope® Probe- V-HCV-GT4 (Cat#423251, target region: 103–2529), RNAscope® Probe- V-HCV-GT5 (Cat#423251, target region: 2–2896), RNAscope® Probe- V-HCV-GT6 (Cat#423271, target region: 2–2623), RNAscope® Positive Control Probe-Hs-PPIB (Cat#313901, target region: 139–989 of NM_000942·4), and RNAscope® Negative Control Probe-DapB (Cat#310043, target region: 414–862 of EF191515). The images were acquired using an Aperio AT2 digital slide scanner equipped with a 40× objective (Leica Biosystems Inc., Buffalo Grove, IL, USA). The performance procedure was provided in supplementary information.

2.4. Validation of RNAscope assay to detect HCV-RNA in liver biopsies

Prior to applying RNAscope in situ hybridization assay for OCI prediction, we validated the sensitivity and specificity of HCV-specific probes in liver specimens from 74 randomly selected patients with or without HCV, as proven by serological test. Among these patients, 14 had genotype 1b HCV, 8 had genotype 1a HCV, 10 had genotype 2a HCV, 8 had genotype 2 ac HCV, 4 had genotype 3b HCV, 4 had genotype 3a HCV, 4 had genotype 6ab HCV, and 22 had non-HCV liver diseases consisting of 7 chronic hepatitis B, 5 autoimmune hepatitis, 5 alcoholic liver disease and 5 nonalcoholic fatty liver disease. Each liver biopsy was subjected to RNAscope tests with 6 specific probes that target HCV genotype 1–6, respectively. As a result, RNA signals were detected in 50 of 52 HCV-positive patients. Among the 50 samples, 47 showed exclusively genotypic signal coinciding with serological result and the remaining 3 showed positive signal for two genotypes probes, one of which coincided with the genotype by serological test. No signal was detected by RNAscope in any liver biopsies from 22 non-HCV patients

(Table S1). Fig. S2 showed representative images of HCV-RNA signals. These results suggested RNAscope a robust method to detect HCV-RNA in situ.

2.5. Testing and genotyping HCV-RNA in PBMC

Total RNA was isolated from 5×10^6 PBMC with RNeasy plus mini kit and HCV-RNA was then quantitated by qRT-PCR following instructions offered by the Diagnostic kit Quantification of Hepatitis C Virus RNA (PCR-Fluorescence Probing) from KHB, Shanghai, China. HCV-RNA positive samples were genotyped using the new VERSANT HCV Genotype 2.0 Assay (Line Probe Assay (LiPA)) based on the simultaneous detection of 5'UTR and core regions.

2.6. Histological evaluation

Paired liver biopsies at baseline and SVR24 were obtained in all patients with antiviral treatment. The hepatic inflammation activity (HAI) (Table S2) and fibrosis stage (S) were scored according to the Ishak classification. Liver biopsies were read and staged by two pathologists blinded from treatment and OCI status. Evaluation of fibrosis according to changes in histology (S score) were classified as follows: a) Improvement: decrease of ≥1stage in the S score at post-SVR biopsy; b) Maintenance: same S score in the post-SVR biopsy compared to pre-treatment biopsy; c) Progression: increase of ≥1 stage in the S score at follow-up.

2.7. Statistics

Mean ± standard deviation (SD) or median (range) were used to describe quantitative variables, while qualitative variables were presented as counts (percentage). Statistical significance for paired design groups was analyzed by paired samples t-test or Wilcoxon signed-rank test. For unpaired samples, student t-test or Mann-Whitney U test were used as appropriate. Differences in categorical variables were analyzed by Chi-square test or Fisher's exact test, whenever appropriate. Logistic regression was used to analyze the risk factors for HAI regression and fibrosis regression. The estimated odds ratio (OR) with their 95% confidence interval (CI) were calculated. Data were analyzed using SAS version 9.2. All statistical tests are based on two-side and the significance level was set at $P < .05$.

2.8. Ethics approval and consent to participate

This study was done in accordance with the ethical principles of the Declaration of Helsinki as revised in 2013 and was approved by the Ethics Committee by each institution's human research committee, Peking University People's Hospital, the first Hospital of Jilin University and Chinese PLA General Hospital. Informed consent was obtained from all participants prior to enrolment. Written informed consent was received from participants prior to inclusion in the study.

3. Results

3.1. Baseline clinical characters of study population

The detailed information of the enrolled 140 subjects was summarized in Table 1. As expect, there is substantial improvement of serum transaminitis and liver histology in term of both scores of inflammatory (HAI) and fibrosis stage (S) at SVR24 in both DAA-based and PR treatment groups (Table 1 and Fig. 1). Before therapy, S 0–2, S 3–4, S 5–6 present in 33%, 59%, and 8% of the patients, while the prevalence of the same stages at SVR24 was 65%, 26%, and 9%, respectively. The median HAI score significantly decreased from 6.0 to 3.5 in DAA-based group ($P < .001$) and from 6.5 to 3.1 in PR group ($P < .001$), and the

Table 1
Clinical characteristics of participants[*].

Parameters	DAA N = 60	P	PR N = 50	P	SR N = 30
Sex (male/female)	33/27	0.001[†]	42/8		12/18
Age (years)	45 (21–67)	0.166[†]	48 (35–66)		53 (34–68)
Pre-treatment serum HCV-RNA (IU/ml)	2.41×10^6 (4.40×10^4–2.71×10^7)	0.151[†]	2.35×10^6 (1.73×10^2–3.19×10^7)		–
Laboratory values					
ALT at baseline	49 (15–228)	< 0.001[‡]	68.8 (19.2–514.7)	< 0.001[‡]	22.4 (8–289)
ALT at SVR24	15.5 (5–81)		25.7 (8–174)		–
AST at baseline	40.5 (16–143)	< 0.001[‡]	51.3 (14.3–331.2)	< 0.001[‡]	25.3 (17–150)
AST at SVR24	20 (11–150)		27.5 (16–183)		–
AST/ALT at baseline	0.8 (0.4–1.9)	< 0.001[‡]	0.7 (0.4–1.7)	< 0.001[‡]	1.2 (0.4–2.4)
AST/ALT at SVR24	1.3 (0.7–4.1)		1.1 (0.5–2.6)		–
TBiL at baseline	11.5 (6–38)	0.95[‡]	23 (2–80)	< 0.001[‡]	10.8 (5.8–70.7)
TBiL at SVR24	11.3 (5–33.4)		16.2 (6.2–67.1)		–
PLT at baseline	171.5 (42–298)	0.08[‡]	154 (72–283)	0.005[‡]	216.5 (64–341)
PLT at SVR24	189 (47–328)		177 (57–356)		–
Histological tests[#]					
HAI score					
Baseline	6.0±2.6	< 0.001	6.5±2.1	< 0.001	
SVR24	3.5±1.8		3.1±1.3		
Fibrosis scoring					
Baseline	2.8±1.7	< 0.001	3.0±0.9	< 0.001	–
SVR24	2.2±1.7		2.0±1.2		
HCV genotype, N					
1a/b	41		30		
2a/c	3		18	< 0.001	
3a/b	14		2		
6ab	2		0		
Treatment regimen categories, N	DCV + ASV 24 weeks (N = 16) Grazoprevir/elbasvir 12 weeks (N = 7) SOF + PR 12 weeks (N = 4) SOF + PR 24 weeks (N = 1) SOF + RBV 12 weeks (N = 3) SOF + RBV 24 weeks (N = 16) Simeprevir 100 mg + PR 24 weeks (N = 5) Simeprevir 150 mg + PR 24 weeks (N = 8)		PR 48 weeks (N = 50)		
IL-28B Genotype[¶], N					
CC	25		46		
CT	8		4		

[*] Continuous variables were expressed in mean ± standard deviation (SD) or median (range). Categorical variables were presented as number (percentage).

[†] P-value was obtained by comparisons between DAA and PR groups.

[‡] P-value was obtained by comparisons between pre- and post-treatment.

[#] One subject's histological tests between DAA group was not available. 59 subjects with histological test in DAA group were used for statistical analysis.

[¶] IL-28B genotype data were available in 33 subjects in DAA group.

fibrosis scores decreased from 2.8 to 2.2 in DAA group (P < .001) and from 3.0 to 2.0 in PR group (P < .001).

3.2. Incidence of OCI

All the subjects from the study cohort were simultaneously subjected to HCV-RNA detection. Fig. 2A is representative image of RNAscope assay for detecting HCV-RNA in liver biopsy of an OCI patient at baseline and post-SVR. Abundant black dots representing specific hybridization signals as readout of HCV-RNA were shown in hepatocytes at baseline. At SVR24, HCV-RNA was still detectable with presenting sporadic dots in a part of hepatocytes. Negative control was obtained by applying an unrelated probe on the same biopsy. Additional three paired liver biopsies randomly selected from patients with OCI were shown in Fig. S3.

Overall, HCV-RNA was detected in a total of 16 subjects (11.4%): nine (15.0%) in DAA-based group, five (10.0%) in PR group, and two (6.7%) in SR group (Fig. 2B-E). The distribution of OCI in DAA-based subgroups

was shown in Table S3. For DAA-based group, eight subjects were positive for HCV in both PBMC and liver tissues, and one subject was detected with HCV-RNA only in PBMC. For PR treatment group, four subjects were positive for HCV-RNA in both PBMC and liver tissues, and one subject was only detected with HCV-RNA in liver tissue. Sequencing the HCV-RNA showed the identity with serum HCV-RNA at baseline, indicating a protracting infection other than reinfection. Intriguingly, the proportion of OCI was found higher in peg-IFN-free (solely DAA or DAA plus RBV) treated individuals than in patients who received PR treatment with or without combination with DAA, suggesting the trend of high frequency of OCI in DAA-based group is likely due to the absence of peg-IFN, other than the presence of DAAs per se (Fig. 2F).

3.3. Clinical relevance of OCI

The incidence of OCI was found not related to baseline viral load, treatment regimens, baseline transaminitis, and the IL28B-genotype,
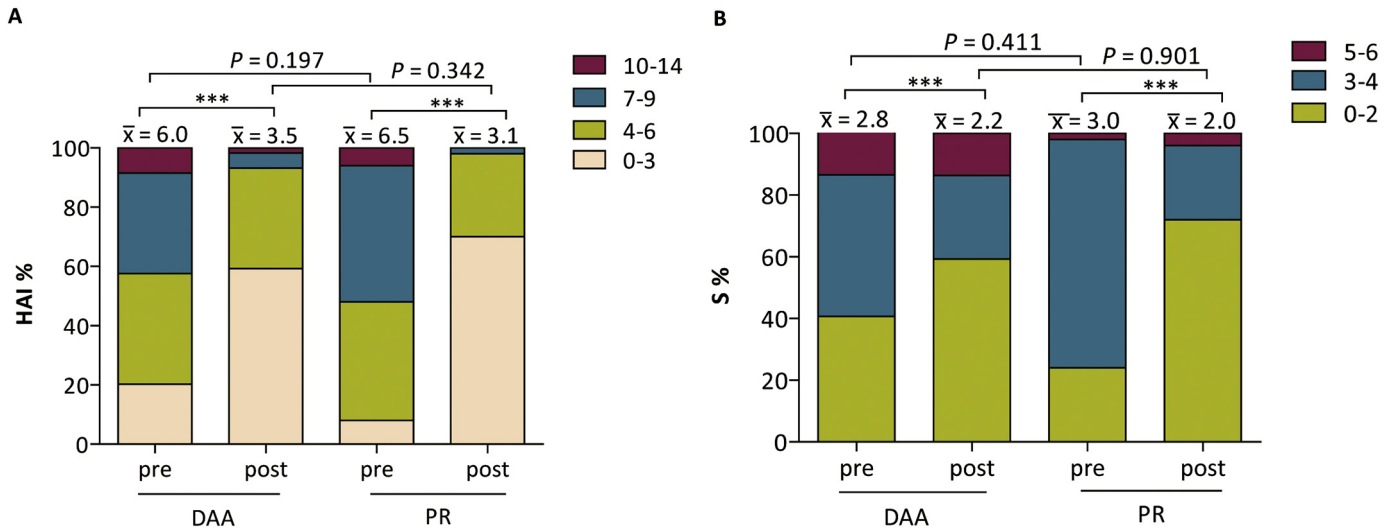
Fig. 1. Histological evaluation at baseline and post-SVR in HCV patients with SVR. HAI (A) and fibrosis (S) (B) score were analyzed at pre- and post-treatment in DAA and PR subgroups. Chi-square test or Fisher's exact test was used to compare pathology between DAA and PR groups. For comparing pathology between pre- and post-treatment, McNemar test was used.
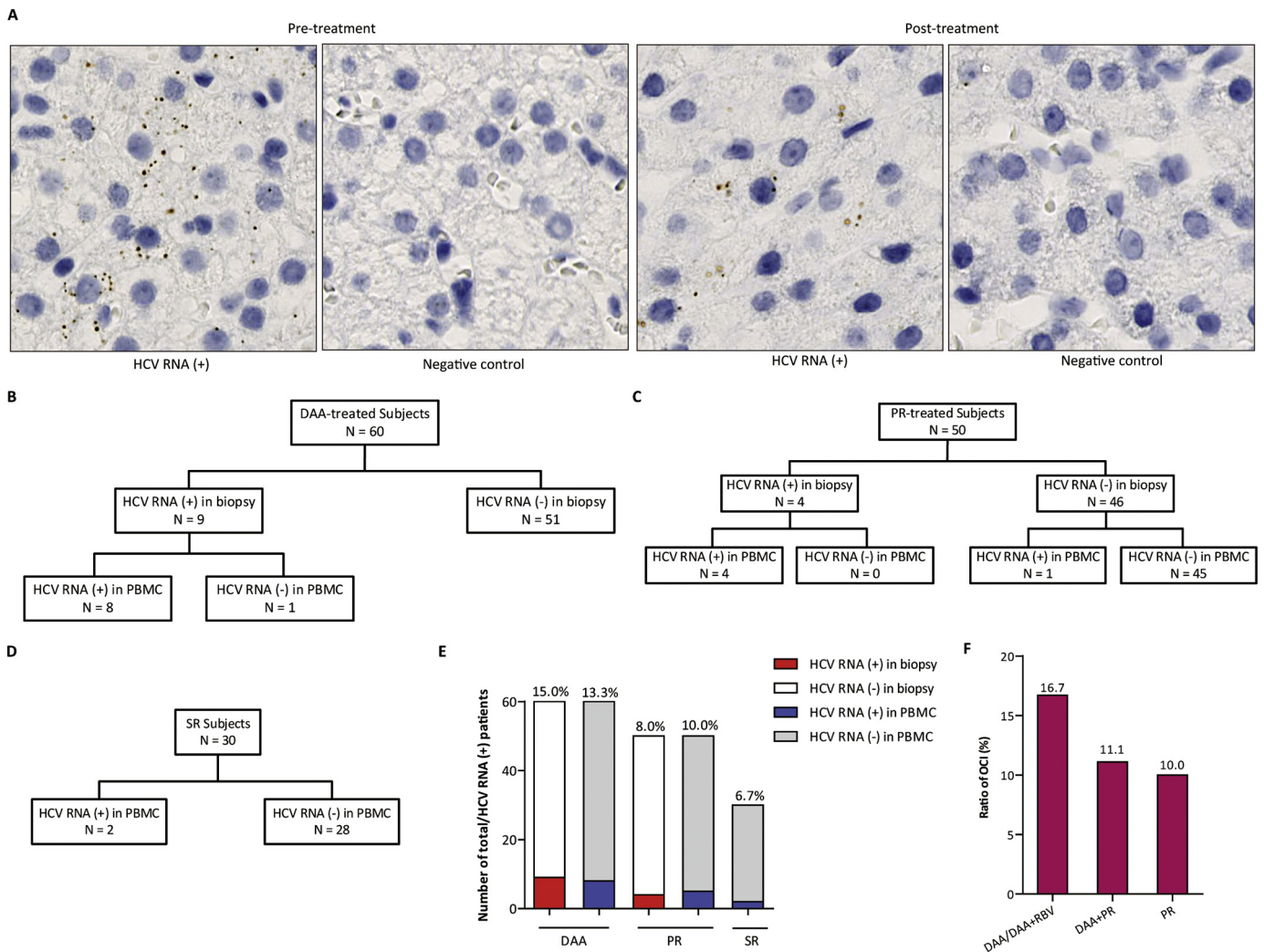
Fig. 2. Incidence of OCI in patients with treatment-induced or spontaneous HCV resolution. (A) Representative images of HCV-RNA detection by RNAScope assay in one OCI patient. The number of OCI cases in subgroups was showed in (B) (C) (D) and the proportion of OCI was summarized in (E). (F) The ratio of OCI in subsets of peg-IFN-free (including solely DAA and DAA plus ribavirin) ($N = 42$), combination of PR and DDA ($N = 18$), and PR ($N = 50$) treatment groups.

Table 2
Clinical characteristics of participants with or without OCI[a].

Parameters	Presence of OCI N = 14	Absence of OCI N = 96	P
Sex (male/female)	7/7	68/28	0.21
Age (years)	43.5 (27–65)	48 (21–67)	0.74
Serum HCV-RNA at baseline (IU/ml)	2.82×10^6 $(1.00 \times 10^5$–$1.02 \times 10^7)$	2.20×10^6 $(1.73 \times 10^2$–$3.19 \times 10^7)$	0.97
Treatment regimen category, N (%)			
DAA	9 (64.3)	51 (53.1)	0.43
PR	5 (35.7)	45 (46.9)	
Laboratory values			
ALT at baseline	52.5 (19.3–502.2)	54.9 (15–514.7)	0.89
ALT at SVR24	17 (9–47)	19.9 (5–174)	0.51
AST at baseline	40.0 (14.3–327.5)	45.2 (16–331.2)	0.59
AST at SVR24	21 (15–47)	22.5 (11–183)	0.29
AST/ALT at baseline	0.7 (0.4–1.3)	0.8 (0.4–1.9)	0.67
AST/ALT at SVR24	1.2 (0.8–1.9)	1.2 (0.5–4.1)	0.83
TBiL at baseline	15 (9–80)	16 (2–73)	0.98
TBiL at SVR24	15.4 (8.5–26.5)	13.4 (5–67.1)	0.38
PLT at baseline	165.5 (45–268)	162 (42–298)	0.64
PLT at SVR24	201.5 (98–317)	177 (47–356)	0.18
HCV genotype, N (%)			
1a/b	3 (21.4)	68 (70.8)	<
2a/c	2 (14.3)	19 (19.8)	0.001
3	8 (57.14)	8 (8.33)	
6ab	1 (7.1)	1 (1.0)	
IL-28B Genotype, N (%)			
CC	12 (85.7)	59 (85.5)	1.000
CT	2 (14.3)	10 (14.5)	
Histological evaluation[b]			
Inflammation (HAI) scoring			
Baseline	7.9±3.2	6.0±2.1	0.03
SVR24	4.2±1.1	3.2±1.6	< 0.001
[c]HAI Change	3.7±2.8	2.8±1.9	0.49
Fibrosis (S) scoring			
Baseline	3.4±0.9	2.8±1.4	0.065
SVR24	3.2±1.3	2.0±1.5	0.003
[c]Fibrosis Change	0.2±0.9	0.8±1.2	0.030

  [a] Continuous variables were expressed in mean ± standard deviation (SD) or median (range). Categorical variables were presented as number (percentage).
  [b] One subject's histological tests in absence of OCI group was not available. 95 subjects with histological tests in absence of OCI group were used for statistical analysis.
  [c] These were calculated by Scoring at baseline minus Scoring at SVR24.

but associated with genotype (Table 2). OCI occurred most frequently in patients with genotype 3. The incidence of OCI was lowest in patients with genotype 1 HCV, respectively. Moreover, the basal scoring of necroinflammatory activity and fibrosis were higher in patients with OCI than in patients without OCI (Table 2, Fig. 3A-B).

It is well noted that HCV clearance generally improves clinical outcomes of chronic hepatitis C, but a subset of HCV patients still exhibit liver histological injury after SVR. We then examined whether OCI is a potential factor compatible with protracted hepatic alteration. Our results revealed that OCI subjects presented significantly more severe HAI ($P < .001$) and hepatic fibrosis ($P = .003$) at SVR24 compared to non-OCI patients (Table 2, Fig. 3A-B). Further analysis revealed that the proportion of unfavorable fibrosis status (S score ≥ 2) at SVR24 was significantly higher in OCI patients (Table S4). To further elucidate whether the higher fibrosis score at SVR24 in OCI subjects is the result of severe fibrosis at baseline or the involvement of residual HCV, we performed subgroup analysis by selecting the patients with comparable fibrosis status at baseline (S score 3–4) to assess the treatment consequence. The fibrosis at SVR24 was still more favorable in non-OCI patients (Table S5) than in OCI patients, strongly supporting the potential role of OCI in hepatic abnormality.

More importantly, the fibrosis was significantly improved only in patients of non-OCI ($P < .001$), but not in OCI subjects ($P = .385$) (Fig. 3B). Fig. 3C-D showed representative paired liver biopsies of pre-

and post- therapy from two OCI patients with DAA or PR treatment, in whom HCV-RNA was 1.02×10^7 IU/m and 8.94×10^6 U/mL at baseline. Hematoxylin-eosin (H&E) staining showed severe interface hepatitis (piecemeal necrosis) and moderate lobular necroinflammtion at baseline. The HAI score of Ishak system was 13 and 15, respectively. Masson's and Gordon-Sweets's silver double-staining displayed marked bridging fibrosis with occasional nodules with fibrosis score 5 and 4, respectively. At post-SVR, HAI score decreased to 5 while fibrosis score were unchanged compared with baseline level in both patients. Overall, we found only five of 14 (35.7%) OCI patients presented fibrosis regression (decrease of fibrosis stage by at least 1 score) and the remaining nine OCI patients exhibited either stabilization or worsening of liver fibrosis, whereas the frequency of fibrosis regression was 61.0% in non-OCI patients ($P = .07$), reflecting a trend towards persisting liver histological alteration in OCI patients.

In the multivariate analysis (Table 3), PR therapy was identified the only independent negative prognostic factor for both hepatic inflammation regression ($P = .022$) and fibrosis regression ($P = .015$). OCI had no impact on inflammation regression, but seemed negatively associated with fibrosis regression, even when considering treatment regimens, although no statistical significance ($P = .106$).

We subsequently investigated whether OCI was associated with abnormal aminotransferases, because OCI has been commonly reported in patients in whom serum levels of aminotransferases did not normalize persistently after achieving SVR. Of the 14 OCI patients, one (7.1%) had abnormal transaminase, in which the ratio is comparable to that in patients of non-OCI (17.7%) ($P = .54$), implying that the presence of OCI seemed not to associate with abnormal aminotransferases (Table S4). Moreover, in the setting of OCI patients, no correlation between the viral titer and liver function tests was found (Fig. S4).

During follow up, one of 16 the OCI cases relapsed at 48 weeks after end of PR treatment (subject 13, Fig. S4), while no reactivation was found in non-OCI individuals.

4. Discussion

This study, with samples from various treatment regimens in a multicenter cohort, unique paired biopsy-proven hepatic evaluation, long-term follow-up of antiviral treatment, assessed the prevalence of OCI based on both PBMC and liver tissue in the immunocompetent individuals who resolved HCV infection either spontaneously or after antiviral treatment. Overall, 11.42% subjects were found to have OCI, with the highest (15.0%) rate in DAA treated subjects, 10.0% in PR treated subjects and 6.7% in spontaneous revolved cases. OCI is more frequent in patients with genotype 3 HCV but less in patients with genotype 1. No correlation between baseline viral load, interleukin-28B genotype, baseline transaminases, post transaminases and OCI was found. Patients with OCI had more severe fibrosis and active necroinflammation at SVR24. The existence of OCI is associated with less frequency and magnitude of fibrosis regression. Finally, we found HCV relapse in one of the OCI patients.

The prevalence of OCI varied among studies depending on the study population, follow-up duration, sample materials that were used for test and the detection assay employed [23]. In our study, the results were guaranteed by the evidence of the relatively high concurrence of detection of HCV-RNA in PBMC and liver tissue obtained at same time point. In particularly, RNAscope technique with confirmed outstanding sensitivity and specificity was innovatively applied for in situ detection of low-level HCV-RNA in hepatocytes. The signal is able to be appeared upon dual targeting probes combining HCV genome simultaneously, thus guaranteeing the specificity. Moreover, some studies argue against the presence of OCI by attributing the undetectable serum HCV genome to the low sensitivity of conventional clinical detection assay at that time [3,24]. However, this is not applicable in our case because clinical test employed in our studies has high sensitivity (15 IU/mL), precluding the false negativity.

Fig. 3. Histological evaluation between patients with or without OCI. HAI (A) and S (B) score were analyzed at pre- and post-treatment in patients with and without OCI. (C) and (D) are representative images of liver biopsies from two OCI patients. Chi-square test or Fisher's exact test was used to compare pathology between between OCI and non-OCI groups. For pre- and post-treatment comparison, McNemar test were used.
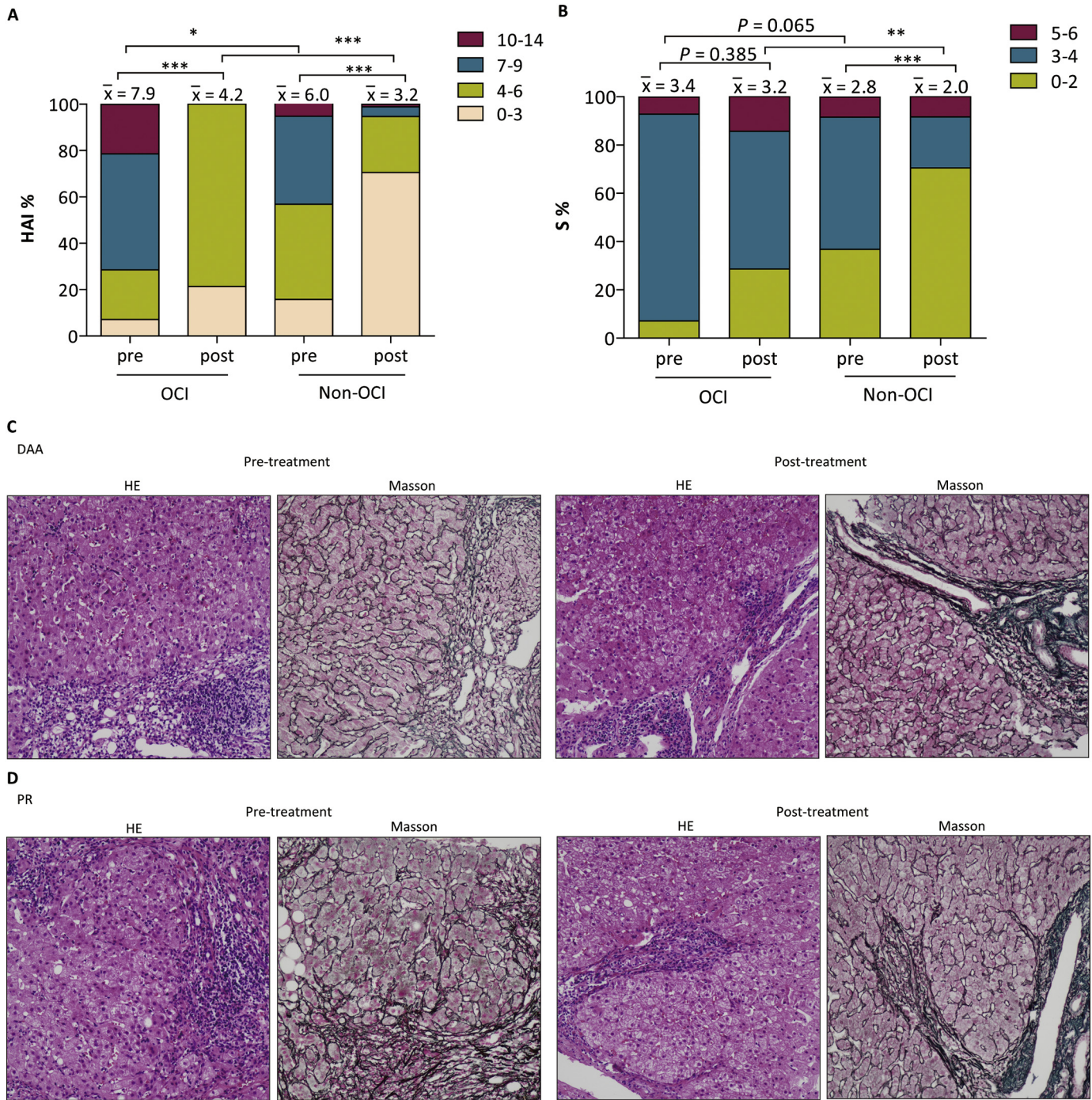
Our results showed that HCV-RNA was detected in the PBMCs of 12 (92.3%) of the 13 patients with intrahepatic HCV-RNA, indicating a high agreement rate of HCV-RNA detection in these two specimens. This finding has important diagnostic implication that HCV-RNA in PBMC can be routinely tested in patients to identify OCI, rather than in liver biopsy specimens. However, previous evidences indicated that HCV-RNA in PBMC was only detectable in 70% patients who had occult HCV-RNA in liver. It has to be stated that a negative result in PBMCs does not exclude the existence of HCV-RNA in liver cells [25]. On the other hand, detection of HCV-RNA in liver biopsy is also able to provide the distribution of HCV-RNA in liver tissue and the association with pathology in addition to simply indicating presence of HCV-RNA.

In this study, combination of DAA(s) with ribavirin does not reduce the incidence of OCI. Intriguingly, OCI seems to occur less frequently in patients with PR alone or combination with DAA treatment than in those with peg-IFN-free regimens, despite an overwhelming SVR rate of DAA. The exact mechanism is not fully understood but might be explained by the distinct pharmacodynamics of DAA and PR. The antiviral activity of interferon is commandingly known through immunomodulation of host cells by stimulating a number of innate

Table 3

Logistic regression analyses of HAI regression and fibrosis regression in patients with hepatitis C and achieved SVR.

	Univariate analysis			Multivariable analysis		
	OR	95 CI	P	aOR	95% CI	P
HAI regression						
Presence of OCI	1.666	0.487–5.701	0.416			
Age ≥ 47 years	1.493	0.688–3.242	0.311			
Male gender	1.555	0.686–3.525	0.291			
Baseline Fibrosis ≥3	2.430	1.070–5.515	0.034	1.858	0.785–4.394	0.159
ALT ≥55 U/L	1.030	0.476–2.229	0.940			
AST ≥ 45 U/L	1.586	0.730–3.448	0.244			
TBiL ≥16 umol/L	1.747	0.802–3.808	0.160	0.795	0.270–2.343	0.678
GT3 HCV	1.454	0.467–4.526	0.519			
HCV RNA ≥ 2.22 × 10^6	1.927	0.881–4.216	0.101	1.881	0.812–4.358	0.140
PR therapy	0.305	0.134–0.697	0.005	0.270	0.088–0.828	0.022
Fibrosis regression						
Presence of OCI	0.354	0.110–1.140	0.082	0.363	0.106–1.238	0.106
Age ≥ 47 years	0.699	0.326–1.502	0.359			
Male gender	0.875	0.386–1.982	0.749			
Baseline HAI ≥ 6	1.489	0.669–3.313	0.330			
ALT ≥55 U/L	0.563	0.261–1.215	0.143	0.478	0.211–1.083	0.077
AST ≥ 45 U/L	0.745	0.347–1.600	0.451			
TBiL ≥16 umol/L	1.279	0.597–2.739	0.527			
GT3 HCV	0.691	0.239–2.001	0.496			
HCV RNA ≥ 2.22 × 10^6	0.764	0.356–1.637	0.488			
PR therapy	0.387	0.175–0.855	0.019	0.359	0.157–0.820	0.015

Abbreviations: aOR: adjusted odds ratio; ALT: alanine aminotransferase; AST: aspartate aminotransferase; TBiL: total bilirubin.

antiviral effectors [26], or indirectly promoting cytotoxic T cell clearance of cells infected by HCV [27]. In contrast, the inhibitory approach of DAA is directly targeting HCV genome by inhibiting HCV protease for viral synthesis and replication, other than modulating cellular defense status. The immune microenvironment by HCV infection might be abrogated after withdraw of DAA therapy, providing the chance for HCV persisting.

Although hepatic fibrosis and inflammation was noted in 81.8% of OCI patients in previous study [28], whether OCI is an involving factor for the unfavorable liver histology after SVR remains on debating, because alternative factors, such as treatment course, follow-up duration, baseline fibrosis, cannot be precluded to impact histology changes. In the current study, the acquisition of paired liver biopsies from 110 patients provided the possibility to analyze the involvement of OCI in pathological changes. Though liver histology showed general and significant improvement after SVR, patients with OCI is likely to associate with persistent histological impairment compared with non-OCI patients regardless of other variants, implying potential pathogenic role of OCI. Our finding seems congruent with previous study that protracting hepatic damage was more remarkable in individuals with detectable HCV-RNA in liver specimens after SVR [2,4]. The intriguing finding is somehow anticipated because many studies suggest that patients with elimination of serum HCV are perceived to achieve regression of liver fibrosis or cirrhosis than non-SVR patients [29,30]. Nevertheless, argument against the clinical relevance of OCI proposed that the persisting histological alteration in a small portal of individuals after SVR may bear the reverse of immune intolerance after withdraw of antiviral therapy [14]. However, this seems unlikely, because one study investigating OCI in patients with unknown cause of persistent abnormal liver tests suggested that occult intrahepatic HCV-RNA was independently linked to inflammatory and fibrosis [13]. The patients in their study cohort were all negative for anti-HCV antibodies and for serum HCV-RNA, and never received antiviral therapy, precluding the role of antiviral therapy mediated immune imbalance in histological abnormality. Recently study in animal indicated that occult hepacivirus re-infections occur and that new liver pathological lesions, including lymphocyte infiltration and fibrosis, are appeared even in the absence of

high viraemia [31]. Refer to the underline explanation, there are two ways of interpreting the association between OCI and persistent histological change: a) A mechanistically cause-effect relationship may exist. Residual HCV-RNA is capable of triggering fibrotic progression constantly or halting fibrosis alleviation. b) It can also be objected that the more advanced and obstinate fibrosis, the less possibilities of HCV-RNA elimination. More molecular pathway studies with animal models are warranted to clarify whether and how the causal relationship exists.

In addition to the revealing minor fibrosis improvement, OCI patients appear to present more severe basal fibrosis, leaving a question whether subjects with advanced fibrosis are more susceptible to development of OCI. It is worth to note that presence of cirrhosis has been proposed to associate with lower rate of SVR and relapse after DAA based treatment [32,33]. Meanwhile, contrasting evidence has been brought forward by a meta-analysis demonstrating that the clearance of serum HCV was not associated with basal fibrosis severity [30]. Additional work is needed to adequately recognize how and what extend the severe fibrosis involves residual HCV protracting.

OCI has been reported to link with persistent elevated transaminases in patients who achieved spontaneous or treatment-induced HCV clearance from serum. Previous studies showed that 55% subjects who presented persistent abnormal aminotransferases after SVR had OCI [5]. However, the incidence in patients with normal liver function tests is not well determined owing to lack of justification for acquisition of liver tissues. In the current study, the access of the liver biopsies legally for evaluating histological changes from all patients enabled the comparison. We found the frequency of transaminases abnormality and the media ALT and AST level were all comparable between patients with and without OCI. This is congruent with an investigation which suggested that OCI is unrelated to serum ALT/AST [34]. Whether OCI and deregulated liver function are causally associated or just coincident events need to be precisely addressed further. Another intriguing result is that OCI was noted in a significant minority of patients with abnormal liver tests (2/19) in our study compared to 5/9 subjects in previous reports. The discrepancy is probably attributed to different immune statuses because the patients in their study were all immunocompromised.

There are some limitations in the current study. First, the patients' inclusions are non-homogeneous, especially in DAA-based treatment group, which contains 8 subgroups of various regimens, leading to intricate context when analyzing the association of OCI and treatment strategies. In fact, this study was initially designed as an exploratory "pilot" study, and at the time of study inception it was felt that DAA-based and PR-based regimens settings are appropriate to investigate and compare the incidence of OCI. Investigation of OCI in populations with defined treatment regimens are required to establish the association. Secondly, T lymphocytes from individuals were not subjected for profiling cytokine pattern and gene expression pattern, thus lacking their relevance of OCI. Thirdly, additional course of antiviral therapy was not applied for patients with OCI, leading to a query of its benefit.

From a clinical perspective, our findings provide new insight into cure of HCV and could influence the following-up scenario after SVR. A complete elimination of virus after clinical diagnosis of SVR seems unlikely despite of substantially improved anti-HCV efficacy. Given the profound link between OCI and persistent hepatic alteration from this study, the chronic liver injury concomitant residual HCV-RNA could remain an issue after SVR. Longitudinal outcomes of OCI are required to be followed up by monitoring of virus reactivation, liver function tests and fibrosis evolution and diseases progression. Large cohort studies are needed to corroborate the histological complication in OCI patients and to identify the variants for predicating development of OCI. Certainly, additional work on how to deal with such patients is in urgent. Adequate and extended treatment of patients with OCI should be considered, even if it is unclear if this strategy could improve histological outcome and further study are needed to elucidate this issue.

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Authors' contributions

Y.W. contributed to study design, analysis and interpretation of data, literature search and writing of the manuscript; H.R. and X.C. contributed to collecting samples and clinical data, analysis and interpretation of data; H.L. contributed to statistical analysis, making tables and figures; B.L., L.W., H.Z., S.L. and G.Z. contributed to acquisition of data; N.L. contributed to technique support; J.N. and L.W. contributed to study design, providing samples and critical revision of the manuscript; J.Z. contributed to study concept, study design, study supervision and critical revision of the manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.07.043.

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