Prevalence of occult hepatitis C virus infection in patients who achieved sustained virologic response to direct-acting antiviral agents

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SUMMARY

The reappearance of HCV infection months or years after sustained virologic response (SVR) may be due to the persistence of HCV in tissue cells in spite of being undetected in serum. This situation is known as occult hepatitis C infection (OCI). We aimed to assess the prevalence of OCI in Egyptian patients with chronic hepatitis C (CHC) who achieved SVR after treatment with direct-acting antiviral agents (DAA). We carried out a cross-sectional study at the Advanced Center for Liver Diseases of Zagazig University Hospitals and Al-Ahrar Viral Hepatitis Treatment Center, Sharkia Governorate, Egypt. One hundred and fifty adult patients with CHC, who achieved SVR 12-24 weeks after end of treatment with sofosbuvir/daclatasvir ± ribavirin (139 patients, 92.67%), sofosbuvir/ledipasvir ± ribavirin (eight patients, 5.33%), sofosbuvir/simeprevir (two patients, 1.33%), and ombitasvir/ paritaprevir/ ritonavir + ribavirin (one patient, 0.67%), according to the Egyptian National Committee for Control of Viral Hepatitis, were included in the study. We tested these patients for HCV RNA in peripheral blood mononuclear cells (PBMCs) immediately after confirmation of SVR12-24 weeks. Statistical analysis was performed by means of the Shapiro-Wilk test, Mann-Whitney U test, Chi-square test, and Fisher's exact test. Seventeen patients (11.33%) were positive for PBMNCs HCV RNA. The prevalence of OCI was highest in patients treated with simeprevir/sofosbuvir (2/2 patients). There is a substantially high prevalence of OCI after treatment with DAAs. We recommend dual testing for HCV RNA in both serum and PBMCs at the end of treatment of HCV infection with DAAs and during validation of the SVR following the initial response.

Keywords: direct acting antiviral agents, occult hepatitis C infection, sustained virologic response.

INTRODUCTION

Egypt is among countries with the highest prevalence and incidence of HCV infection all over the world. Egypt Health Issues Survey in 2015 revealed that 4.4% or around 3.5 million Egyptians from 1-59 years old have an evidence of active hepatitis C infection. HCV infection rates increased sharply with age, with around 1 of each 6 women and 1 of each 4 men having an active HCV infection in the age range from 50-59 at the time of the survey [1]. The recently discovered interferon-free directly acting antiviral drugs (DAAs) achieved approximately around 95% sustained virologic response (SVR) [2].

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Later recurrence of active infection due to either reinfection or late relapse represents a real problem. The majority of relapse occurs within 1-4 weeks after the end of treatment. A minority of cases relapses months to years later [3-5]. Activation of occult HCV infection (OCI) may be the origin of these late relapses [6-10].

OCI is defined as the existence of HCV RNA in hepatocytes or peripheral blood mononuclear cells (PBMCs) of individuals whose serum samples tested negative for HCV RNA by conventional PCR assays, with or without the presence of anti-HCV antibodies [11]. Pham and his colleagues were the first to report OCI [5]. Thereafter, many reports suggested that OCI could be present in many individuals who virtually have "cleared" the virus from their serum, either spontaneously or after antiviral treatment [12, 13].

The detection of HCV RNA in the liver cells is the reference method or the gold standard for the identification of an occult HCV infection, however; liver biopsies are not readily available [5]. The alternative way for diagnosing an OCI is to detect HCV RNA in peripheral blood mononuclear cells or by using Ultrasensitive PCR assays in the plasma or serum [14].

Identification of evidence of persistent infection such as detection of HCV RNA in PBMCs (particularly the negative sense strand) following apparent SVR after treatment with DAAs might be an important diagnostic tool of non-cure that raises the probability of later relapse.

We aimed to identify the frequency of occult HCV in patients who achieved SVR after DAA therapy by detection of viral RNA in their peripheral blood mononuclear cells and to evaluate the need of this test for assessment of the real cure of these patients.

PATIENTS AND METHODS

Study design

We carried out a cross-sectional study.

Settings

We did this work in the period from March 2017 to September 2017. We had recruited the patients from the Outpatient Clinic of The Advanced Center for Liver Diseases of Zagazig University Hospitals and Al-Ahrar Viral Hepatitis Treatment Center (VHTC) located in Zagazig, Sharkia Governorate, Egypt. We performed laboratory work in Microbiology and Immunology Department in collaboration with the Zagazig Research Scientific Center, Faculty of Medicine, Zagazig University.

Participants

The study included 150 patients with chronic hepatitis C. All patients had finished antiviral therapy and achieved SVR (undetected serum HCV RNA 12-24 weeks after the end of treatment), according to Egyptian National Protocol Guidance for Treatment of CHC that was updated in November 2015 by the "National Committee for Control of Viral Hepatitis".

Sampling

The estimated sample size was 150 patients at 80% power and 95% Confidence Interval (CI) (Open Epi).

Ethical Clearance

We gained Institutional Review Board (IRB) approval of the Zagazig Faculty of Medicine, under the number of 3339-14-2-2017 prior to the study. We conducted all procedures according to the ethical principles expressed in the Declaration of Helsinki. We obtained written informed consents from all patients.

Inclusion criteria

We included adult patients who were aged ≥18 years old, of both gender, naïve and treatment experienced, and non-cirrhotic or cirrhotic.

Exclusion criteria

We excluded patients with combined infections (HCV/HBV or HCV/HIV), hepatocellular carcinoma or other malignancies, and patients using immunosuppressive therapy.

Basic data process

We performed full history taking and thorough clinical examination. We revised the initial data of the patients including age, sex, body mass index (BMI), any associated comorbidities, history of previous treatment with pegylated interferon/ribavirin, sofosbuvir/ribavirin ± pegylated interferon, HCV RNA load by sensitive real-time PCR technique, liver function tests, complete blood count, prothrombin time, serum creatinine level,

alpha-fetoprotein, fasting blood sugar, as well as abdominal ultrasonography. We calculated the Child-Pugh score, MELD score and FIB-4 score to assess the severity of liver disease. Although genotyping was not performed at baseline, it is known that more than 94% of the Egyptian patients with chronic hepatitis C are infected by genotype 4 regardless of population type according to the most recent meta-analysis by Kouyoumjian et al. who stated that genotype diversity was low with a relative Shannon Diversity Index of only 14.4% (score: 0.27 out of a maximum of 1.95) [15]. Therefore, we consider this work representative to HCV genotype 4.

Peripheral Blood Monocyte Separation

Immediately after confirmation of 12-24 weeks SVR after the end of treatment, we collected two separate 3 mL samples of peripheral blood from each patient into EDTA tube. Samples were diluted 1:1 using phosphate buffer saline (PBS). Diluted samples were layered onto 4 mL of density gradient Histopaque® 1,077 (Sigma Aldrich, Germany) and centrifuged at 400 xg for 30 minutes at room temperature. Buffy coat at the interphase was carefully transferred to another tube and washed twice, using phosphate buffer saline. Pellet was resuspended in 0.5 mL PBS and transferred into 1.5 mL tube. The number of cells was counted and adjusted to a concentration of approximately 2-3 x 10^6 cells/mL using the hemocytometer. Diluted plasma was carefully aspirated from the upper layer and kept at -80 C for further work.

RNA extraction and qRT-PCR

Total RNA extraction from mononuclear cells was performed using TRIzol™ Reagent (Invitrogen) following manufacturer's instructions. A positive and negative control serum samples were included each time. For lysis of ~1-2 X 10^6 cells, 0.75 ml of TRIzol™ Reagent was used in 1.5 mL microcentrifuge tube. An internal positive control and carrier RNA was added for each sample at the lysis step of extraction. RNA pellet was dissolved into 40 µl of RNase free water. RNA extract was kept at -80 C until PCR reaction. For Viral RNA extraction from diluted plasma samples, Instant viral RNA extraction kit (Analytic Jena, Germany) was used. The Robogene HCV RNA quantification kit 3.0 (Analytic Jena, Germany) was used for PCR detection of HCV RNA from all samples using Mx3005P qPCR System (Agilent Genomics). Target HCV RNA signal was detected at FAM channel while internal positive control signal was detected at Joe/Hex Channel. The machine Stratagene Mx3005P is one of the real-time PCR machines validated for use with many IVD approved HCV quantification kits. The count and limits of detection cannot be determined in PBMC since the available standards are prescribed for serum or plasma samples only. Standards and detection limit vary with the starting volume of serum or plasma used for extraction, the elution volume and the volume of RNA added to the reaction. However, in this study, we tried to standardize the method by adjusting the number of cells to be 1-2 million cells to begin extraction with. To check for the sensibility, we also made a serial dilution of a serum sample with a known count. Fortunately, RNA was still detectable to a level of about 10 IU/mL using the same methodology applied to the cells.

Statistical analysis

We performed all statistics by using SPSS 22.0 for Windows (SPSS Inc., Chicago, IL, USA), MedCalc Windows (MedCalc Software bvba 13, Ostend, Belgium), and Microsoft Office Excel 2010 for Windows (Microsoft Cor., Redmond, WA, USA). We expressed continuous variables as mean ± SD and categorical variables as number and percentage. We checked continuous data for normality by using Shapiro Walk test. Mann-Whitney U test was used to compare two groups of non-normally distributed data. Percent of categorical variables were compared using Chi-square test or Fisher's exact test when appropriate. A two-sided (α=2) $p < 0.05$ was considered statistically significant.

RESULTS

This study included 150 patients with age ranging from 21 to 70 years, 88 of the patients were females and 62 of them were males. One hundred and forty-four patients were treatment naive and six of them were treatment experienced. All the patients achieved SVR with one of the following regimens: sofosbuvir/daclatasvir ± ribavirin (SOF/DCV±RBV) in 139 patients (92.67%), sofosbuvir/ledipasvir ± ribavirin (SOF/LDV±RBV) in eight patients (5.33%), simeprevir/sofosbuvir

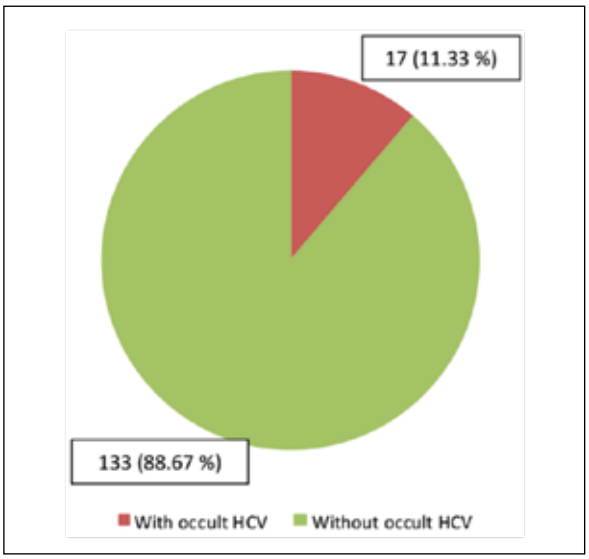

Figure 1 - Pie diagram show prevalence of occult HCV among 150 studied patients.

(SIM/SOF) in two patients (1.33%), and ombitasvir/paritaprevir/ritonavir + ribavirin (OBV/PTV/RTV+RBV) in one patient (0.67%) for 12 or 24 weeks.

As illustrated in Figure 1, the prevalence rate of OCI among all studied patients was 17 patients (11.33%). The prevalence of OCI was not significantly related to all baseline demographic, clinical, and laboratory characteristics of study population (Tables 1 and 2).

Table 3 shows a statistically non-significant association between the prevalence rate of OCI and the two largest HCV treatment regimen groups SOF/DCV12 and SOF/DCV/RBV, other treatment regimen groups are excluded from the comparison due to low number of treatments (≤5) [OCI was present in both the two patients in SIM/SOF regimen and in one of two patients in SOF/DCV24 regimen whereas it was absent in patients in SOF/LDV/RBV and OMB/PTV/RTV/RBV regimens].

Table 1 - Comparison of baseline demographic, clinical and antiviral therapy data between patients with and without occult HCV infection

Variables		*All studied patients (N=150)*		*Without occult HCV (N=133)*		*With occult HCV (N=17)*		*p*
		No.	*(%)*	*No.*	*(%)*	*No.*	*(%)*	
Gender	Female	88	58.7%	78	58.6%	10	58.8%	0.99*
	Male	62	41.3%	55	41.4%	7	41.2%	
Age (years) (Mean ± SD)		48.86±10.57		49±10.27		47.76±12.97		0.72†
BMI (kg/m²) (Mean ± SD)		31.84±6.29		31.93±6.44		31.18±5.07		0.92†
Clinical data	Ascites	4	2.7%	4	3%	0	0%	1.00‡
	HE	0	0%	0	0%	0	0%	---
	DM	22	14.7%	20	15%	2	11.8%	1.00‡
	HTN	16	10.7%	14	10.5%	2	11.8%	1.00‡
	IHD	2	1.3%	2	1.5%	0	0%	1.00‡
CP score (Mean ± SD)		5.25±0.56		5.25±0.58		5.24±0.43		0.69†
Score 5		122	81.3%	109	82%	13	76.5%	0.46*
Score 6		20	13.3%	16	12%	4	23.5%	
Score 7		7	4.7%	7	5.3%	0	0%	
Score 8		1	0.7%	1	0.8%	0	0%	
Meld score		8.23±2.29		8.29±2.29		7.76±2.27		0.26†
Fib4 score (Mean ± SD)		2.82±2.59		2.89±2.68		2.26±1.67		0.3†
Treat-ment	Naïve	144	96%	127	95.5%	17	100%	1.00‡
	Experi-enced	6	4%	6	4.5%	0	0%	

*Chi-square test, †Mann Whitney U test, ‡Fischer's exact test.
BMI: Body mass index, HE: Hepatic encephalopathy, DM: Diabetes mellitus, HTN: Hypertension, IHD: Ischemic heart disease, CP: Child-Pugh.

Table 2 - Comparison of baseline biochemical data between patients with and without occult HCV infection

Variables	*All studied patients (N=150)*	*Without occult HCV (N=133)*	*With occult HCV (N=17)*	*p**
	Mean ±SD	*Mean ±SD*	*Mean ±SD*	
Hemoglobin (g/dl)	12.95±1.68	12.92±1.71	13.18±1.50	0.41
Platelet count (x10^3/mm^3)	175.04±65.03	175.49±66.44	171.47±54.29	0.82
WBCs count (x10^3/mm^3)	6.15±2.40	6.16±2.42	6.05±2.25	0.94
Total bilirubin (mg/dl)	0.82±0.33	0.83±0.33	0.77±0.29	0.57
Albumin (g/dl)	3.96±0.52	3.95±0.52	4.02±0.50	0.73
ALT (U/L)	54.40±32.90	53.59±31.59	60.71±42.41	0.6
AST (U/L)	56.43±30.90	57.17±31.07	50.61±29.83	0.34
INR	1.15±0.22	1.15±0.23	1.10±0.15	0.22
Serum creatinine (mg/dl)	0.86±0.23	0.86±0.23	0.82±0.21	0.2
Fasting Blood Sugar (mg/dl)	109.78±45.29	110.77±47.71	101.96±15.58	0.77
Alpha-fetoprotein (ng/dl)	7.73±10.68	6.75±9.03	15.15±17.73	0.08
HCV RNA viral load (x10^6IU/ml)	1.218±1.796	1.251±1.850	0.961±1.315	0.72

*Mann Whitney U test.

Table 3 - Distribution of occult HCV infection (OCI) cases in different categories of treatment regimens

Treatment regimen (N)		*Without OCI (N=133)*		*With OCI (N=17)*		*p**
		No.	*(%)*	*No.*	*(%)*	
SOF/DCV12	(67)	62	92.5%	5	7.5%	0.43
SOF/DCV/RBV12	(70)	62	88.6%	8	11.4%	

*Chi-square test.
SOF/DCV12: Sofosbuvir (400 mg) plus daclatasvir (60 mg) daily for 12 weeks. SOF/DCV/RBV12: Sofosbuvir (400 mg) plus daclatasvir (60 mg) plus ribavirin (1000-1200 mg) daily for 12 weeks.

DISCUSSION

In spite of the great progress achieved in HCV treatment in the last few years, chronic HCV infection is still a major global health problem, particularly in Egypt [1]. In the era of orally acting DAAs therapy for HCV infection, more than 90% of treated patients achieved SVR. However, a varying number of patients have experienced relapses later [16, 17]. Post-treatment persistence of intracellular HCV infection (occult infection) possibly acts as a reservoir and hence might be related to later serologic relapse, and even to the ongoing hepatocellular damage, fibrosis and cirrhosis [18, 19]. Replication of genomic HCV-RNA inside PBMCs was established and was followed, in some reported cases, by overt viremia after initial serologic clearance [18, 20]. Therefore, testing for HCV RNA both in serum and PBMCs at the end of treatment and at the follow up will be more informative regarding concordant clearance of viral RNA from both serum and cells [19].

In our study, OCI was diagnosed in 17 out of 150 patients (11.33%) who achieved SVR based on negative serum HCV viremia. Prevalence of OCI was 10.1% in patients treated with SOF/DCV±RBV and 12.5% in patients treated with SOF/LDV±RBV, while the two patients who were treated with SIM/SOF in the study got OCI (100%). However, we can't link the prevalence of OCI to the specific regimen of treatment due to the small number of patients included in this study.

Prevalence of OCI in general population is around 3.3% [8], and it is higher in those who have a spontaneous or post-treatment clearance of HCV infection [8, 18, 19, 21, 22]. In the study carried out

by Aboalam et al., aiming to determine the prevalence of OCI in PBMCs of 25 patients who spontaneously cleared HCV infection and presenting with a positive serologic test for anti-HCV Ab and negative serum HCV RNA PCR, OCI was detected in three patients (12%). On follow up every 6 months for 18 months using highly sensitive serum RT PCR, only one patient (4%) got overt HCV with a low level of viremia [22]. OCI was reported after HCV treatment in sustained responders to interferon-based therapy in multiple studies. McHutchison and co-workers (2002) found that only 4.1% (7/170) of sustained responders to standard INF and ribavirin had detectable intrahepatic HCV RNA, 24 weeks after treatment [23]. In another study from Egypt, Hanno and his colleagues explored the relationship between the HCV RNA in the PBMCs and response to IFN therapy in 25 chronic HCV patients [18]. At the end of treatment, all patients had negative serum PCR test for HCV RNA, nevertheless, HCV RNA was detected in PBMCs (OCI) of eight patients (32%). Patients with OCI had an overall significantly higher relapse rate after two years (50%) when compared with those without (6%). Patients with OCI who continued to receive interferon-based treatment for further six months (4/8) had a lower relapse rate (25%) when compared with other patients with OCI (4/8) who stopped interferon treatment at the 48th week (75%). A recent study conducted in Egypt by Abd Alla and El Awady reported a considerably high prevalence of OCI (18%) using PBMCs PCR in sustained responders to DAAs [19].

The OCI prevalence in our work had no statistically significant relation to any of the baseline demographic, clinical or laboratory characteristics of the study population. Elmasry et al. studied 134 patients who received DAAs for treatment of recurrent HCV infection after liver transplantation [24]. Out of 129 patients who achieved SVR12 in their study, nine patients with persistently elevated serum aminotransferases after DAAs therapy were assessed for OCI by looking for HCV RNA in hepatocytes and PMNCs using sensitive reverse transcription quantitative PCR technique. They identified OCI in five out of the 9 patients (55%). The same authors detected the negative HCV RNA strand in 4 of these 5 patients with OCI (80%) which reflects viral genome replication and they concluded that the possible existence of occult HCV infection may explain the elevated serum aminotransferases in some patients regardless of SVR to HCV treatment with DAAs.

Persistence of HCV as occult infection following natural clearance or clinically successful antiviral therapy has a substantial pathogenic and epidemiologic importance to the patient himself and the community. This warrants a comprehensive investigation and consideration of new therapeutic practices against HCV infection, which continues beyond a clinically apparent recovery [21, 22]. Regarding the possibility of treating patients with post antiviral therapy OCI and the expected outcome, no available trials yet involving those who finished oral DAAs, and there is only a few data concerning those who received IFN based therapy like the aforementioned significant reduction of late relapse rate [18]. Therefore, to use an extended treatment course or to give a consolidation course of DAAs in those who achieved SVR after the standard course of DAAs therapy but still have OCI could be an option.

Limitations to this work include lack of short term and long-term outcome for patients with OCI, the small number of the patients included in the study and the lack of assay for the presence of HCV RNA negative strands in PMNCs in patients with OCI to verify the viral replication activity in these patients and its impact on disease outcome. In addition, testing for HCV RNA in PBMC was not evaluated at baseline. This issue should be considered in further studies for better understanding of the dynamics of HCV infection.

In conclusion, OCI is encountered substantially in patients with CHC who achieved SVR with DAA therapy. Therefore, we recommend dual testing of HCV RNA in both serum and PBMCs at the end of treatment with DAAs and during validation of SVR. Further trials are needed to find out the possible predictors for persistence of OCI after DAAs. Long-term observational studies using a larger number of patients are warranted to confirm our conclusions. Modulation of treatment protocols with the aim of preventing OCI needs further work, especially with the newer more potent DAA generations. The need for retreatment in such patients is another issue has to be evaluated.

Conflicting interest

None

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