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DIRECT ACTING ANTIVIRAL THERAPY AND OCCULT HEPATITIS C IN UPPER EGYPT: RECENT ADVANCES IN HEPATITIS C VIRUS MANAGEMENT

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ABSTRACT

Background: depending on current estimates, the prevalence of the hepatitis C virus (HCV), one of the leading causes of death and illness worldwide, has increased over the past ten years to 2.8%, or more than 185 million infections globally. Although there is no vaccination, the infection can be healed with short-term, eight to twelve weeks therapies by direct acing antiviral drugs (DAAs) that are very successful and curative. Occult HCV infection (OCI) is described by HCV RNA being present in peripheral blood mononuclear cells or hepatocytes but remains undetected in serum.
Objectives: To detect occurrence of OCI in upper Egypt among chronic HCV cases in who achieved sustained virologic response (SVR) following management with DAA by 24 weeks later.
Methods: the study contains 301 HCV cases who achieved SVR following management with DAA (group1), and 100 healthy controls (group2) have been involved in this research. Routine laboratory investigations (complete blood count (CBC), kidney and liver function tests and alpha-feto protein) and HCV RNA in serum and peripheral blood mononuclear cells were done for all participants in both groups.
Results: Despite achieving SVR after DAA treatment and maintaining undetectable serum HCV RNA, persistence of HCV RNA in PBMCs was identified in 3 out of 301 cases (1%).
Conclusion: Simultaneous measurement of HCV RNA in both serum and PBMCs at the end of management with DAAs and through validation of SVR is recommended. Further studies are required to find out the potential predictors for persistence of OCI after treatment.

KEYWORDS: Occult HCV, Direct Acting Antivirals, Sustained virologic response

INTRODUCTION:

Around the world, fifty-eight million persons have chronic hepatitis C infection. With regard to the 2015 Egypt Health Issues Survey, 4.4% of Egyptians between the ages of 1 and 59, or around 3.5 million people, had evidence of an active hepatitis C infection. The WHO gold tier targets of identifying at least eighty percent of persons with hepatitis C and treating at least seventy percent of those identified have been exceeded by Egypt, which has diagnosed eighty-seven percent of persons infected with the virus and given curative treatment to ninety-three percent of those diagnosed [(1)].

The hepatitis C virus was a major public health problem in Upper Egypt governorates like Beni Suef, Minya, and Assiut. Historical seroprevalence is detected up to 28–29%, largely due to iatrogenic transmission from past schistosomiasis campaigns and unsafe medical practices. National screening programs and direct-acting antiviral therapies have contributed to a progressive decline in HCV infection across the country [(2)].

The advent of interferon-free direct-acting antiviral agents (DAAs) represents a major breakthrough in the management of hepatitis C virus infection. These therapies have transformed the management landscape of chronic HCV, providing a highly effective and well-tolerated alternative to traditional interferon-based regimens. With sustained virologic response rates approaching 95%, DAAs demonstrate remarkable efficacy in viral eradication, even among cases with advanced liver disease, including cirrhosis. This therapeutic success has translated into significant improvements in clinical outcomes and a marked reduction in the need for invasive interventions such as liver transplantation [(2)]

OCI denotes the existence of HCV in individuals deemed cured of the infection, marked by the lack of detectable HCV RNA in serum; nonetheless, highly sensitive PCR methodologies may still uncover minute quantities of HCV RNA in the liver or peripheral blood mononuclear cells (PBMCs) [(3)]. Two distinct types of OCI have been identified: (i) persons who are anti-HCV positive but HCV-RNA negative, following a resolved infection, and (ii) individuals who are both anti-HCV negative and HCV-RNA negative, often referred to as having cryptogenic HCV infection [(4)].

The clinical importance of OCI remains uncertain, as concerns persist that complete viral eradication may not be achieved even after a sustained virologic response. This raises the possibility that OCI could contribute to ongoing low-grade hepatic injury or viral reactivation, particularly in immunocompromised individuals, although definitive evidence is still lacking. [5, 6].

Finding of HCV RNA in hepatic cells is considered the reference technique, or gold standard, for diagnosing an occult HCV infection but, liver biopsies aren't usually easily available [7]. Detecting HCV RNA in peripheral blood mononuclear cells or utilizing ultrasensitive PCR tests in plasma or serum are two alternate methods for identifying an occult HCV infection. [8].

Although HCV is primarily hepatotropic, substantial evidence indicates that the virus can also replicate in extrahepatic compartments, including peripheral blood mononuclear cells. The detection of the negative-strand HCV RNA, a replicative intermediate, within infected cells provides strong support for active viral replication beyond the liver. [9]. It has been recommended that HCV may persist in PBMCs, which could serve as an extrahepatic viral reservoir. Persistence in these cellular compartments, even after clearance of serum HCV RNA, has been suggested as a potential mechanism underlying viral relapses and continued pathogenic effects, although the clinical implications of this phenomenon remain incompletely understood. [10].

However, in certain individuals who have attained HCV RNA clearance, the persistence of HCV-RNA in PBMCs is linked to persistent histological abnormalities. Dual testing in serum and PBMCs may be necessary to achieve SVR after direct-acting antiviral (DAA) treatment [11].

This research aimed to examine the potential presence of OCI in upper Egypt in individuals who achieved SVR 24 weeks after receiving direct-acting antiviral treatment.

PARTICIPANTS AND METHODS:

Study design and population

This cross-sectional research included 301 patients as group 1 (G1) who were HCV infected and achieved SVR, which is described as the absence of detectable serum HCV RNA. The study also included 100 healthy individuals as group 2 (G2) were taken as control group who were age and sex matched to group 1.

a. Inclusion Criteria:

HCV infected and achieved SVR for at least 24 weeks (SVR24) after completing DAA treatment

b. Exclusion Criteria

Cases with co-infections (HCV/HBV or HCV/HIV), hepatocellular carcinoma, other malignancies, and those undergoing immunosuppressive therapy were excluded.

Study Setting: (Including All Collaborating Centers)

This study was conducted at the Molecular Biology Research and Studies Institute at Assiut University, Assiut, Egypt in collaboration with the Clinical Pathology Department at Faculty of Medicine Assiut University, Assiut, Egypt and the Centre for Liver Disease, Ministry of Health in the period from April 2024 to March 2025.

Ethical considerations

Ethical approval has been gathered from the Ethics Committee of the Molecular Biology Researches & Studies Institute (MBRSI) (IRB no: 22-2023-0020), Assiut University, Egypt. All participants provided written informed consent prior to their inclusion in the research.

Study protocol:

All participants were subjected to the following conditions:

1- **Comprehensive history and thorough clinical examination** were conducted for all participant

2- Laboratory investigations:

Blood Sampling: A total of 10 mL of blood was collected from each participant and separated into two aliquots. One aliquot was collected without anticoagulant to yield serum separation were utilized for routine investigations and serum HCV- RNA for all participants , while the second aliquot contained ethylenediaminetetraacetic acid (EDTA) for complete blood count (CBC) and the preparation of PBMCs to determine HCV-RNA levels.

Routine laboratory investigation

-***Complete blood count***, including Red Blood cells (RBCs), Hemoglobin (Hb), Hematocrit (HCT), white Blood cells (WBCs) and Platelets (PLT), was performed utilizing the ADVIA 2120i (Siemens, Boston, USA).

-***Liver function tests***, comprising Direct Bilirubin (DBil), Total Bilirubin (TBil), Aspartate Aminotransferase (AST), Albumin (Alb.), Alanine Aminotransferase (ALT), and Alkaline Phosphatase (ALP), and ***kidney function tests***, including serum urea and creatinine, were conducted utilizing an automated blood chemistry analyzer (Siemens Advia 1800 Chemistry Analyzer, Siemens Boston, USA).

-***Alpha-fetoprotein test*** was determined on a high-performance ADVIA Centaur® XP system using chemiluminescence (Siemens, Boston, USA).

Specific Laboratory investigations

1- Separation of PBMCs

Three ml of EDTA-treated blood were mixed with 6 ml of lymphocyte separation solution in a 15 ml Falcon tube. Cells were separated within 24 hours of sample collection through Ficoll-Hypaque density gradient centrifugation using (Fecoll, Serana company, 14641Pessin, Germany) with regard to manufacturer's instructions. PBMCs count was adjusted to 5-10 $x10^3/\mu L$

2- Detection of HCV in Serum and PBMCs

Extraction of viral ribonucleic acid, reverse transcription, and real-time PCR were subsequently performed utilizing the automated Roche Molecular Systems Cobas AmpliPrep and Cobas TaqMan 48 (CAP/CTM), adhering to the manufacturer's protocol in serum and PBMCs for all participants.

Verification experiment: It was done using 30 samples with previously documented plasma viremia, categorized into three groups:

(1) 10 samples previously reported as HCV-RNA undetectable in plasma,
(2) 10 samples with low-level viremia
(3) 10 samples with moderate-level viremia.

Blood was collected into EDTA tubes for PBMC isolation and plain tubes for serum separation. PBMCs were isolated utilizing the standardized density-gradient protocol described earlier. Both serum and PBMC suspensions subsequently underwent automated nucleic acid extraction, reverse transcription, and real-time PCR using Roche Molecular Systems, with all assays performed in duplicate to ensure analytical precision

Statistical Analysis:

Statistical Analysis: Information are presented as mean ± SD or as counts. Comparisons between categorical variables have been done utilizing the Fisher's exact test or chi-square test, as appropriate. For comparing numerical variables between two groups, the Mann-Whitney U test was used. Data analysis was conducted utilizing the Statistical Package for Social Sciences (SPSS), version 22 for Windows. A p-value of < 0.05 was considered statistically significant**.**

RESULTS

Table 1 represents the demographic data for the study group and control group

Table (1): Demographic data in study groups.

Item	**G1 post treatment "n=301"**	**G2 Control "n=100"**	**P-value**
Age "years" (Mean ± SD)	52.91 ± 11.05	40.25±8.76	**P<0.03***
Sex:			
Male (Mean ± SD)	174(57.8%)	54(54.0%)	**P** > 0.05
Female (Mean ± SD)	127(42.2%)	46(46.0%)	

Table 2. summarized the results of liver and kidney function tests, along with serum alpha-fetoprotein (AFP) concentration, for both the study and control groups.

Table (2): Liver, Kidney function and AFP test results in study groups.

Item	**Units**	**Normal range**	**G1 post treatment "n=301"**	**G2 Control "n=100"**	**P-value**
Tbil	mg/dl	Less than 1.2	0.76 ± 0.179	0.62±0.34	**P<0.001****
Dbil	mg/dl	Less than 0.2	0.21 ± 0.084	0.21±0.02	**P=0.575**
ALB	g/dl	3.5 – 5.5	4.08 ± 0.43	4.21±0.28	**P=0.436**
AST	IU/L	male: Less than 39 female: less than 37	23.33 ± 10.58	19.53±2.76	**P=0.273**
ALT	IU/L	male: Less than 45 female: less than 41	31.98 ± 10.48	23.78±3.47	**P<0.001****
ALP	IU/L	Less than 295	211.75 ± 63.20	102.33±12.43	**P<0.01***
Creatinine	mmol/L	male: 62 -115	75.35 ± 16.12	83.43±6.80	**P<0.000*****

		Female**:** 53 - 97			
Urea	µmol/L	male: 2.5 – 7.1 Female**:** 2.1 – 6.8	5.35 ± 1.30	3.30±0.79	**P<0.000**
AFP	ng/ml	less than 10	7.56 ± 4.11	3.76±1.33	**P<0.001****

Table 3 represents the complete blood count (CBC) results for both the study and control groups.

Table (3): CBC results in study groups

Item	**Units**	**Normal Range**	**G1 post treatment "n=301"**	**G2Control "n=100"**	**P-value**
RBCs	10^6/µl	male: 4.7 – 6.1 female: 3.8 – 4.8	4.10 ± 0.32	4.21±0.43	P=0.241
Hb	g/dl	male: 12 - 16 female: 11 - 15	11.50 ± 0.11	12.46±1.46	**P<0.02***
HCT	%	male: 36 - 48 female: 36 - 46	30.14 ±0.14	39.45±3.67	P=0.584
WBCs	10^3/µl	4 – 11	6.22 ± 1.78	5.78±1.83	P=0.563
Platelets	10^3/µl	150 – 450	254.47±18.76	342.27±14.27	P<0.001**

In the research, a total of 301 cases with chronic hepatitis C virus infection who were treated within the national HCV control program in Egypt using direct-acting antivirals (DAAs) were included. Following completion of DAA treatment, they achieved a sustained virologic response, with undetectable serum HCV- RNA after 24 weeks. Among these patients, three individuals (two males and one female) were identified as having occult HCV infection.

Table 4 shows the descriptive data of the these 3 patients.

Table (4): Descriptive data of OCI patients in post DAA treatment group.

Item	**Case No.1**	**Case No.2**	**Case No.3**
Age	**58**	**49years**	49years
Sex	**Male**	**Female**	male
TBil	**0.8**	**0.5**	0.6
- **DBil**	**0.2**	**0.2**	0.2
- **ALb**	**4.4**	**4.0**	4.7
- **AST**	**16**	**10**	10
- **ALT**	**49**	**22**	23
- **ALP**	**285**	**268**	312
- **Creatinine**	**61.6**	**96.9**	70.4
- **Urea**	**5.67**	**5.83**	6.83
- **AFP**	**12.7**	**9.9**	4.7
- **HCV RNA**	**$1.14x10^3$**	**$7.25x10^3$**	$1.61x10^3$

DISCUSSION

From a clinical and public health standpoint, Egypt has achieved remarkable progress in hepatitis C control through the implementation of a comprehensive nationwide screening and treatment campaign. Given that HCV is an RNA virus that doesn't integrate into the host genome, complete viral eradication is biologically achievable following effective antiviral therapy. Interferon (IFN) was introduced in 1990 as the first approved treatment for chronic HCV infection, although its efficacy and tolerability were limited. The subsequent development of direct-acting antivirals has revolutionized HCV management, dramatically improving treatment outcomes. Contemporary DAA regimens now achieve sustained virologic response (SVR) rates exceeding ninety-five percent across most genotypes in non-cirrhotic patients, and approximately 80–90% even among patients with established cirrhosis These advances have transformed HCV from a chronic progressive disease into one that is largely curable at the population level. [(12).]

Furthermore, the advent of direct-acting antivirals has enabled the management of patients previously ineligible for IFN-based regimens, including those with autoimmune diseases, psychiatric disorders, and decompensated cirrhosis.

The extensive genetic diversity of HCV posed challenges that necessitated the development of multiple therapeutic regimens. Leveraging the high efficacy of DAAs, the national program has successfully screened and treated over four million individuals, advancing Egypt toward the World Health Organization's (WHO) objective of HCV elimination. [14].
In the context of post-hepatitis monitoring, occult hepatitis C infection has emerged as a complex clinical entity. OCI is diagnosed when HCV-RNA is detectable in hepatic tissue or peripheral blood mononuclear cells of cases whose serum HCV-RNA remains undetectable. Consequently, in cases who have attained sustained virological response, OCI may represent a latent reservoir for reinfection or a concealed source of HCV recurrence. [15].

Furthermore, Yousif et al. reported that the persistence of intracellular HCV infection (occult HCV infection, OCI) following therapy may act as a viral reservoir, potentially contributing to subsequent serologic relapse, as well as ongoing cirrhosis, fibrosis, and hepatocellular injury[16]. This phenomenon remains a subject of debate, particularly in regions with an elevated occurrence of HCV infection. In this context, the current research aimed to examine the occurrence of OCI, as indicated by persistent HCV-RNA in peripheral blood mononuclear cells.
In the present study, we found that 3 patients (1.0%) in the post-DAA treatment group had occult HCV infection. These findings are consistent with those of Austria and Wu (2018) and Wroblewska et al. (2021), who reported that OCI is diagnosed by the recognition of HCV/OCI in PBMC samples of cases, despite being undetectable in serum samples using conventional PCR assays [17,18]. Additionally, Silva et al. (2023) reported that OCI is characterized by serum-negative and PBMC-positive outcomes using ddPCR, highlighting the potential for HCV/OCI transmission in different study populations [19].
Habeeb et al. also noted that various researches have reported the occurrence of OCI in many populations; nevertheless, the associated risk factors haven't yet been established [20].

Our results were contradicted by a study by Martinez et al. (2018) that found that 3.4% of blood donors had OCI (seronegative for HCV antibodies) where in our study we did not detect OCI in control group, as assessed by analyzing HCV- RNA in both hepatocytes and PBMCs [21]. However, Bernardin et al. (2008) examined HCV- RNA in PBMCs from cases who had either naturally or through treatment eradicated the virus and discovered that HCV was completely removed from PBMCs [22].

This outcome is also supported by Saad et al., who stated that OCI is more common in people who had a spontaneous or post-treatment clearance of HCV infection, with an occurrence of about 3.3% in the general population [23].
Sood et al., who tracked about 100 patients who experienced sustained virologic response (SVR) for periods varying from six months to eight years, similarly documented a greater proportion of late recurrence. Among these, 8 cases (8%) developed late recurrence. Late relapses were more common in cases with cirrhosis (5/28, or 18%) compared to those without cirrhosis (3/72, or 4%) (P = 0.037) [24]. Similarly, an OCI rate of 11.33% was recorded in PBMCs by Yousif et al. in their study that looked at the occurrence of OCI in PBMCs in cases managed with DAAs and reaching SVR 12–24 weeks post-treatment. Nevertheless, no liver biopsy was performed on any of the research participants to evaluate OCI in their hepatocytes [16].
In the present study, three cases of OCI were identified, two of which were male. These outcomes are in line with those of Habeeb et al., who evaluated several independent predictor variables for the likelihood of OCI, including age, male sex, present smoking status, and the existence of diabetes mellitus [20]. Of these factors, male sex was found to be statistically significant, However, the virus's survival in PBMCs was unrelated to variables such pretreatment viral load, treatment method, and treatment period. [25].

The ability of the virus to replicate in these extrahepatic areas presents a potential danger of transmission to other tissues or individuals, even if the significance of discovering HCV-RNA in PBMCs and/or liver tissue in the absence of blood RNA positive is not entirely known. Raising awareness of occult HCV infection in clinical settings is crucial as a result [26].
It's yet unknown what persisting HCV- RNA in PBMCs means for DAA-treated individuals who have attained SVR. It is unclear if this discovery shows a slow reduction of the virus within tissues or if it signifies a genuine, long-term persistence of the virus in PBMCs, which could result in problems and/or subsequent relapses [27].
Regarding liver function tests shown in table (2), our results showed insignificant variance among both groups with regard to direct bilirubin, albumin, and AST. However, there was a significant increase in total TBil, ALP, and ALT in the post-treatment group in comparison with the control group. A previous study by Ali et al. (2020) confirmed that after DAA treatment, serum albumin, total bilirubin, and ALT levels significantly improved. This improvement was attributed to the fact that the eradication of infection with HCV can reverse hepatic function anomalies [28].
Our findings, as shown in table (2), showed significantly increased creatinine levels in the control group than in the post-treatment group, although results in both groups were within normal. Nevola et al. (2020) found that SVR patients illustrated a significant decline in serum creatinine concentration and a significant rise in eGFR. This means that DAAs After the virus is eradicated, liver function recovers and reduces the inflammation, which can enhance renal perfusion

and improve overall kidney function [29]. In contrast, the Chui et al. 2020 study has shown a defined renal function deterioration following DAA treatment. That was a novel result that had not been reported before [30].
Regarding platelet count Sayyar et al. (2019) found that platelet count significantly elevated in cases with HCV who attained SVR irrespective of the presence or absence of cirrhosis. [31]
Also, Ali et al. (2020) found that platelet count improved after DAA treatment. [28] In our present study, in table (3), there is a significant decrease in platelet count in the post-treatment group than the control group, despite both groups remaining within the normal range, and this could be attributed to several factors. After successful HCV eradication with DAA treatment, the liver and spleen undergo a recovery phase, which can affect platelet dynamics. In cases with chronic liver disease or cirrhosis, platelet production may remain suppressed due to residual liver dysfunction or impaired thrombopoietin production, even after viral clearance. Additionally, transient inflammation and the resolution of HCV-related immune responses post-treatment may contribute to fluctuations in platelet levels [32]. These factors could explain the observed decrease in platelet count in the post-treatment.
Our results illustrated a significant rise in AFP levels in the post-treatment group than the control group, although both groups remained within the normal range, see table (4). In contrast, Ali et al. found that AFP levels significantly decreased after DAA treatment, which confirms the ability of DAAs to improve liver function [28]. Furthermore, Yang et al. (2020) noted that increased serum alpha-fetoprotein (AFP) levels in cases with chronic HCV on direct-acting antiviral therapy (DAAs) can happen for a number of reasons. Persistent liver damage, such as cirrhosis, may continue to elevate AFP levels even after successful viral eradication. Additionally, AFP can be elevated in cases of hepatocellular carcinoma (HCC), which may develop in cases with advanced liver illness, even if the HCV has been cleared. Liver regeneration following DAA treatment, particularly in patients with cirrhosis, can also cause transient AFP increases, as AFP is associated with liver cell turnover. Finally, extrahepatic causes, such as tumors or other conditions, may also lead to elevated AFP levels, though these are less common in the context of HCV treatment. [33]

***Study Limitations**:* Being a *s*ingle center study and absence of liver biopsy may underestimate OCI prevalence. Also lack of long-term follow-up restricts insight into its clinical significance.

CONCLUSION

Taken together, our results illustrate that in Upper Egypt cases with chronic HCV who attain sustained virologic response following direct-acting antiviral therapy may nonetheless harbor occult HCV infection. This highlights that the persistence of viral RNA in PBMCs despite undetectable serum HCV RNA, emphasizing the need for dual testing in both serum and PBMCs during SVR validation. Identification of factors predicting OCI persistence post-DAA therapy remains a critical area for future research. Long-term observational studies with larger patient cohorts are essential to corroborate these findings and to inform clinical guidelines. Moreover, optimizing antiviral regimens, particularly with the latest generations of highly potent DAAs, may be necessary to prevent OCI. Finally, the potential requirement for retreatment in affected individuals warrants careful evaluation to ensure complete viral eradication.

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