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Age and Gender are Predictors for Occult Hepatitis C in Egyptian Sustained Responders to Directly Acting Antivirals

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ABSTRACT

Background: Occult HCV infection (OCI) describes detectable viral RNA in the hepatocytes and in peripheral blood mononuclear cells (PBMCs) in the absence of conventional markers for HCV. The present study aimed to detect the prevalence of occult hepatitis C virus infection in patients who achieved a sustained virologic response (SVR) to direct-acting antiviral agents (DAAs) and to outline predictors of OCI. **Patients and methods:** This multicenter cross-sectional study included 50 males and 50 females seeking treatment for chronic hepatitis C patients without liver cirrhosis who were treated with combination of sofosbuvir (SOF) and daclatasvir (DCV) with or without ribavirin for three months and achieved SVR as proved by negative PCR after 12 weeks of the end of treatment. Twelve weeks after the end of treatment, HCV RNA was tested in PBMCs to detect OCI in patients with SVR. Logistic regression was used to find out factors affecting the presence of OCI. ROC curve was used to evaluate the performance of different tests to predict OCI. **Results:** Of the 100 patients, 12 (12%) had detectable HCV RNA in PBMCs after achieving SVR12 denoting presence of occult HCV infection. Age ≤45 years and male gender were significant predictors for occurrence of OCI. However, direct bilirubin ≥0.40 mg/dL was a significant predictor with low diagnostic performance.

Conclusion: OCI can persist in sustained responders to DAA therapy and the risk of OCI is more prevalent in males younger than 45 years. Age and direct bilirubin can be possible predictors for OCI.

Keywords: Directly acting antivirals, sofosbuvir, daclatasvir, Hepatitis C, Occult hepatitis C, Ain Shams University.

INTRODUCTION

Hepatitis C Virus (HCV) infection is known to pose a serious public health problem all over the world; therefore, efforts have been made worldwide to eliminate HCV by 2030 [(1)]. In Egypt, the latest demographic health survey reported a seroprevalence of HCV of about 10% [(2)] and the Egyptian government has exerted enormous efforts to screen 49.6 million people and treat nearly 2.2 million [(3)]. In 2004, a new category of patients was described to have occult HCV infection (OCI) where they had persistently deranged liver functions despite being negative for anti-HCV antibodies and HCV RNA tested by polymerase chain reaction (PCR) [(4)]. OCI describes detectable viral RNA in the hepatocytes and in peripheral blood mononuclear cells (PBMCs) in the absence of conventional markers for HCV. It has also been described in asymptomatic carriers with normal liver enzymes and in patients who showed spontaneous or post antiviral clearance of infection [(5)]. OCI is best diagnosed by liver biopsy but due to the invasive nature of the procedure, HCV RNA can be alternatively tested in PBMCs which can detect 70% of cases [(6)]. After treatment, chronic hepatitis C cure is defined as sustained virologic response with negative results of HCV PCR in serum samples [(7)]. This was found to be associated with clinical and histological improvement even in patients with advanced fibrosis [(8)]. In OCI, although the hepatic injury is considered milder, an association with hepatic necroinflammation and fibrosis has been described and is thought to be related to the persistence of viral RNA in hepatocytes of patients treated for HCV [(9)]. Moreover, development of hepatocellular carcinoma (HCC) after treatment of HCV was linked to the presence of OCI in liver tissue [(10)] which can lead to induction of immunosenescence, T cell exhaustion, promotion of immune cell apoptosis and stimulation of carcinogenesis [(11)]. Accordingly, persistence of OCI in hepatocytes of patients treated for HCV may lead to residual histological abnormality and HCC [(12)], risk of reactivation of HCV on exposure to immunosuppression [(13)], risk of transmission of HCV by blood donors [(5)] or in hemodialysis centers [(14)].

The present study aimed to detect the prevalence of occult hepatitis C virus infection in patients who achieved a sustained virologic response (SVR) to direct-acting antiviral agents (DAAs) and to outline predictors of OCI.

PATIENTS AND METHODS

This multicenter cross-sectional study was carried out in Ain Shams University Hospital, Ahmed Maher Teaching hospital and Elgomhorya Teaching Hospital on 100 patients attending hepatitis C clinics over the period of 1 year, treated for chronic hepatitis C with sofosbuvir (400mg) plus daclatasvir (60mg) daily with or without ribavirin for 12 weeks according to NCCVH protocol [(15)].

Inclusion and exclusion criteria:

Participants included in our study were chronic hepatitis C patients without liver cirrhosis who were treated with combination of sofosbuvir (SOF) and daclatasvir (DCV) with or without ribavirin for three months and achieved SVR as proved by negative PCR after 12 weeks of the end of treatment.

Patients with liver cirrhosis, hepatitis B or HIV co-infection, chronic kidney disease, alcohol intake, history of HCC and patients on treatment with hepatotoxic medications were excluded from the study.

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Choice of treatment:

According to National committee to combat viral hepatitis (NCCVH) protocol [15], Peg-IFN treatment naive patients, and patients with total bilirubin ≤1.2 mg/dl, serum albumin ≥3.5 g/dl, INR ≤1.2 and platelet count ≥150.000/mm^3 were treated with sofosbuvir, daclatasvir without ribavirin for 12 weeks. All patients in our study were non-cirrhotic, however some patients were treated with sofosbuvir, daclatasvir and ribavirin for 12 weeks if they were difficult to treat according to the national protocol [15] with any of the following criteria; Peg-IFN experienced patients, patients with total bilirubin >1.2 mg/dl, serum albumin <3.5 g/dl, INR >1.2 and platelet count <150.000/mm^3.

Selection of patients, baseline and follow up investigations:

We randomly chose 50 males and 50 females from the patients attending the clinics. History, clinical examination, abdominal ultrasonography and baseline lab workup was done before start of treatment. Pre-treatment laboratory tests included complete blood count (CBC), HCV antibodies, hepatitis B surface antigen (HBsAg), hepatitis B core antibody (HBcAb), HCV RT-PCR at time of diagnosis, alanine transaminase (ALT), aspartate transaminase (AST), serum bilirubin (direct and indirect), serum albumin and prothrombin time, α fetoprotein, serum creatinine, fasting blood sugar and glycated hemoglobin (HbA1c). Twelve weeks after the end of treatment, post-treatment tests were performed. They included CBC, ALT, AST, bilirubin (direct and indirect), serum albumin and prothrombin time, α fetoprotein, serum creatinine, HCV RNA by RT-PCR and HCV RNA in PBMCs by RT-PCR.

Serum sampling and testing

Serum samples were collected to detect HCV RNA via RT-PCR:

Samples of peripheral blood from each patient were collected into EDTA tubes (1.5 ml) and blood samples were processed within a few hours of collection, PBMCs were separated from the blood using standard Ficoll–Hypaque density gradient centrifugation method [16] as follows: Two mL of anticoagulant-treated blood and an equal volume of balanced salt solution (final volume 4 mL) were mixed. After withdrawal of Ficoll-Paque media using a pipette, 3 mL were added to the centrifuge tube. Then 4 mL of the diluted blood sample were layered onto the Ficoll-Paque media solution without mixing, and centrifuged at 400 g for 30 to 40 min at 18 ºC to 20 °C. The upper layer containing plasma and platelets was drawn off using a sterile pipette, leaving the mononuclear cell layer undisturbed at the interface and the layer of mononuclear cells was moved to a sterile centrifuge tube using a sterile pipette. After washing the cell isolate, isolation of PBMCs was done. Plasma RNA extraction was done using QIAamp® RNA Blood mini kit (QIAGEN cat# 52304) according to the manufacturer's instructions. Total RNA was extracted from PBMCs using the miRNeasy® Mini Kit (QIAGEN cat# 217004) according to the manufacturer's instructions.

Ethical consent:

Patients signed an informed consent after full explanation of the study procedures and their right to withdraw from the study anytime. The study had been performed in accordance with the ethical standards and has been approved by Ain Shams University Ethical Committee. The study protocol conforms to the ethical guidelines of the 2013 Declaration of Helsinki.

Statistical analysis

Coding, and statistical analysis was done using IBM SPSS statistics (Statistical Package for Social Sciences) software version 28.0, IBM Corp., Chicago, USA, 2021. Quantitative data was described as mean±SD (standard deviation), then compared using independent student's t-test after being tested for normality using Shapiro-Wilk test. Qualitative data was described as numbers and percentages, then compared using Chi-square test. Logistic regression was used to find out factors affecting the presence of occult HCV. ROC curve was used to evaluate the performance of different tests to predict occult HCV. P-value <0.050 was considered significant, otherwise it was considered insignificant.

Diagnostic characteristics were calculated as follows:

- Sensitivity = (True positive test / Total positive) x 100.
- Specificity = (True negative test / Total negative) x 100.
- Diagnostic accuracy = ([True positive test + True negative test] / Total cases) x 100.
- Youden's index = sensitivity + specificity – 1.
- Predictive positive value = (True positive test / Total positive test) x 100.
- Predictive negative value = (True negative test / Total negative test) x 100.
- LR+ = (sensitivity/ 1-specificity).
- LR- = (1- sensitivity / specificity)

RESULTS

We randomly selected 50 males and 50 females seeking treatment for HCV. The mean age of participants was 48.9 (SD 15) years. Pre-treatment initial viral RNA ranged from 0.008–4.0x10^6/mL with a mean of 2.85 x10^6/mL (SD 1.7). All of the studied patients achieved SVR12, where serum HCV RT-PCR was negative 3 months after the end of treatment.

Of the 100 patients, 12 (12%) had detectable HCV RNA in PBMCs after achieving SVR12 denoting presence of occult HCV infection. Patients with OCI were predominantly males and were significantly younger than non-OCI patients; however, both groups showed comparable levels of pretreatment HCV viremia. Occult HCV cases had significantly higher total& direct bilirubin, AST and ALT while no statistically significant difference was found in other laboratory tests **(Table 1).**

Table (1): Collective demographic data and pretreatment laboratory tests as opposed to post-treatment tests subdivided according to occult HCV status.

Variables		All cases (N=100)	Occult HCV		P-value
			Positive (N=12)	Negative (N=88)	
Age (years)		48.9±15	34.2±8.3	50.5±14.7	**^0.001***
Sex (n, %)	**Male**	50 (50%)	8 (80%)	42 (46.7%)	**#<0.001***
	Female	50 (50%)	2 (20%)	48 (53.3%)	
Initial RNA (x10^6/mL)		2.9±1.7	3.2±1.6	2.8±1.7	^0.467
Hemoglobin (gm/dL)		12.3±1.4	11.6±1.5	12.4±1.4	^0.110
WBC (x103/mL)		7.3±1.8	7.5±1.4	7.3±1.8	^0.712
Platelets (x103/mL)		220.3±51.6	213.4±49.5	221±47	^0.842
FBG (gm/dL)		93.9±8.4	91.2±5.6	94.2±8.6	^0.151
HbA1c (%)		5.5±0.4	5.3±0.4	5.6±0.4	^0.149
Creatinine (mg/dL)		0.92±0.23	0.85±0.14	0.93±0.24	^0.322
Direct bilirubin		0.28±0.01	0.39±0.07	0.27±0.01	**^<0.001***
Total bilirubin		0.76±0.14	0.92±0.29	0.74±0.14	**^0.035***
AST (IU/L)		30.2±7.2	39.0±9.1	29.2±7.4	**^<0.001***
ALT (IU/L)		28.8±6.2	35.1±8.4	28.1±6.5	**^0.020***
Albumin (gm/dL)		4.1±0.2	4.1±0.2	4.1±0.2	^0.469
AFP (ng/mL)		4.0±0.8	3.1±0.7	4.1±0.9	^0.422
PC		95.9±5.6	92.9±8.1	96.2±5.2	^0.236
INR		1.02±0.06	1.05±0.06	1.02±0.06	^0.171

Data presented as Mean±SD unless mentioned otherwise. ^Independent t-test. #Chi square test. *Significant (<0.05)

By assessing the diagnostic performance of the studied factors for prediction of occult HCV, age of the patient ≤45 years and direct bilirubin ≥0.40 mg/dL were significant predictors of HCV with moderate and low diagnostic performance respectively **(Table 2 and Figure 1).**

Table (2): Diagnostic performance of significant variables and liver enzymes in prediction of occult HCV

Predictive Factors	AUC	SE	P-value	95% CI	Cut off
Age	0.820	0.047	**0.001***	0.727−0.913	≤45 years
Direct bilirubin	0.696	0.069	**0.043***	0.496−0.869	≥0.40 mg/dL
Total bilirubin	0.562	0.069	0.520	0.680−0.916	NA
AST	0.509	0.055	0.927	0.664−0.936	NA
ALT	0.640	0.060	0.148	0.508−0.864	NA

AUC: Area under curve. SE: Standard error. CI: Confidence interval. NA: Not applicable. *significant

Age ≤45 years had highest sensitivity (100%) for prediction of occult HCV while specificity and diagnostic accuracy of direct bilirubin ≥0.40 mg/dL were non-remarkably higher. Male gender has also shown high sensitivity (80%) for prediction of OCI **(Table 3).**

Table (3): Diagnostic characteristics of suggested cutoff points in prediction of occult HCV.

Characteristics	Sex (Males)	Age (≤45.0 years)	Direct bilirubin (≥0.40 mg/dL)
Sensitivity	80% (44.4%–97.5%)	100% (69.2%–100%)	70% (34.8%–93.3%)
Specificity	53.3% (42.5%–63.9%)	68.9% (58.3%–78.2%)	73.3% (63.0%–82.1%)
DA	56% (45.7%–65.9%)	72% (62.1%–80.5%)	73% (63.2%–81.4%)
Youden's index	33.3% (6.5%–60.2%)	68.9% (59.3%–78.5%)	43.3% (13.5%–73.2%)
PPV	16% (7.2%–29.1%)	26.3% (13.4%–43.1%)	22.6% (9.6%–41.1%)
NPV	96% (86.3%–99.5%)	100% (94.2%–100.0%)	95.7% (87.8%–99.1%)
LR+	1.71 (1.17–2.51)	3.21 (2.36–4.37)	2.63 (1.54–4.46)
LR-	0.38 (0.11–1.31)	0.00 (0.00–0.00)	0.41 (0.16–1.06)

Data were presented as value (95% CI: Confidence interval). DA: Diagnostic accuracy. PPV: Positive Predictive value. NPV: Negative Predictive value. LR+: Positive likelihood ratio. LR-: Negative likelihood ratio.

Logistic regression analysis of the significant variables was done to study predictors of OCI in treated patients; with a Confidence interval of 95%, age and male gender were significant predictors for occurrence of OCI with odds ratio of 7.742 (1.20–50.01) and 35.941 (5.93–217.77) and a p-Value of 0.032 and <0.001 respectively **(Table 4).**

Table (4): Logistic regression for factors affecting having occult HCV

Predictive Factors	B	SE	P-value	OR (95% CI)
Sex (Male)	3.58	0.92	**<0.001***	**35.94 (5.93–217.77)**
Age ≤45.0 years	2.05	0.95	**0.032***	**7.74 (1.20–50.01)**
Constant	-5.12	1.10	**<0.001***	---

β: Regression coefficient, SE: Standard error, OR: Odds ratio, CI: Confidence interval, *significant

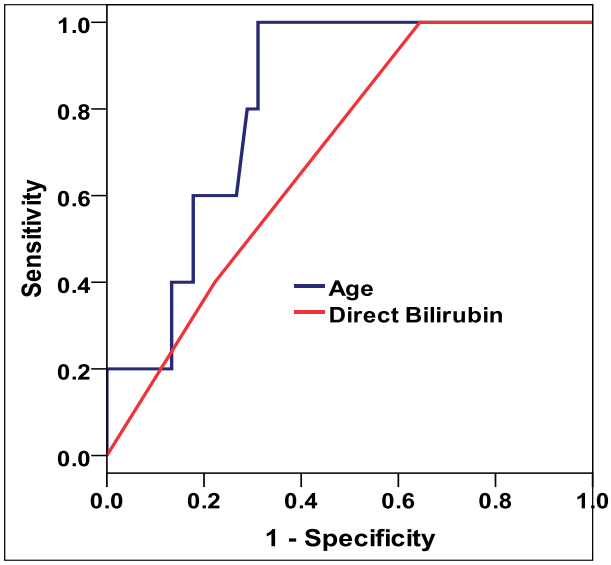

Figure (1): ROC curve for the significant variables in prediction of occult HCV

DISCUSSION

Occult HCV infection is a novel challenge to patients treated with directly acting antivirals. Although viral clearance by serum PCR used to be the target of therapy, studies show that viral RNA can persist in the liver tissue in a proportion of patients [17] and lead to progressive hepatocellular injury [18]**.** Detection of HCV RNA in PBMCs is an alternative non-invasive method to test for OCI [6]**.**

Several Egyptian studies were conducted to detect the prevalence of occult HCV in different populations with numbers starting from 4% [19] to 20% [20] and 40.7% [21]. A metanalysis of different Middle Eastern studies showed that Egypt had an estimated prevalence ranging from 3.33-44.44% in different populations [22]**.**

Many studies reported persistence of OCI after serum viral clearance. In a study of 25 patients who spontaneously cleared HCV infection, 12% of cases showed detectable RNA in PBMCs denoting OCI and one patient developed overt HCV with detectable serum PCR after 18 months of follow up [23]**.** Additionally, in 2002, **McHutchison and colleagues** [24] found that around 4% (7/170) of patients who achieved SVR on interferon-based therapy had detectable intrahepatic viral RNA 24 weeks after treatment. A smaller study on only 25 Egyptian patients revealed OCI in eight patients (32%) after 48 weeks of treatment with interferon and ribavirin [25].

In this study, we tested for OCI in patients treated with DAAs. HCV RNA was detected in PBMCs of 12 out of 100 (12%) chronic hepatitis C patients who achieved SVR 12 after being treated with the combination of sofosbuvir (SOF) and daclatasvir (DAC) with or without ribavirin (RBV) for 3 months.

Similar studies reported a considerably higher prevalence of OCI in sustained responders to DAAs who received SOF 400 mg and DAC 60 mg with and without weight-based RBV daily for 12 weeks. By testing HCV PCR in PBMCs, OCI rates of 22.7% (49 out of 215 patients) and 25% (10 out of 40 patients) were reported by **Abd Alla *et al.*** [26] **and Abu Khadr *et al.*** [27] respectively.

According to presence of OCI, we divided the patients into two groups; OCI negative patients and OCI positive patients. We found that male gender and age younger than 45 years were significant predictors of occult HCV. Likewise, **Mekky *et al.*** [28] found that younger age was significantly related to the presence of OCI in sustained responders to treatment with sofosbuvir and daclatasvir with or without ribavirin.

OCI patients had similar age range in other studies [29,30]**.** The Pakistani study done by **Idrees and colleagues** [29] stated that chances of OCI increased in male patients and age above 30 years. Occult hepatitis C was found to be more predominant in males in a number of other studies [27,30]. Additionally, in a study by **Habeeb *et al.*** [31]**,** male sex was a predictor of OCI in patients with anti-HCV antibodies.

A recent study examined the persistence of OCI in patients after 8 versus 12 weeks of DAA therapy and found that patients younger than 52 years had early viral clearance unlike older patients who couldn't clear the virus in 8 weeks of therapy [17]**.**

In the current study, patients who tested positive for OCI after antiviral treatment were found to have significantly higher ALT, AST and bilirubin in patients negative for OCI. Direct bilirubin at a level ≥0.40 mg/dL was a statistically significant predictor of HCV with low diagnostic performance. Due to the small number of patients with OCI in the study, further studies with larger number of patients are needed to confirm this finding and accurately calculate the cut-off value for prediction of OCI.

Presence of OCI was associated with higher fibrosis score, abnormal liver function tests, elevated

ALT and serum bilirubin level [28]. Furthermore, higher aminotransferase levels were found in liver transplant recipients with OCI after achieving SVR on DAA therapy [32]. However, other studies revealed no significant differences in the prevalence of occult HCV infection between patients with normal or high liver enzymes[18].

Another study found that there were statistically significant differences between the positive and negative patients with OCI regarding direct bilirubin but not indirect bilirubin [27].

This higher level of bilirubin and hepatic transaminases in OCI patients can be explained by the presence of a degree of microinflammation in the infected hepatocytes as was evident by research [18]. It was noticed that highest FIB-4 score was seen in patients with relapsing HCV (positive in PBMC but negative serum RNA PCR) than in patients with SVR (0.03373) [26].

By comparing the liver biopsies of chronic hepatitis C patients and patients with OCI, studies [33, 34] found that although chronic hepatitis C biopsies had more necroinflammation and fibrosis, patients of both groups had similar frequencies of liver cirrhosis.

The persistence of a hepatocellular inflammatory state even after 3 months of DAAs should make us question the proper duration of therapy to eliminate OCI [25] or even consider changes in the drug regimen, as it was suggested that adding ribavirin might help in clearing OCI [26]. This might also shed the light upon using proper methods to ensure the viral eradication from hepatocytes and the possible utilization of viral RNA testing in PBMCs to confirm SVR after treatment. **Kamhawy and colleagues** [17] suggested that easy to treat patients can take a shorter course of treatment of 8 weeks provided that they confirm their cure by testing for viral RNA in PBMCs.

Patients with detectable OCI after treatment of HCV should also undergo strict follow-up due to their higher risk of relapse to overt HCV [25]. On a molecular level, the stress implemented by antiviral treatment on intracellular HCV-RNA strands is associated with disappearance of the antisense strand interrupting the virus life cycle [35]. Therefore, persisting intra-PBMCs strands or the presence of non-responding antisense strand to antiviral therapy can predict non-response or relapse after treatment [5].

The main limitation of this study is the short duration of follow-up and relatively small sample size. Further studies on different populations and larger numbers of participants are warranted to validate our findings.

In conclusion, occult hepatitis C can persist in sustained responders to DAA therapy for HCV and the risk of OCI is more prevalent in males younger than 45 years where age and level of direct bilirubin can be possible predictors for OCI. Predicting and testing for OCI will protect patients from developing progressive hepatic injury, decrease viral transmission in the community and ensure safety of immunosuppressed individuals from viral reactivation. Hence, revision of diagnostic and therapeutic interventions for chronic hepatitis C is strongly advised as explained.

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