EGYPTIAN PHARMACEUTICAL JOURNAL EPJ
Original Article 171

Detection of occult hepatitis C virus infection among Egyptian patients with hepatocellular carcinoma who achieved sustained virologic response to direct-acting antiviral therapy

Fatma E.M.A. Kassem[a], Emtethal E. El-Kholy[b], Amira H. Mohamed[a], Abeer M. Abdul-Mohaymen[b], Asmaa I. Gomaa[c], Ayat S.M. Hassan[a]

[a]Department of Electron Microscopy, Theodore Bilharz Research Institute, Giza, [b]Department of Clinical Pathology, Faculty of Medicine for Girls, Al-Azhar University, Cairo, [c]Department of Tropical Medicine, National Liver Institute, Menoufia University, Menoufia, Egypt

Correspondence to Ayat S.M. Hassan, MD, Theodor Bilharz Research Institute, El-Nile Street, Warrak El-Hader, P.O. Box 30, Giza 12411, Egypt. Tel: +20 100 516 4954, 0023 540 1019 – 0023 540 9670; Facsimile numbers: +00235408125; e-mail: drayatsalah@yahoo.com

Received: 19 August 2024
Revised: 7 September 2024
Accepted: 8 September 2024
Published: 28 January 2025

Egyptian Pharmaceutical Journal 2025, 24:171–180

Background
Occult hepatitis C virus infection (OCI) is defined as the presence of hepatitis C virus (HCV) RNA in nonserum reservoirs without any detectable HCV-RNA in serum by standard assays. The association between OCI and hepatocellular carcinoma (HCC) is controversial. Direct-acting antiviral (DAA) therapy effectively controls HCV infection and proves sustained virological response (SVR). However, HCC after DAA therapy is debatable.
Objective
In this study, we tried to detect OCI in HCC Egyptian patients who achieved SVR post-DAA therapy for possible correlation and prediction for HCC.
Patients and methods
A cross–sectional study included seventy HCC Egyptian patients who achieved SVR post sofosbuvir/daclatasvir (SOF/DACLA) +/–ribavirin therapy. HCV-RNA was detected in peripheral blood mononuclear cells (PBMCs) by RT-PCR. Immune transmission electron microscopy (TEM) was done to study OCI in PBMCs using specific anti-HCV antibodies.
Results and conclusion
TEM examination revealed positive OCI in PBMCs of all patients (70/70) with 3 stages of grading reflecting viral particle load in PBMCs, with 68.6% of the patients having a marked OCI grade, while PCR only detected 5.7% (4/70) of OCI. Nearly 67.1% of the cases received dual anti-HCV therapy (sofosbuvir/daclatasvir). Ribavirin-inclusive DAA treatment shows a higher grade of OCI (P=0.02). The average duration from SVR to HCC development was 29.8±13.4 months. In conclusion, DAA drugs effectively eliminate HCV in serum, but OCI is still considered a risk for developing HCC, recommending a re-definition for SVR through detecting HCV-RNA in serum, PBMCs, and TEM studies. Our study is the first to provide electron microscopy as a sensitive tool for OCI detection with established superiority over the PCR technique.

Keywords:
direct acting antiviral drugs, hepatocellular carcinoma, immune electron microscopy, occult hepatitis C infection

Egypt Pharmaceut J 24:171–180

1687-4315

Introduction

Hepatitis C virus (HCV) has a substantial role in hepatocellular carcinoma (HCC) and chronic liver disease [1]. Direct-acting antiviral (DAA) therapy effectively eliminates HCV-RNA from serum, but is still present as an occult hepatitis C virus infection (OCI) in liver tissue and/or peripheral blood mononuclear cells (PBMCs). OCI has been considered a new pathological entity described in patients with chronic liver disease of unknown etiology, and it is characterized by the presence of HCV-RNA in liver cells and PBMCs in the absence of both HCV-RNA and anti-HCV antibodies in serum by standard assays [2]. It might contribute to resistance to anti-viral treatment and may be the cause of viral persistence and relapse after achieving the sustained virological response (SVR) [3]. Moreover, it is also thought to be the primary reason for reinfection following liver transplantation. So the risk of re-infection in these patients could be prevented by close monitoring and treatment [4]. HCV infection treatment has made considerable progress with a high cure rate since the release of DAA drugs without the use of interferon in 2014. However, there was a percentage (1–15%) of patients who failed to complete elimination of infection [5]. It has been observed that interferon-induced viral

DOI: 10.21608/epj.2025.407585

clearance significantly lowers the risk of HCC incidence. On the other hand, after SVR using DAA, there was a documented rise in newly developed and recurrent HCC cases [6]. Since anti-HCV medication cannot completely prevent hepatocarcinogenesis, consideration should be given to the several variables that are linked to the disease's development, including OCI. The association between OCI and HCC is controversial; while many studies indicated the contribution of OCI in the development of HCC, other studies denied the significance of the impact of OCI [7,8]. Several methods for OCI diagnosis are reported, the standard one is detecting HCV RNA in liver tissue, which is an invasive procedure. Other noninvasive methods, such as detecting HCV RNA in PBMCs or ultra-centrifuged serum, have successfully diagnosed more than 60% of OCI cases. Combining these techniques improves OCI diagnosis in over 90% of cases [2].

Virology research is closely linked to the advancement of microscopy techniques. Transmission electron microscopy (TEM) is crucial for detecting virus particles, which are typically too tiny for traditional light microscopy. HCV viral particles from infected PBMC could be visualized by immune electron microscopy through labeling with specific anti-viral immunoglobulin [9]. In this respect, this study aims to detect occult HCV RNA in Egyptian patients with post-HCV hepatocellular carcinoma who achieved SVR after DAAs therapy for possible correlation and prediction of HCC.

Patients and methods

Study design and patients' criteria

Seventy HCC Patients with sustained viral response following DAA regimens (Sofosbuvir (SOF) 400 mg + Daclatasvir (DACLA) 60 mg once daily) ±ribavirin (RIB) (1200 mg for patients up to 75 kg body weight and 1400 mg for patients exceeding 75 kg) for 12 weeks were collected from different Egyptian centers dedicated for HCV management in a cross-sectional study. All patients were primarily diagnosed as HCV genotype 4. HCV genotyping by HybProbe probes with the lightcycler carousel-based system was used. Patients were subjected to a full history, clinical examination, triphasic computed tomography abdomen, and laboratory testing (complete blood picture, liver functions tests, and alpha fetoprotein level).

Collection of samples

Under completely aseptic conditions, ten milliliters of venous blood were obtained from participants. The blood was then divided into four tubes, as follows: 8 ml were collected in three sterile EDTA-containing tubes (Vacutainer; BD Biosciences) for PBMC separation for further HCV-RNA detection by real-time PCR and TEM study. A 2 ml were collected in plain tubes (Vacutainer; BD Biosciences) for the detection of serum HCV by PCR.

Peripheral blood mononuclear cells (PBMCs) preparation

The PBMCs were isolated from blood samples by density gradient separation on Ficoll-hypaque (FH) liquid (Sigma, USA). In a 15-ml conical tube (Falcon; NJ), 4 ml of blood was carefully layered over 3 ml of FH and then centrifuged at 400 g for 20 min. The buffy coat at the interface between the Ficoll solution and plasma was then collected and washed twice in phosphate-buffered saline (PBS) (pH = 7.2).

Hepatitis C virus (HCV)-RNA detection by PCR in serum and peripheral blood mononuclear cells (PBMCs)

Real-time PCR was performed first on patients' serum samples, and HCV-RNA negative cases were subjected to further assessment of occult HCV in PBMCs by RT-PCR. The viral RNA extraction from PBMCs and plasma was done following the procedure of the Promega magnetic bead RNA extraction kit (Promega Co., USA). Then HCV-RNA PCR was carried out using Abbott real time detection kit for HCV (Abbott Inc, Germany) on ABI 7500 real time PCR instrument (Applied Biosystems, USA).

Conventional electron microscopy

The PBMCs were immediately fixed in buffered 2.5% glutaraldehyde in PBS (BDH chemicals, Poole, England) for 30 min at room temperature and postfixed in 1% OsO_4 (BDH chemicals, Poole, England) in PBS for 30 min at 4°C. This was followed by dehydration in ascending grades of ethyl alcohol (50, 70, 90, and 100%) at a 5 min gap, substitution in epon/ethanol mixture (1 : 1) for 1 h, infiltration in three overnight baths of epoxy resin at room temperature (Epon 812 kit, TAAB Laboratories Equipment Ltd., Aldermaston, Berkshire, UK), and finally embedded in Epon/DMP30 and polymerized at 37°C for 12 h and at 60°C for 2 days. Semi-thin sections were prepared using an ultramicrotome (Leica, Vienna, Austria) and stained with methylene blue and Azur II for detection of the region of choice for performing ultrathin sections. After that, the ultrathin sections cut at a thickness of 70 nm using an Ultracut R ultramicrotome (Leica, Vienna, Austria), were mounted on copper grid mesh. And then

contrasted with uranyl acetate and lead citrate before being examined under a Philips EM 208S transmission electron microscope TEM (Philips Optics, Eindhoven, Netherlands).

Immune-electron microscopy

Serial unstained ultrathin sections were mounted on copper grid mesh and then processed for post-embedding immune peroxidase labeling technique (indirect technique) to detect HCV in resin-embedded PBMCs using monoclonal anti-HCV antibody [10]. Deosmification was done by incubation of the grids in 0.5% periodic acid for 4 min at room temperature, followed by blocking of endogenous peroxidase activity for 5 min in 2% bovine serum albumin, then labeled overnight at 4°C with a primary anti-HCV (NS4B region) antibody # ab13832 (abcam, England). Then it was washed and incubated with biotinylated goat anti-mouse IgG for 30 min, followed by washing and incubation with streptavidin peroxidase (Horseradish peroxidase detection kit # ab64261, Abcam, England) for 10 min, followed by washing and incubating the sample with DAB substrate-chromogen for 10 minutes. Both positive and negative controls (supplied with the kit) were used to validate our results.

Statistical analysis

Data were analyzed with SPSS Inc.'s version 28.0.1.1 (IBM Corp., Armonk, New York, USA). Qualitative data was represented as frequency and percentages. Quantitative data were expressed as mean± SD. Determination of the data's normality was done using the Kolmogorov–Smirnov single-sample test. Independent *t*-test or Mann–Whitney *U* test were used to compare quantitative data between groups with parametric distribution or nonparametric distribution, respectively. Pearson χ^2 test and likelihood-ratio χ^2 was used where appropriate. The influence of variables on duration from SVR to HCC development was analyzed using Pearson's correlation. The *P* value was considered significant at *P* value less than 0.05.

Results

Sociodemographic and disease characteristics

The study included 70 HCC cases, of which 60 (85.7%) were males; the average age of the cases was 59.9±6.0 years. Nearly 2/3 of the cases (67.1%) received dual anti-HCV therapy (SOF+ DACLA). Four out of 70 (5.7%) cases were positive for HCV by PBMNCs PCR (all of them were marked grade for viral load by TEM); however, all cases (100%) were positive by TEM. The average months from SVR to HCC development were 29.8±13.4 months. More than two-thirds (68.6%) of the cases had severe OCI grade in PBMNCs by TEM examination, and 68.6% had right hepatic focal lesions (Table 1).

Transmission electron microscopic examination of occult hepatitis C virus (OCI) in peripheral blood mononuclear cells (PBMCs)

Semi-quantitative analysis of OCI in PBMCs by conventional and immune-electron microscopic examination revealed positive OCI in PBMCs of all (100%) patients ($N=70$) with three stages of grading reflecting viral particle load in mononuclear cells, with more than two-thirds (68.6%) of the cases having marked OCI grade by TEM examination (Table 1), Figs 1–3 as follows:

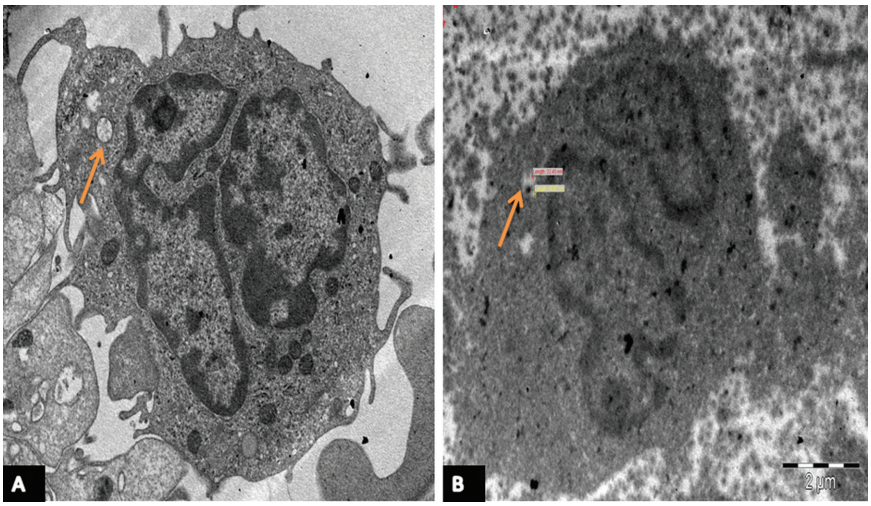
Grade I (mild): Few mononuclear cells showed few numbers of vesicles with minimal virus-like particles (VLP) in conventional electron microscopy. Immune

Table 1 Sociodemographic and disease characteristics among the studied group

Characteristics	Total (*n*=70)
Age (years) Mean±SD	59.9±6.0
Sex	
Female	10 (14.3)
Male	60 (85.7)
Anti-HCV treatment	
Double (SOF/DACLA)	47 (67.1)
Triple (SOF/DACLA/RIB)	23 (32.9)
Duration from SVR to HCC development (months) Mean±SD	29.8±13.4
OCI IN PBMCs by PCR	
Negative	66 (94.3)
Positive	4 (5.7)
OCI IN PBMCs by TEM	
Positive	70 (100)
Negative	0
OCI grade by TEM	
Mild	2 (2.9)
Moderate	20 (28.6)
Marked	48 (68.6)
Site of hepatic focal lesion*	
Left	14 (20.0)
Right	48 (68.6)
Left+ Right	8 (11.4)
Number of hepatic focal lesions	
Single	47 (67.1)
Multiple	23 (32.9)

*One case may have more than one site. Quantitative data was expressed as mean±standard deviation (SD). Qualitative data was represented as frequency and percentages. DACLA, daclatasvir; HCC, hepatocellular carcinoma; OCI, occult HCV infection; PBMCs, peripheral blood mononuclear cells; RIB, ribavirin; SD, standard deviation; SOF, sofosbuvir; SVR, sustained virologic response; TEM, transmission electron microscopy.

Figure 1



Transmission electron microscopy (TEM) micrograph of peripheral blood mononuclear cells (PBMCs) showing vacuoles containing viral – like particles (VLP) (Mild grade of occult hepatitis C infection (OCI) (a): Conventional electron micrograph reveals a moderate amount of cytoplasm and intracytoplasmic vacuoles which contain viral – like particles (VLP), magnification 8900x, (b): Immune electron micrograph showing VLP positivity (arrow) of grade I by TEM, magnification 5600x.

electron microscopy confirmed VLP positivity, as shown in Fig. 1.

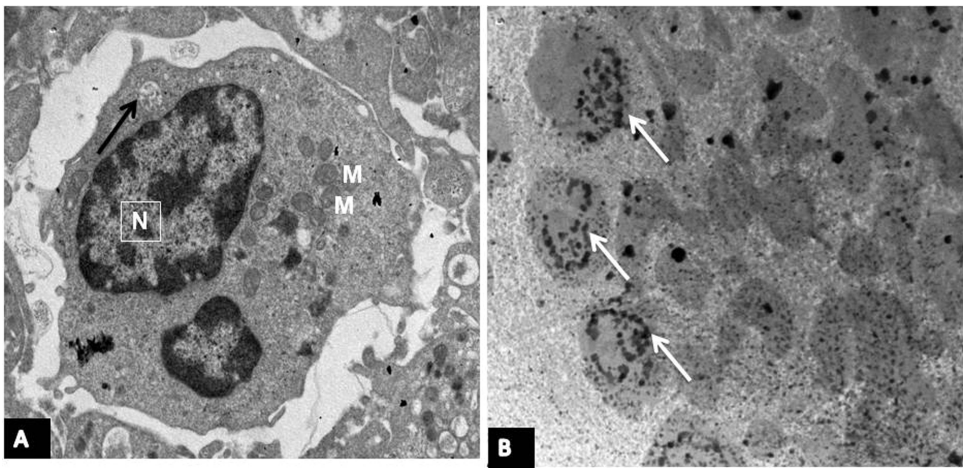
Grade II (moderate): Most mononuclear cells showed a moderate number of vesicles with VLP in conventional electron microscopy. Immune electron microscopy confirmed VLP with a peri-nuclear pattern of positivity, as shown in Fig. 2.

Grade III (marked): All mononuclear cells showed marked numbers of vesicles with marked VLP in conventional electron microscopy. Immune electron microscopy showed a diffuse pattern of positivity, as shown in Fig. 3.

Hepatitis C virus (HCV)-RNA by PCR in serum and peripheral blood mononuclear cells (PBMCs)

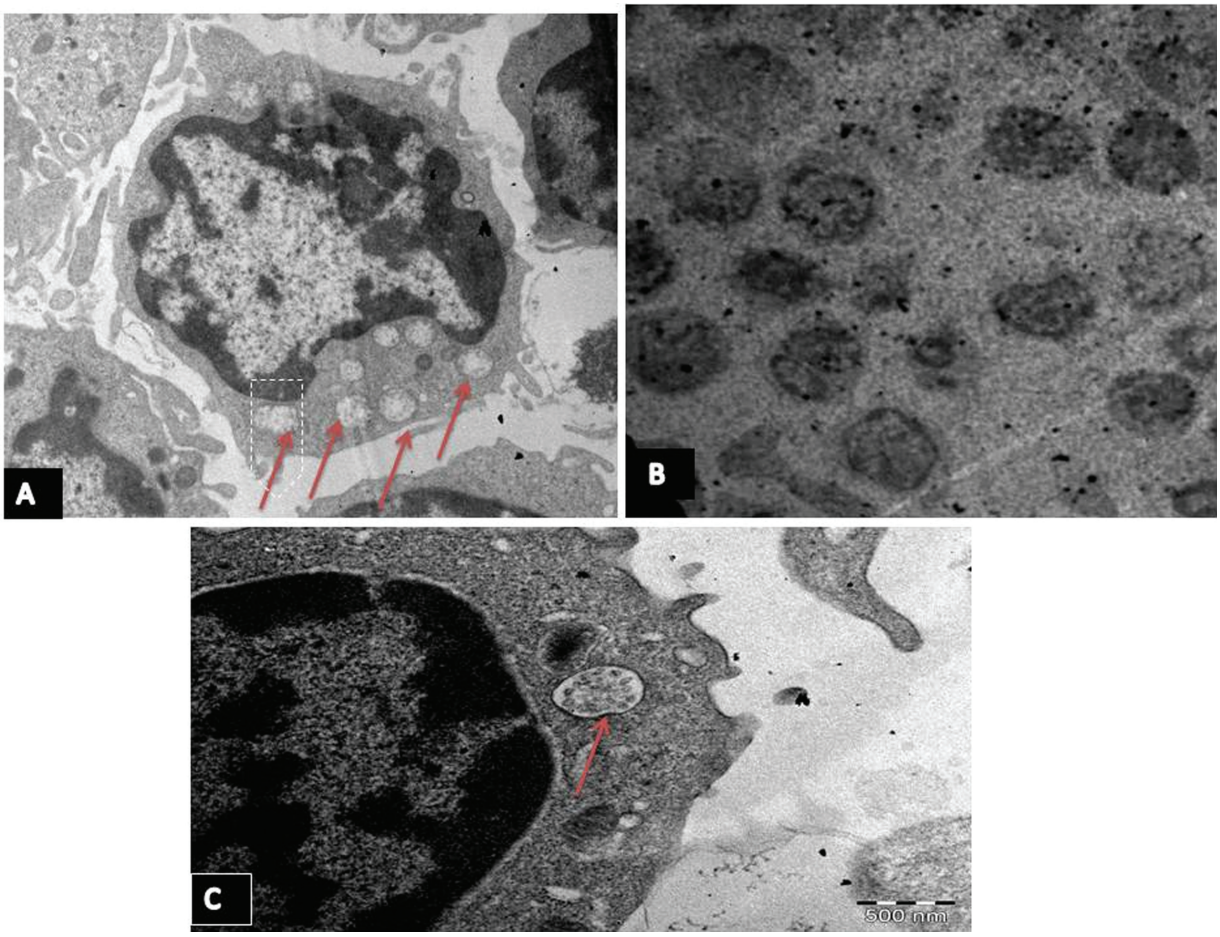
Serum HCV-RNA detection by PCR revealed negativity in all enrolled patients (*N*=70) denoting SVR. Four out of the 70 (5.7%) HCC patients showed positive OCI in PBMCs by PCR compared with 70 (100%) patients who showed positive OCI by EM, as shown in Table 1. This could denote that EM is more sensitive to the detection of OCI than PCR in PBMCs.

Figure 2



Transmission electron microscopy (TEM) micrograph of peripheral blood mononuclear cells (PBMCs) shows moderate grade of occult hepatitis C infection (OCI) (a): Conventional electron micrograph showing vacuole containing viral – like particles (VLP) (black arrow), N: Nucleus, M: Mitochondria. Magnification 8900x, (b): Immune electron micrograph showing a perinuclear pattern of grade II by transmission electron microscopy (TEM) (white arrows), magnification 2800x.

Figure 3



Transmission electron microscopy (TEM) micrograph of peripheral blood mononuclear cells (PBMCs) shows marked grade of occult hepatitis C infection (OCI) (a & c): Conventional electron micrograph showing vacuoles containing viral-like particles (VLP) (arrow), magnification 8900x, (b): immune electron micrograph showing a diffuse pattern of grade III by transmission electron microscopy (TEM), magnification 1100x.

Relation between sociodemographic, disease characteristics, and occult hepatitis C infection (OCI) grade in peripheral blood mononuclear cells (PBMCs) by transmission electron microscopy (TEM)

Statistical comparison between patients with different grades of OCI in PBMNCs by TEM and the type of antiviral drugs used was done, showing that there was a significantly higher proportion of marked OCI grade among cases who received triple therapy (SOF +DACLA+ RIB) (87% vs. 59.6%, P=0.020). So RIB use with DAAs has no role, neither in decreasing viral load intracellularly nor in the prevention of the development of severe OCI. In addition, there was a significantly higher proportion of severe OCI grades among cases who had right lobe lesions (75% versus 25%, P=0.020). However, no statistically significant difference between age, gender, duration from SVR till the development of HCC, number of lesions, and OCI grade severity by TEM could be detected (Table 2).

Association between occult hepatitis C infection (OCI) grades in peripheral blood mononuclear cells (PBMCs) by transmission electron microscopy (TEM) and laboratory data of the patients

On comparing laboratory data of the patients within the different grades of OCI in PBMNCs by TEM, there was a significantly lower total leucocytic count (TLC) in marked OCI grades compared with mild and moderate grades (P=0.01); however, there was no statistically significant relation between platelet count, hemoglobin level, INR, prothrombin concentration, urea, creatinine, direct bilirubin, total bilirubin, albumin, alpha fetoprotein, ALT, AST and the grade of OCI (P>0.05) (Table 3).

Association of laboratory data and the duration (months) from sustained virological response (SVR) till the development of hepatocellular carcinoma (HCC)

A significant positive correlation with the platelet count of the patients was detected (P=0.032); denoting that there is an association between

Table 2 Relation between Sociodemographic, disease characteristics, and occult hepatitis C infection (OCI) grade severity in peripheral blood mononuclear cells (PBMCs) by transmission electron microscopy (TEM)

		OCI grades By TEM		
Characteristics	n	Mild and moderate (*n*=22)	Marked (*n*=48)	*P* value
Age (years) Mean±SD	70	57.5±6.8	61±5.4	0.060
Sex				
Female	10	1 (10)	9 (90)	0.115
Male	60	21 (35)	39 (65)	
HCV treatment				
Double (SOF/DACLA)	47	19 (40.4)	28 (59.6)	0.020
Triple (SOF/DACLA/RIB)	23	3 (13)	20 (87)	
Site of hepatic focal lesion				
Left	22	10 (45.5)	12 (54.5)	0.020
Right	56	14 (25)	42 (75)	
Number of hepatic focal lesions				
Single	47	15 (31.9)	32 (68.1)	0.900
Multiple	23	7 (30.4)	16 (69.6)	

Quantitative data (age) was expressed as mean±standard deviation (SD). Other qualitative data was represented as frequency and percentages. Independent *t*-test was used to compare quantitative data between groups with parametric distribution. Pearson chi-square (χ^2) test and likelihood-ratio chi-square was used where appropriate. A *P* value equal to or less than 0.05 was considered significant. DACLA, daclatasvir; HCC, hepatocellular carcinoma; OCI, occult HCV infection; PBMCs, peripheral blood mononuclear cells; RIB, ribavirin; SOF, sofosbuvir; SVR, sustained virologic response; TEM, transmission electron microscopy.

Table 3 Association between laboratory data and occult hepatitis C infection (OCI) grade severity in peripheral blood mononuclear cells (PBMNCs) by transmission electron microscopy (TEM)

	OCI grades By TEM		
Characteristics	Mild and moderate (*n*=22)	Marked (*n*=48)	*P* value
Platelet count	148.2±56.8	132.1±67.7	0.160
Hemoglobin	12.7±1.6	12.0±1.7	0.113
TLC	6.0±2.1	4.9±2.4	**0.010**
INR	1.1±0.1	1.17±0.1	0.328
Prothrombin concentration	78.5±14.0	75.3±9.4	0.169
Urea	34.8±9.5	35.1±11.7	0.889
Creatine	0.98±0.13	0.96±0.25	0.307
Direct bilirubin	0.24±0.21	0.35±0.30	0.307
Total bilirubin	1±0.5	1.1±0.57	0.795
Albumin	3.6±0.7	3.6±0.5	0.844
AFP	868.6±2018.5	755.4±1868.2	0.483
ALT	33.4±16.0	41.9±43.6	0.864
AST	45.0±22.1	47.1±42.7	0.399

The values are presented as mean±SD. The independent *t*-test was used to compare two independent groups. A *P*-value equal to or less than 0.05 was considered significant. AFP, alpha-fetoprotein; ALT, alanine transaminase; AST, aspartate transaminase; INR, international normalized ratio; OCI, Occult HCV infection; PBMCs, Peripheral blood mononuclear cells; TEM, transmission electron microscopy; TLC, total leucocyte count.

thrombocytopenia and early development of HCC in the studied cases, however, no statistically significant association of other laboratory data with the early development of HCC could be detected (Table 4).

Discussion

The hepatitis C virus has a significant impact on HCC development. DAA therapy may eliminate the virus from the blood as evaluated by PCR technique but remains occult in PBMCs and/or liver tissue and is defined as OCI. Physicians should be aware that anti-HCV drugs alone cannot totally prevent hepatocarcinogenesis. Other factors, including OCI, should also be considered [11]. OCI is a problematic concept in post-hepatitis care and follow-up, particularly after DAA regimens were implemented [12]. The detection of OCI in HCC patients who achieved SVR and its clinical significance are not well understood, and it is a topic of ongoing research and debate in the field of hepatology [13]. It is crucial to note that the detection of occult HCV RNA may be influenced by the sensitivity of the assay used and the duration of follow-up, among other factors. Further studies are needed for a better understanding of the clinical implications of OCI and for the

Table 4 The correlation between duration (months) from sustained virological response (SVR) till the development of hepatocellular carcinoma (HCC) with measured laboratory data

	The duration from SVR till HCC development	
	r	*P*-value
Platelet count	0.257	**0.032**
Hemoglobin	0.085	0.484
TLC	–0.007	0.956
Prothrombin concentration	0.093	0.442
Urea	0.035	0.774
Creatine	–0.046	0.655
Direct bilirubin	–0.056	0.648
Total bilirubin	0.089	0.466
Albumin	0.009	0.944
AFP	–0.082	0.501
ALT	0.035	0.774
AST	-0.081	0.505

The correlation between duration (months) from SVR till the development of HCC with measured laboratory data in the studied group was analyzed using the Pearson correlation coefficient.
P- value <0.05 was defined as statistically significant. AFP, alpha fetoprotein; ALT, alanine transaminase; AST, aspartate transaminase; HCC, hepatocellular carcinoma; INR, International normalized ratio; SVR, Sustained virologic response; TLC, Total leucocyte count.

development of more sensitive assays for its detection. Moreover, developing guidelines for the management and monitoring of OCI remains challenging due to a lack of precise definition and confusion about its clinical importance, particularly in areas with high HCV prevalence [14]. From this point, we investigated OCI frequency in the PBMCs of HCC patients after SVR by both PCR and electron microscopy techniques, with the novelty of studying the OCI in PBMNCs by immune electron microscopic analysis for the viral antigen load at an ultrastructural level.

In the present study, we were able to detect a high prevalence of 100% (70/70) of OCI in HCC patients with SVR after receiving DAA drugs regimens for HCV by TEM technique, with more than two-thirds of the cases (68.6%) having marked OCI grade. All patients in our study were primarily diagnosed as HCV genotype 4 as this is the prevalent genotype in Egypt [15]. Our study has established the superiority of the electron microscopy (EM) technique over the PCR technique, with higher sensitivity in the detection of OCI than PCR in PBMCs (100% Vs 5.7%, respectively). The superiority of EM over the PCR technique was stated in the studies done by Johnsen and Schramlová *et al*. Their findings comparing the detection of viruses from untreated tissue culture using EM and real-time PCR demonstrated the superiority of EM, which discovered 38 times more viral copies than PCR [16,17]. The prevalence of OCI that could be detected by PCR in PBMCs was 5.7% (4/70). Our results are in accordance with previous studies that reported the prevalence of OCI as (2-11%) by RT-PCR in PBMCs and liver tissue samples in cases with SVR following treatment with DAA drugs for chronic HCV infection [4,12,18], while some studies reported a relatively higher prevalence of OCI by PCR as Hanafy et al., who reported that out of 89 patients who achieved SVR 9% of them developed HCC and all of the HCC patients were OCI positive by RT-PCR in PBMCs [19], and Khattab et al., who reported 20% OCI by PCR, although inclusion criteria were different for each study [20]. The inclusion of RIB in DAA drugs for treating HCV infection has been debated, particularly concerning its impact on OCI. Both RIB-inclusive and RIB-sparing DAA regimens are highly effective in treating HCV. RIB-inclusive regimens may offer additional benefits in reducing OCI due to their enhanced antiviral and immune-modulatory effects, though they come with a higher risk of adverse effects. RIB-sparing regimens are well-tolerated and effective, making them a preferred option for many patients. Personalized treatment decisions and ongoing research are essential to optimize outcomes and address the risk of OCI in HCV patients [21].

In our study, patients who received DAAs with RIB (triple therapy) showed significantly higher grades of OCI in PBMNCs by TEM than those who received double therapy (87% versus 59.6%, P=0.020), denoting that RIB use with DAAs has no role neither in decreasing viral load intracellularly nor in the prevention of the development of severe OCI. In agreement with our findings, previous studies comparing RIB-inclusive DAA regimens to RIB-sparing DAAs show that the addition of RIB often does not significantly increase SVR rates. For instance, the LONESTAR and ION-1 trials did not find any significant difference in SVR rates among patients treated with SOF/ledipasvir (LDV/SOF) with and without RIB. Furthermore, RIB-inclusive regimens are associated with higher rates of adverse events, including anemia and fatigue, suggesting that RIB may not be necessary for most DAA regimens [21]. Other studies have investigated the detection of OCI when using a combination of DAAs with or without RIB by PBMC PCR for HCV RNA and their results do not show a difference with or without RIB [20,22].

There is a complex interplay between OCI and leucopenia in HCC patients. Chronic immune activation due to OCI combined with the effects of cirrhosis and HCC treatments can lead to leucopenia, complicating HCC management by increasing the risk

of infections and limiting the ability to administer full-dose chemotherapy. Regular monitoring and tailored therapeutic strategies are essential to address these challenges and improve patient outcomes [23].

In our study, There was a significantly lower TLC in severe OCI grade by TEM compared with mild and moderate grades (P=0.010), however, there was no statistically significant relation between the other laboratory parameters and the severity of OCI. A possible explanation for the significant reduction of TLC in our study is that persistent low-level HCV infection in the liver may continually stimulate the immune system, leading to immune exhaustion or dysfunction, which can manifest as leucopenia. Moreover, the antiviral therapy may alter the immune response, leading to a decrease in leucocytic count.

On the contrary, Nakano *et al.*, found that an elevated white blood cell count in the initial stage of HCV infection is an independent risk factor for the early development of HCC [24], taking into account that the white blood cell count is a reliable indicator of inflammation and that persistent inflammation may increase the risk of developing hepatocarcinogenesis by activating receptors for advanced glycation end products and chemokines [25]. So, regular monitoring of leucocyte counts in HCC patients is essential, especially those with known OCI. In addition, addressing underlying OCI with antiviral therapy, if applicable, may help mitigate some of the immune dysfunction and improve leucocyte counts.

The platelet count in chronic liver disease patients, especially those with liver cirrhosis, could be an important indicator of liver function and overall health. In the context of HCC, platelet count has been studied as a potential marker for the early development of the disease [26]. In our study, however no statistically significant relation between platelet count and the advanced grade of OCI by TEM could be detected, the onset of HCC development is significantly associated with platelet count (P<0.032); denoting that thrombocytopenia is associated with the early development of HCC in the studied cases. In agreement with our findings, previous studies reported that lower platelet counts were correlated with a higher risk of HCC development, as thrombocytopenia reflects more advanced liver disease, which leads to an increased chance for HCC [27,28]. Conversely, Lu and colleagues suggested that increased platelet is a predictor of HCC recurrence following radical treatment in the presence of circulating tumor cells. This finding implies that platelets can attach to circulating tumor cells, reducing blood flow to them and changing their immunogenicity. Moreover, the platelets interact with the tumor microenvironment by promoting an inflammatory response and creating new blood vessels, which in turn stimulates the shedding and spreading of cancer cells by releasing a significant number of cytokines [29].

In our study, 68.6% of the patients had right hepatic focal lesions. There was a significantly higher proportion of marked OCI grades among cases with right lobe lesions (75% vs. 25%, P=0.020).

The right hepatic lobe is more commonly affected by HCC than the left lobe. This has been reported in numerous studies [30,31]. The incidence of HCC in the right hepatic lobe is likely multifactorial. The right lobe is larger with a higher concentration of hepatocytes, which are the primary cells that can develop into HCC. Additionally, the right lobe receives more blood flow and has a greater exposure to potentially hepatotoxic substances compared with the left lobe. Furthermore, the right lobe of the liver is anatomically located adjacent to the gallbladder, which can harbor gallstones that may lead to inflammation and damage to the liver and finally promote HCC development [31].

Conclusion

In conclusion, DAAs are effective in eliminating HCV in serum, but OCI should be considered, recommending a re-definition for SVR through detection of HCV-RNA in serum and PBMCs by PCR and TEM studies. Our study is the first to provide electron microscopy as a sensitive tool for OCI detection, with established superiority over the PCR technique in the detection of OCI. Therefore, electron microscopy studies should be considered in screening OCI in high-risk groups, especially patients with low TLC and platelet count, to determine the presence and stage of OCI for treatment modifications and follow-up.

Limitation and recommendations

Further studies are needed to confirm such a conclusion of association between OCI and HCC by comparing these patients to other control groups of treated HCV patients who did not develop HCC.

Acknowledgments

This work would not have been possible without the support of the electron microscopy department staff

members, TBRI, Faculty of medicine, Al-Azhar University and National liver institute, Menoufia University.

This work is based upon an internal research project (grant No. 126 D) supported by Theodor Bilharz Research Institute (TBRI).

Source(s) of support: This work is based upon an internal research project (grant No. 126 D) supported by Theodor Bilharz Research Institute (TBRI).

Ethics approval and consent to participate: All procedures followed were in accordance with the ethical standards of ethics committee of Theodor Bilharz Research Institute (under Federal Wide Assurance No. FWA00010609) and with the Helsinki Declaration of 1975, as revised in 2008. Informed consent was obtained from all patients for being included in the study.

Authors' contributions: A.H., Put the conception and design of the study; A.S.E.H. and F.K. performed the practical work of the study; A.H., A.S.E.H., E.E.S.E. K., and A.M.A.M. revised and analyzed the results; A. H., F.K., and A.S.E.H. analyzed and interpreted the ultrastructural data; A.G. was primarily responsible for selection and recruitment of the patients, A.S.E.H. and F.K. was primarily responsible for writing the manuscript, drafting of the paper, revising it critically for intellectual content; and the final approval of the version to be published; and all authors agree to be accountable for all aspects of the work.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

References

1 Finn RS, Ryoo BY, Merle P, Kudo M, Bouattour M, Lim HY, *et al.* Pembrolizumab as second-line therapy in patients with advanced hepatocellular carcinoma in KEYNOTE-240: a randomized, double-blind, phase III trial. J Clin Oncol 2020; 38:193–202.

2 Castillo I, Bartolomé J, Quiroga JA, Barril G, Carreño V. Diagnosis of occult hepatitis C without the need for a liver biopsy. J Med Virol 2010; 82:1554–1559.

3 Zaman N, Asad M, Raza A, Raja G, Akhter S, *et al.* Detection of HCV RNA and NS5A Protein in Peripheral Blood Mononuclear Cells After Sustained Virological Response May Cause Viral Relapse. Pakistan. J Zool 2015; 4:981–987.

4 Saad ZM, Ghany WAE, Khalifa R, Higazi A, Al-Shazly M, *et al.* Occult HCV infection in liver transplanted patients: frequency and consequences. Clin exp hepatol 2022; 2:125–131.

5 European Association for the Study of the Liver. Electronic address: easloffice@easloffice.eu; European Association for the Study of the Liver. EASL Recommendations on Treatment of Hepatitis C 2018. J Hepatol 2018; 69:461–511.

6 Baumert TF, Jühling F, Ono A, Hoshida Y. Hepatitis C related hepatocellular carcinoma in the era of new generation antivirals. BMC Med 2017; 1:52.

7 Carreño, García V, Nebreda JB, Aguilar IC, Quiroga, Estévez JA. Occult hepatitis C virus infection. Enfermedades Infecciosas y Microbiologia Clinica 2011; 29(Suppl 3):14–19.

8 Lok AS, Everhart JE, Di Bisceglie AM, Kim HY, Hussain M, Morgan TR, HALT-C Trial Group. Occult and previous hepatitis B virus infection are not associated with hepatocellular carcinoma in U.S. patients with chronic hepatitis C. HEPATOLOGY 2011; 54:434–442.

9 MacParland SA, Pham TN, Guy CS, Michalak TI. Hepatitis C virus persisting after clinically apparent sustained virological response to antiviral therapy retains infectivity in vitro. Hepatology. 2009; 49:1431–41.

10 Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) inimmunoperoxidasetechniques.A comparisonbetween ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 1981; 29: 577–781.

11 Kishta S, Tabll A, Omanovic, Kolaric T, Smolic R, Smolic M. Risk Factors Contributing to the Occurrence and Recurrence of Hepatocellular Carcinoma in Hepatitis C Virus Patients Treated with Direct-Acting Antivirals. Biomedicines. 2020; 8:175.

12 Mekky MA, Sayed HI, Abdelmalek MO, Saleh MA, Osman OA, Osman HA, *et al.* Prevalence and predictors of occult hepatitis C virus infection among Egyptian patients who achieved sustained virologic response to sofosbuvir/ daclatasvir therapy: a multi-center study. Infection and drug resistance 2019; 12:273–279.

13 Yosef T, Hussein M, Elzohary A, Tari M, Michael G. Age and Gender are Predictors for Occult Hepatitis C in Egyptian Sustained Responders to Directly Acting Antivirals. The Egyptian. J Hosp Med 2022; 89:4552–4557.

14 Wróblewska A, Krzysztof PB, Katarzyna S. Occult Infection with Hepatitis C Virus: Looking for Clear-Cut Boundaries and Methodological Consensus". J Clin Med 2021; 10:5874.

15 Kouyoumjian SP, Chemaitelly H, Abu-Raddad LJ. Characterizing hepatitis C virus epidemiology in Egypt: systematic reviews, meta-analyses, and meta-regressions. Sci Rep 2018; 8:1661.

16 Johnsen CK, Böttiger B, Blom J. Confirmation of electron microscopy results by direct testing of viruses adhered to grids using nucleic acid amplification techniques. J virol methods 2006; 134:92–98.

17 Schramlová J, Arientová S, Hulínská D. The role of electron microscopy in the rapid diagnosis of viral infections-review. Folia Microbiol 2010; 55: 88–101.

18 Mashaal AR, Abd, El-Hameed M, El Ray AA, Mahmoud, Diab T, Dawood RM, Shemis MA, Seyam M. Detection of Occult Hepatitis C Virus Infection in Egyptian Patients Who Achieved a Sustained Virologic Response to Direct-Acting Antiviral Agents. Asian Pacific j cancer prevention: APJCP 2022; 23:2965–2971.

19 Hanafy AS, Seleem WM, Basha MAA, Marei AM. Residual hepatitis C virus in peripheral blood mononuclear cell as a risk factor for hepatocellular carcinoma after achieving a sustained virological response: a dogma or fiction. Eur j gastroenterol hepatol 2019; 31:1275–1282.

20 Khattab MA, Zakaria Y, Sadek E, Abd El Fatah AS, Fouad M, *et al.* Detection of hepatitis C virus (HCV) RNA in the peripheral blood mononuclear cells of HCV-infected patients following sustained virologic response. Clin exp med 2023; 23:131–140.

21 Mathur P, Kottilil S, Wilson E. Use of Ribavirin for Hepatitis C Treatment in the Modern Direct-acting Antiviral Era. J Clin Transl Hepatol 2018; 6:431–437.

22 Wang Y, Rao H, Chi X, Li B, Liu H, *et al.* Detection of residual HCV-RNA in patients who have achieved sustained virological response is associated with persistent histological abnormality. EBioMed. 2019; 46: 227–235.

23 Kedia S, Bhatt VR, Rajan SK, Tandra PK, El Behery RA, Akhtari M. Benign and Malignant Hematological Manifestations of Chronic Hepatitis C Virus Infection. Int J Prev Med 2014; 5(Suppl 3):S179–92.

24 Nakano M, Kawaguchi T, Nakamoto S, Kawaguchi A, Kanda T, Imazeki F, *et al.* Effect of occult hepatitis B virus infection on the early-onset of hepatocellular carcinoma in patients with hepatitis C virus infection. Oncol Reports 2013; 30:2049–2055.

25 Pusterla T, Nèmeth J, Stein I, Wiechert L, Knigin D, *et al.* Receptor for advanced glycation endproducts (RAGE) is a key regulator of oval cell activation and inflammation-associated liver carcinogenesis in mice. Hepatol 2013; 8:363–373.

26 Xia W, Peng T, Guan R, Zhou Y, Zeng C, *et al.* Development of a novel prognostic nomogram for the early recurrence of liver cancer after curative hepatectomy. Ann Transl Med 2021; 9:1541.

27 Celli R, Saffo S, Kamili S, Wiese N, Hayden T, Taddei T, *et al.* Liver Pathologic Changes After Direct-Acting Antiviral Agent Therapy and Sustained Virologic Response in the Setting of Chronic Hepatitis C Virus Infection. Arch pathol laboratory med 2021; 145:419–427.

28 Fujii H, Fujii M, Iwaki M, Hayashi H, Toyoda H, Oeda S, *et al.* Japan Study Group of Nonalcoholic Fatty Liver Disease (JSG-NAFLD). Multicenter, retrospective, cohort study shows platelet counts predict hepatocellular carcinoma development in patients with nonalcoholic fatty liver disease. Hepatol Res 2023; 53:391–400.

29 Lu Z, Huang Y, Huang J, Ni HH, Luo T, *et al.* High Platelet Count is a Potential Prognostic Factor of the Early Recurrence of Hepatocellular Carcinoma in the Presence of Circulating Tumor Cells. J hepatocellular carcinoma 2023; 10:57–68.

30 Ali A, Gamal S, Anwar R, ElZahaf E, Eskandare D. Assessment of clinico-epidemiological profile of Hepatocellular carcinoma in the last two decades. Egypt. J Intern Med 2023; 35:18.

31 Renzulli M, Brandi N, Pecorelli A, Pastore LV, Granito A, *et al.* Segmental Distribution of Hepatocellular Carcinoma in Cirrhotic Livers. Diagnostics (Basel) 2022; 12:834.