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International Journal of Immunopathology & Pharmacology

Original Research Article

Occult hepatitis C virus infection in patients with malignant lymphoproliferative disorders

International Journal of Immunopathology and Pharmacology
Volume 34: 1–8
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DOI: 10.1177/2058738420961202
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Abstract

Despite the link between HCV and malignant lymphoproliferative disorders has been established, the association between occult hepatitis C virus infection and malignant lymphoproliferative disorders remains obscure. The present study intended to identify the possible association between occult HCV infection and malignant lymphoproliferative disorders. Newly diagnosed patients with LPDs were screened for the presence of HCV-RNA in both plasma and PBMCs. PBMCs of the subjects were also, examined by transmission and immuno-electron microscopy. LPD patients showed a high percentage of HCV infection (71.9%): OCI-HCV (37.5%) and HCV (34.38%). Meanwhile, 28.13% of LPD patients did not show any evidence of HCV infection. Ultrastructural examination of PBMCs revealed the presence of intracytoplasmic vacuoles enclosing viral like particles, which were less prominent in occult HCV patients. The possibility of occult HCV should be considered in patients with LPDs which can be helpful in the management of the treatment protocol in order to set up a balance between the control of the tumor progression and minimizing post chemotherapy complications related to HCV infection.

Keywords

hepatitis C virus, lymphoproliferative disorders, occult HCV

Date received: 29 May 2020; accepted: 2 September 2020

Introduction

Lymphoproliferative disorder (LPD) is a term that includes a wide spectrum of pathologies ranging from a minor expansion of a B-cell population (with no clinical significance) to an aggressive high-grade lymphoma.[1] Such proliferations of B cells apparently can be triggered by a number of viruses. Some of these viruses have a direct oncogenic effect, while others cause lymphoma due to chronic antigenic stimulation or due to immunosuppression associated with the virus.[2] A causal association between hepatotropic viruses, especially hepatitis C virus (HCV), and malignant B-cell LPDs has been demonstrated utilizing epidemiologic data, biologic and

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molecular investigations, as well as clinical observations.[3] LPD was not correlated with infection with a certain genotype/subtype of HCV,[4] however, the genotypes 1b and 2a were reported to be among risk factors.[5–7]

A new form of chronic HCV infection named occult HCV infection (OCI) was described by Castillo et al.[8] Occult hepatitis C virus infection (OCI) referred to the existence of genomic HCV RNA in the peripheral blood mononuclear cells (PBMCs) and liver without any detectable HCV RNA in serum by standard assays. OCI be found in anti-HCV positive patients with normal serum levels of liver enzymes and in anti-HCV negative patients with persistently elevated liver enzymes of unknown etiology.[9–11] The estimated frequency of OCI among patients displaying hepatic dysfunction may be as high as 57%.[8,10]

Although the detection of HCV RNA in the liver biopsy is the gold standard method for the diagnosis of an OCI, however, it is an invasive approach. Other non-invasive techniques include the detection of HCV RNA in PBMCs, or in ultracentrifuged serum were successful in diagnosis of >60% OCI cases. The combination of these non-invasive approaches along with the detection of anti-core HCV improve the diagnosis of OCI in more than 90% of the cases.[12] Interestingly, HCV viral particle from HCV infected PBMC could be fixed with specific immunoglobulins against the virus and can be detected by immune electron microscopy.[13,14]

Accumulated experimental and clinical evidence indicates that HCV infects not only hepatocytes but also cells in extrahepatic compartments, particularly those in the immune and the central nervous systems.[15] According to different studies, 40–74% of patients infected with HCV might develop at least one extrahepatic manifestation during the course of the disease. Further, extrahepatic disorders could represent the first signal of an HCV infection, as many patients show no hepatic symptoms.[16] In regard to infection of immune cells, HCV replication was shown in circulating T and B lymphocytes and monocytes from patients with chronic as well as persistent low level (occult) infections.[17]

As a consequence of the lymphatic infection, several LPDs have been associated with HCV infection, including MC and B-NHL.[18] A possible association between HCV infection and B-NHL have been reported.[2,18,19] It was proved that HCV is a risk factor for diffuse large B-cell, marginal zone, and follicular lymphomas in Egypt[19]

An indirect evidence of the oncogenic role of HCV in NHL raised from the finding that PEG-IFN/RBV completely or partially restricted lymphoma in HCV positive, but not in HCV-negative, NHL patients.[18]

Lymphoma-associated viral infections have major therapeutic implications. Treatment of these lymphomas is difficult because of the risk of reactivation after immuno-suppressive treatment which could cause severe scheduled organ-damage, severe hepatitis in the case of HCV reactivation, resulting sometimes in the reduction of the doses or even treatment discontinuation.[2] More interestingly, HCV antiviral treatment lead to loss of the heavy chain of the immunoglobulin and t(14;18) translocation that reflected a possible role of HCV in inducing genetic alterations that are associated with NHL.[20] Recently, recurrent malignant lymphoma was observed after using direct acting HCV antiviral, sofosbuvir/ledipasvir.[21]

In spite of a link between HCV and malignant LPDs is suggested,[22,23] the association between OCI and malignant LPDs still needs further investigation which will be helpful in the choice of treatment modalities, post chemotherapy complications, with subsequent improvement in the survival rate. The current study aimed to find some relationship between OCI and malignant LPDs that may help in clinical treatment of malignant LPDs.

Subjects and methods

Ethical approval

The study protocol and the suggested informed consent were approved by the Institutional Review Board (IRB) of Theodor Bilharz Research Institute. An informed consent was obtained in a written form from each participant in the study.

Subjects

This study includes 32 patients with de novo LPDs who were referred to Medical Oncology department in the National Cancer Institute, Cairo University and Clinical Hematology Unit of Ain Shams University Hospitals period between June 2017 and December 2019 in addition to 27 age and sex matched healthy subjects were also included as

a control group. All the patients were subjected to the following clinical examination, complete blood picture (CBC) with differential white cell count, bone marrow aspirate and immunophenotyping, Bone marrow biopsy and immunohistochemistry, complete liver and kidney function tests, Assay of beta 2 microglobulin, lymph node biopsy, abdominal ultrasound, and CT.

Selection criteria for patients; LPD confirmed patients, negative for infection with hepatitis B virus (HBV), HIV, Epstein-Barr virus (EBV), and cytomegalovirus (CMV). Control group inclusion criteria were apparently healthy individuals, negative for both serum HCV Ab and serum HCV RNA. Exclusion criteria were positive results for HBVsAg, HIV, Epstein-Barr virus (EBV), or cytomegalovirus (CMV). The subjects were grouped into four main categories: HCV classical infection, HCV seronegative [OCI + HCV/OCI Ab (−)/sRNA (+)], [OCI + HCV/Ab (−)/sRNA (−)], and negative HCV as previously described.[9] Samples were taken before starting any treatment and protocol of treatment were selected based on local NCI guidelines.

Screening of HCV, HBV, and HIV

Both patients and control subjects were screened for the possible presence of HCV IgG (Murex Anti-HCV version 4.0, DiaSorin, South Africa), HBsAg (HBsAg ELISA version 1, Autobio Diagnostics Co., Ltd, China), and HIV Ag-Ab (Genscreen™ ULTRA HIV Ag-Ab assay, Bio-Rad, France) according to the manufacturers' instructions.

Preparation of PBMCs

Briefly, 5 ml of prediluted blood (1:2 in PBS, pH 7.2) was carefully layered over 5 ml of Ficoll-Paque liquid (Sigma, USA) in a 15-ml tube (Falcon; NJ). Tubes were centrifuged at 400 g for 30 min. The mononuclear cells were collected at interphase layer, then washed twice with three volumes of PBS.

Detection of HCV-RNA by PCR

Viral RNA was extracted from plasma and PBMCs followed by real time PCR detection of HCV-RNA (Abbott real time detection kit for HCV, Abbott Inc, Germany) using ABI 7500 real time PCR (Applied Biosystems, USA).

Transmission electron microscopy

PBMCs were fixed with buffered glutaraldehyde (2.5%) for 30 min. then dehydrated using a series of different concentrations of ethanol (50%,70%, 90%, 100%). Substitution was carried out in epon/ethanol mixture (1:1) for 1 h, followed by infiltration in an overnight bath of epon at RT. Embedding was conducted using Epon/DMP30 (1.7%DMP30 in epon) which followed by polymerization at 37°C for 12 h and at 60°C for 2 days. Ultramicrotome was used to make an ultrathin section that was placed on copper grids. Staining was conducted with uranyl acetate and lead citrate, prior to examination with a TEM (Phillips 208S, Germany).

Immuno-electron microscopy

The PBMCs were fixed in 1.25% glutaraldehyde in PBS for 30 min at RT. Following blocking of endogenous peroxidase activity, the cells were labeled by the primary anti-HCV (NS4B region) antibody overnight at 4°C. After washing, detecting biotinylated goat anti mouse IgG was incubated for 10 min followed by washing and incubation with streptavidin peroxidase for 10 min. After washing, DAB substrate-chromogen was incubated with specimen for 10 min. This step was followed by washing and post fixation in Oso4 solution in PBS (v/v, Oso_4 2%: PBS) for 30 min at 4°C and dehydrated using variable ethanol concentrations (50%, 70%, 90%, and 100%). Substitution was carried out in epon/ethanol mixture (1:1) for 1 h, followed by infiltration in an overnight bath of epon at RT. Embedding was conducted using epon/DMP30 (1.7%DMP30 in epon) which followed by polymerization at 37°C for 12 h and at 60°C for 2 days. Ultramicrotome was used to make an ultrathin section that was placed on copper grids. Staining was conducted with uranyl acetate and lead citrate, prior to examination with a TEM (Phillips 208S, Germany).

Statistical analysis

Data were analyzed using the Statistical Program for Social Science (SPSS) version 18.0. Both Chi-square and Spearman's rho correlation were used to analyze the data.

Results

This study was conducted on 32 newly diagnosed patients with LPDs. Cases included (i) diffuse large

Table 1. Clinical and laboratory findings of lymphoproliferative disorders patients.

Age (yr)	Sex	Computerized tomography			Bone marrow biopsy		Diagnosis	HCV Ab	rRT-PCR-plasma		rRT-PCR-PBMCs		Type
		LN	HM	SM	Infiltration	Pattern			Result	Quantity	Result	Quantity	
62	M				−	−	NHL	+	+	101,139	+	5562	HCV classical infection
52	M	+	−	−	−	−	NHL	+	+	56,112	+	3232	HCV classical infection
66	F	+	+	+	+	Patchy	NHL	+	+	47,534	+	31,476	HCV classical infection
60	F	+	+	+	−	−	NHL	+	+	29,331	+	657	HCV classical infection
65	F	+	+	+	+	Diffuse	CLL	+	+	80,735	+	52	HCV classical infection
50	M	−	−	−	−	−	NHL	+	+	5655	+	132	HCV classical infection
47	M	+	+	+	+	Diffuse	NHL	−	+	161,020	+	47	HCV seronegative/OCI[a]
45	M	+	+	+	+	Patchy	NHL	−	+	201,970	+	9795	HCV seronegative/OCI[a]
55	F	+	+	+	−	−	NHL	−	+	1280	+	22,559	HCV seronegative/OCI[a]
49	M	+	−	−	−	−	NHL	−	+	2698	+	4707	HCV seronegative/OCI[a]
47	M	+	+	−	−	−	NHL	−	+	8988	+	104	HCV seronegative/OCI[a]
62	M	+	−	+	+	Diffuse	CLL	−	−	−	+	50	OCI[b]
50	M	+	+	+	−	−	NHL	−	−	−	+	1110	OCI[b]
52	M	+	+	+	+	Diffuse	CLL	−	−	−	+	26	OCI[b]
80	M	+	+	+	+	Diffuse	NHL	−	−	−	+	124	OCI[b]
33	M	+	−	−	−	−	NHL	−	−	−	+	76,444	OCI[b]
46	M	+	+	+	+	Diffuse	CLL	−	−	−	+	45	OCI[b]
62	M	−	−	+	+	Diffuse	CLL	−	−	−	+	80	OCI[b]
58	M	−	+	+	+	Patchy	CLL	−	−	−	+	67	OCI[b]
60	F	+	+	+	+	Patchy	NHL	−	−	−	+	3248	OCI[b]
72	M	+	+	+	+	Diffuse	CLL	−	−	−	+	49	OCI[b]
67	M	+	+	+	−	−	NHL	−	−	−	+	650	OCI[b]
43	F	+	+	+	−	−	NHL	−	−	−	+	987	OCI[b]
38	M	+	+	+	−	−	NHL	−	−	−	−	−	Negative HCV
45	M	+	−	+	+	Patchy	NHL	−	−	−	−	−	Negative HCV
60	F	−	−	+	+	Diffuse	CLL	−	−	−	−	−	Negative HCV
64	M	+	−	−	−	−	NHL	−	−	−	−	−	Negative HCV
48	M	+	−	−	−	−	NHL	−	−	−	−	−	Negative HCV
44	M	+	−	−	−	−	NHL	−	−	−	−	−	Negative HCV
47	M	+	−	+	−	−	NHL	−	−	−	−	−	Negative HCV
65	F	−	−	−	−	−	NHL	−	−	−	−	−	Negative HCV
29	M	−	−	−	−	−	NHL	+	−	−	−	−	Negative HCV

LN: lymph node; HM: hepatomegaly; SM: splenomegaly; NHL: non Hodgkin lymphoma; CLL: chronic lymphocytic leukemia.
[a]OCI + HCV/OCI Ab (−)/sRNA (+).
[b]OCI + HCV/Ab (−)/sRNA (−) based on Abdel-Moneim.[9]

B-cell lymphoma (DLBCL) (n=8), (ii) chronic lymphocytic leukemia (CLL/SLL) (n=8), (iii) MALT and marginal zone lymphoma (n=7), (iv) follicular lymphoma (n=3), (v) T-cell lymphoma (n=3), (vi) lymphoblastic T-cell lymphoma (n=2) and (vii) Mantle cell lymphoma (n=1).

The mean age of the patients was 53.84 ± 11.34 years. Among the 32 patients, 24 (75%) were males and 8 (25%) were females with a male: female ratio of 3:1. Twenty-four patients (75%) were NHL and 8 patients (25%) were CLL. Twenty-seven age and sex matched healthy subjects were also included as a control group. The mean age of the control group was 45.73 ± 11.14 years. Among the control group, 18 (66.7%) were males and 9 (33.3%) were females with a male: female ratio of 2:1. They were enrolled, in order to screen the prevalence of OCI in LPD patients versus healthy subjects (Table 1). Bone marrow biopsy showed infiltration in 14/32 samples of the LPD patients: five patchy and nine diffuse patterns.

The 32 examined LPD patients included nine HCV negative subjects (28.13%) and the rest were positive for HCV (23; 71.87%) (Table 1). All HCV infected cases infected with genotype 4a (data not shown). Six subjects presented with classical HCV infection. OCI(OCI + HCV/Ab (−)/sRNA (−)) and + HCV/seronegative(OCI + HCV/OCI

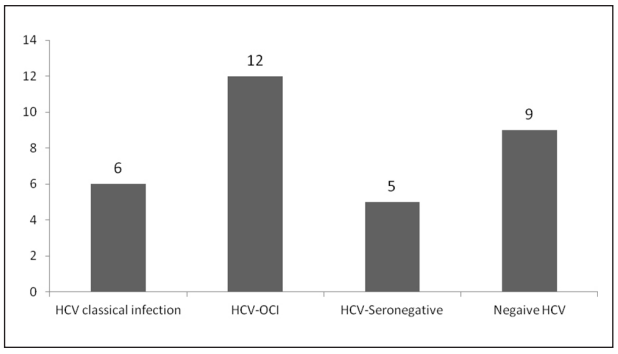

Figure 1. Number of LPD patients clustered based on the type of the HCV infection.

Ab (−)/sRNA (+)) subjects were detected in twelve (37.5%) and five (15.6%) patients, respectively (Figure 1) with no significant variation among the copy numbers of the three HCV infected clusters of subjects. None of the control group showed evidence of OCI or HCV seronegative infections (data not shown). There was a high correlation between the bone marrow biopsy findings and the type of the tumor ($r=0.774$) Spearman's rho correlation, $P>0.01$ (2-tailed) while no correlation was found between the HCV infection type and diagnosis or the bone marrow biopsy findings.

Evaluation of clinical data obtained in our study demonstrated variable percentage of patients with hepatosplenomegaly in all three groups. However, the positive OCI group showed a significantly higher number of patients with hepatomegaly and splenomegaly compared to positive HCV and negative OCI/negative HCV LPD patient groups.

There is a wide variation of the viral load in different HCV positive groups, hence there no significant statistical difference between the viral load presented as copy numbers of the HCV in PBMCs among positive HCV clusters. The electron microscopic analysis of PBMCs showed more abundance viral like particles (Figure 2) in classical positive HCV patients more than those seen in HCV OCI or seronegative HCV-positive patients (data not shown).

The viral load in PBMCs in HCV infected patients showed a higher frequency in HCV (3/6) and seronegative (OCI Ab (−)/sRNA (+)) groups (3/5) compared to OCI (OCI Ab (−)/sRNA (−)) group (3/12) (Table 1). This result was confirmed by ultrastructural examination of PBMCs that revealed the presence of intracytoplasmic vacuoles enclosing viral like particles (Figure 2), which were more abundant in positive HCV patients and seronegative HCV patients in comparison to positive OCI ones.

Discussion

In the current study high percentage (23/32, 71.9%) of LPD subjects were infected with HCV. High result was also recorded previously 20% of LPD patients were HCV OCI and 26% were positive HCV.[24] Meanwhile, the percentage of OCI in the LPD patients disagrees with the findings of Farahani et al.[25] who reported a low percentage of LPD patients were infected with HCV (1.9%). Such discrepancies in the results could be related to the difference in the overall prevalence of HCV infection in the general population in Egypt and Iran. Egypt has the highest HCV prevalence worldwide (14.7%) while the prevalence of HCV infection in the Iranian population is $<0.5\%$.[25–27]

The variable percentage of patients with hepatosplenomegaly in all the three groups in the current study agrees with the fact that hepatic and splenic involvement vary with different types and subtypes of LPDs.[28,29] Additionally, chronic liver disease and portal hypertension as well as hematological disease are among the potential causes that result in abnormal liver and spleen functions and imaging including hepatosplenomegaly.[28] Metabolic, infiltrative, and connective tissue disorders are among other causes of hepatosplenomegaly.[30]

There is a wide variation of the viral load in different HCV positive groups, hence there no significant statistical difference between the viral load presented as copy numbers of the HCV in PBMCs among positive HCV clusters. It is known that the viral RNA load is detected in low concentration in comparison to the classical form of HCV infection.[9,11] The electron microscopic analysis of PBMCs revealed the abundance HCV viruses in classical HCV patients in comparison to HCV OCI or seronegative HCV-positive patients. The cellular immune response in OCI patients was much more frequent and efficient than in those with typical clinical course. It is suspected that this stronger immune response may be involved in viral replication control to undetectable levels.[31] Consequently, it must be taken into consideration that OCI may predispose to

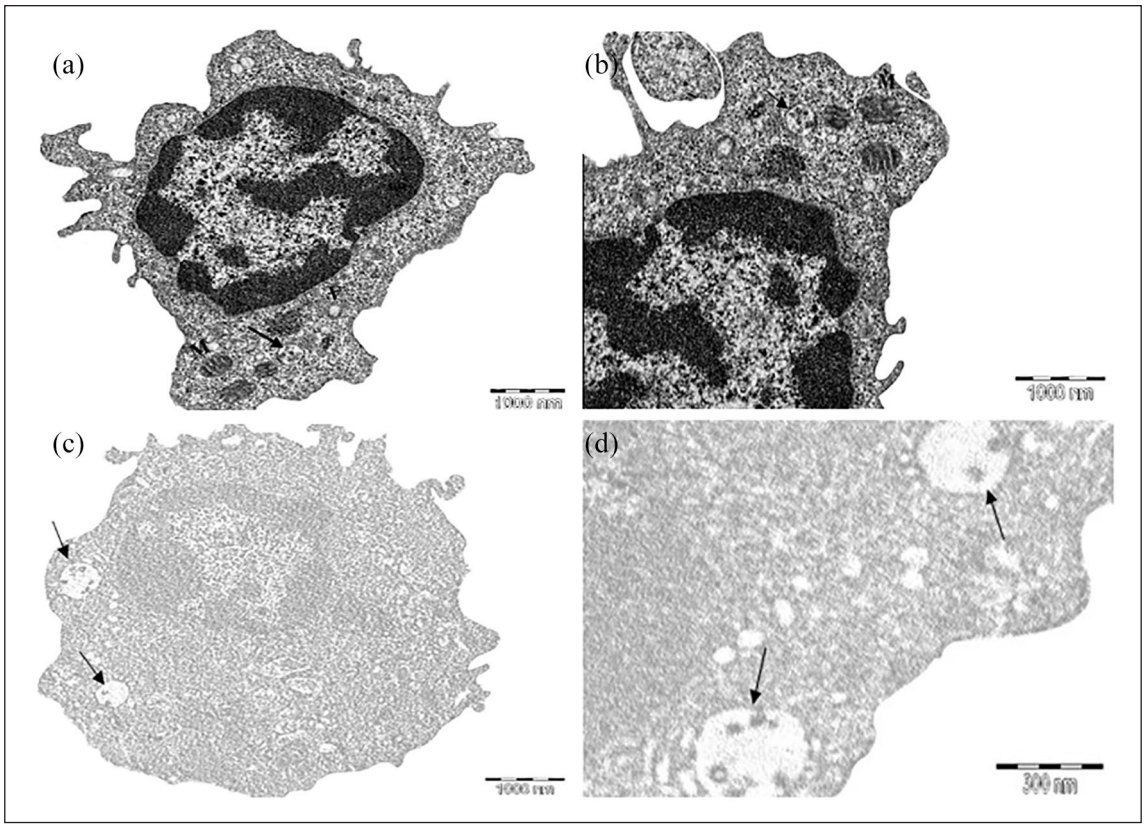

Figure 2. TEM micrograph of a mononuclear cell isolated from a patient with OCI. (a) TEM micrograph reveals the moderate amount of cytoplasm, including many intact mitochondria (M), and many intracytoplasmic vacuoles. One of these vacuoles contains viral-like particles (VLPs) (↑) while the rest appear empty (V). (Original magnification × 11000). (b) A higher magnification showing the intact mitochondria (M) and intracytoplasmic electron dense VLPs (–50–60 mm in diameter) (↑) surrounded by degenerated material enclosed in a vacuole. (Original magnification × 14000). (c) Immunoelectron microscopy of mononuclear cell stained with anti-HCV peroxidase-labeled from HCV-infected patient showing intracytoplasmic positively-stained VLPs (↑) (Original magnification × 11000). (d) A higher magnification showing the vacuoles studded with VLPs (–80 nm in diameter) (↑) in more details. (Original magnification × 36000).

reactivation of clinically evident HCV infection, especially in situations where the immune system is compromised because of comorbid disease or suppressive treatment.[32,33]

The main limitation of the current study was the unavailability for an involvement number of patients, and we do not do any power analysis to calculate the sample size selected for this study.

Conclusion

Screening for OCI should be considered in individuals at high risk for developing LPD. Prospective studies to assess OCI as a causal agent for LPDs are needed.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The authors work was supported by Taif University Researchers Supporting Project Number (TURSP-2020/11), Taif University, Taif, Saudi Arabia and Theodor Bilharz Research Institute, Project Number T-15.

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