

RESEARCH ARTICLE

Hepatitis C Virus Associations with Non Hodgkin's Lymphoma: Insights on Inflammation/Angiogenesis and CD Markers

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Abstract

We aimed to investigate any association between hepatitis C virus (HCV) infection and non-Hodgkin's lymphoma (NHL) in the view of cytokines that control inflammation/angiogenesis and their correlation with certain CD markers. NHL patients with or without HCV infection were studied. CD5, CD30, CD3, CD20 and CD45 were immunohistochemically evaluated. Plasma levels of vascular endothelial and platelet derived growth factors (VEGF, and PDGF), tumor necrosis factor (TNF- α), transforming growth factor (TGF- β), interleukin-6 (IL-6), IL-8, IL-4, IL-12 and interferon gamma (IFN- γ) were detected by enzyme-linked immunosorbent assay (ELISA). HCV+ve NHL patients showed a significant reduction in VEGF, PDGF, IFN- γ , CD5 and CD45 and a significant increase in IL-12 and IL-8. In conclusion, there was a significant change in cytokine secretion and expression of CD markers in HCV+ve NHL patients. Based on our results, HCV infection in NHL patients requires more in-depth investigations to explore any role in lymphoma progression.

Keywords: NHL - HCV - inflammation - CD - angiogenesis

Asian Pac J Cancer Prev, 17 (9), 4415-4420

Introduction

Non-Hodgkin lymphoma (NHL), the hematologic malignancy with the highest prevalence worldwide, represents the fifth most common cancer, in terms of incidence rates, which have grown faster with an annual percentage increase of nearly 3% (Marcucci and Mele, 2011; Vendrame and Martinez, 2011). Malignant lymphomas are caused by the abnormal growth of lymphocytes in the lymphoid organs or tissues (Pei et al., 2011). Worldwide, one of the highest incidence rates of lymphoma, mainly NHL is in Egypt (Freedman, 2006; Curado et al., 2007). NHLs are classified as B-cell or T-cell lymphoma, depending on the lymphocyte lineage that gave rise to the malignancy. Approximately 90% of NHLs represented by B-cell lymphomas whereas T-cell lymphomas represent approximately 10% (Kasamon and Swinnen, 2004)

HCV is a worldwide health problem, approximately 14.7% of Egyptians, have serological evidence of HCV infection (Miller and Abu, 2010). It is a hepatotropic, sialotropic and lymphotropic virus, capable of replicating in extrahepatic sites within the B cells and triggering malignant transformation (Goldman et al., 2009; Rastin et al., 2013). HCV has been postulated to be an etiological agent for lymphoid malignancies (Farawela, et al., 2012).

Numerous population-based studies have demonstrated an association between HCV infection and NHL points to a finding that has now been assured that HCV plays a role in the development of this malignancy (Mele et al., 2003; Duberg et al., 2005; Anderson et al., 2008; Anderson and Engels, 2008; Marcucci and Mele, 2011; Hamdy et al., 2015).

Cowgill et al. (2004) reported the association of HCV with increased risk of B-cell NHL in Egyptian as a whole (OR=2.3, 95% CI 1.5-3.5) (Cowgill et al., 2004). The role of HCV infection in lymphomagenesis may be related to the chronic antigenic stimulation of B-cell immunologic response of the virus (Arcaini et al., 2012). The long-term infection can continuously stimulate and expand lymphocyte clones resulting in further mutation, transformation and ultimately aggressive malignancies such as NHL (Rastin et al., 2013).

Cytokines can contribute to the etiology of NHL having the prospect to serve as biomarkers for an immune system environment that consolidate the emergence of these tumors (Vendrame and Martinez, 2011). The host cytokine environment may promote the development of lymphoma by increased levels of B cell stimulatory cytokines promoting B cell activation, apoptosis, V(D)J recombination and isotype switching, which collectively enhance the chromosome translocations that are a hallmark

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of B-NHL (Gu et al., 2010). During viral infection, diverse cytokines play a role both in viral clearance and tissue damage (Steinke and Borish, 2006). HCV interferes with cytokines at various levels and escapes the immune response by inducing a T-helper (Th) 2/T cytotoxic 2 cytokine profile. The disability to control this infection leads to the recruitment of inflammatory cytokines (Fallahi et al., 2010).

Consequently, this study was conducted to investigate the prevalence of HCV infection in NHL Egyptian patients in view of its potential contribution to the pathogenesis of NHL. Plasma levels of cytokines (VEGF, PDGF, IL-6, IL-4, IFN- γ , TNF- α , TGF- β , IL-8 and IL-12) which have roles in inflammation and angiogenesis of the disease, were measured in all patients to determine whether the change in the level of these cytokines is associated or independent on the HCV infection and its correlation with CD markers on NHL cells.

Materials and Methods

Subjects

Ninety Seven patients with NHL were recruited from the Oncology Hospital, Menoufia University (Shebein El-kom, Menoufia Governorate, Egypt) (56 men and 41 women; mean age 50.64 ± 14.27 years; range 17-75 years), classified according to HCV infection into two groups; NHL-HCV positive and NHL-HCV negative groups. Informed consent was obtained from all the study participants. All investigations were performed in accordance with the Menoufia University. Health and Human Ethical Clearance Committee guidelines for Clinical Researches. Local ethics committee approved the study protocol.

Diagnosis of NHL

The diagnosis of NHL was confirmed by histology and immunohistochemistry tests. The initial medical evaluation consisted of a complete history and physical examination; chest radiographic examination; computed tomographic scan of the chest, abdomen, and pelvis; bone marrow biopsy or aspirate blood chemistry; were performed at diagnosis. The extent of the disease was categorized according to the Ann Arbor classification and performance status was assessed using criteria of the Eastern Cooperative Oncology Group (ECOG) (Lech et al., 2004). Routine laboratory investigations were collected from the patients' records.

Diagnosis of HCV infection

The diagnosis of hepatitis C was based on a positive HCV antibody test (third-generation enzyme immunoassay) (Murex Biotech Ltd.) and confirmed by reverse transcription-polymerase chain reaction using the Amplicore HCV assay (Roche Diagnostics Corp., Indianapolis, IN).

Detection of CD markers: For this study, sections prepared from the available tissue blocks and subjected to immunostaining with monoclonal antibodies for CD markers include Leucocyte Common Antigen (LCA or CD45), CD20 (B cell marker), CD30 (T and B cell

marker), CD5 (B and T cell marker) and CD3 (T cell marker) were studied. This part was performed at Pathology Department, Faculty of Medicine, Menoufia University.

Measurement of cytokine plasma levels by ELISA

Plasma was collected from patients, separated by centrifugation at 2000xg for 3 min at 4°C, aliquotted, and stored at -80°C until analysis. Total concentrations of VEGF, PDGF, IL-6, IL-8, TNF- α , TGF- β 1, IL-4, IL-12 and IFN- γ using a quantitative ELISA (R&D Systems, Minneapolis, MN) as previously described (Talaat et al., 2007; Talaat, 2010; Talaat et al., 2014). The absorbance measured spectrophotometrically at 450nm using a microplate reader (Sunrise™, Tecan Group Ltd., Männedorf, Switzerland) and plotted against a standard curve with standard levels expressed as pg/ml. Each plasma sample was analyzed in duplicate. The ELISA reader-controlling software (Softmax) readily processes the digital raw absorbance data into a standard curve from which cytokine concentrations of samples were derived directly and expressed as pg/ml plasma.

Statistical analysis

All statistical analyses were performed using SPSS version 19 (LEAD Technology Inc). Data were presented as means with corresponding standard error (SE). Comparisons among groups were performed using independent T test. The chi-square test was used to compare the frequency of variables between groups. Correlation among variables was determined using Spearman's correlation test. A P-value of less than 0.05 was considered significant.

Results

Patients' characteristics

This study includes 97 NHL patients, 36 patients were positive HCV-RNA and 61 were negative; the prevalence of HCV infection is about 37.11% of cases. The mean age for HCV-positive patients was 52.00 ± 12.84 years, whereas the HCV-negative patients were 49.85 ± 15.10 years. There was no age or sex difference between the HCV-RNA positive and HCV-RNA negative groups in

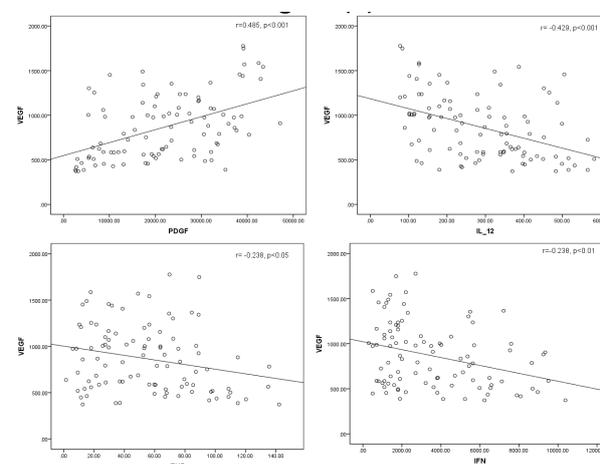


Figure 1. Correlation between Plasma Level of VEGF and PDGF, IL-12, IFN- γ and TNF- α Levels

Table 1. Demographical and Biochemical Data for NHL (HCV-Negative versus HCV-Positive) Patients

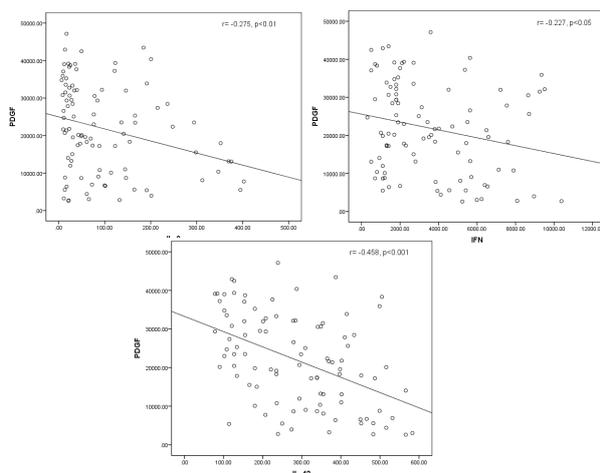
| Parameter | NHL -ve HCV group (Mean± SD) | NHL +ve HCV group (Mean± SD) | P- value |
|----------------------------------|------------------------------|------------------------------|----------|
| Age (year) | 49.8±15.1 | 52.0±12.8 | NS |
| Gender (male/female) | 35/26 | 21/15 | NS |
| WBCs (1000/mm ³) | 8.11±3.53 | 7.14±3.39 | NS |
| Hemoglobin (mmol/L) | 11.02±1.66 | 11.55±1.52 | NS |
| Platelet (1000/mm ³) | 256.1±114.7 | 212.2±103.8 | NS |
| AST (IU/L) | 36.4±21.7 | 47.3±27.0 | P<0.05 |
| ALT (IU/L) | 35.7±20.0 | 50.5±31.5 | P<0.01 |
| Bilirubin (mol/L) | 0.88± 0.37 | 0.95±0.38 | NS |
| Albumin (mol/L) | 3.54±0.51 | 3.48±0.48 | NS |
| Creatinine (mol/L) | 1.02±0.19 | 1.08±0.24 | NS |
| LDH (IU/L) | 402.8±107.73.4 | 492.72±192.3 | P<0.01 |

All data are presented as Mean ± Standard Deviation; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; NS, not significant

Table 2. Mean Plasma Levels of Cytokines for NHL (HCV-Negative versus HCV-Positive) Patients.

| Cytokine (pg/ml) | NHL -ve HCV Group (N=61) (Mean± SE) | NHL +ve HCV Group (N=61) (Mean± SE) | P- value |
|------------------|-------------------------------------|-------------------------------------|----------|
| VEGF | 969.7 ± 48.8 | 689.30 ± 37.2 | P<0.001 |
| PDGF | 24585.2 ± 1571.3 | 17322.80 ± 1648.6 | P<0.01 |
| IL-6 | 103.7 ± 14.7 | 102.8 ± 14.5 | NS |
| IL-4 | 12140.6 ± 886.4 | 12439.5 ± 1052.7 | NS |
| IFN-γ | 3954.3 ± 375.5 | 2904.7 ± 296.8 | P<0.05 |
| TNF-α | 57.4 ± 4.5 | 50.8 ± 5.3 | NS |
| TGF-β | 29864.0±1899.4 | 28802.8 ± 2657.0 | NS |
| IL-12 | 264.7 ± 16.0 | 327.6 ± 25.3 | P<0.05 |
| IL-8 | 64.5 ± 7.3 | 149.3 ± 22.4 | P<0.001 |

Results are expressed as Mean ± Standard Error; VEGF, vascular endothelial growth factor; PDGF, platelet derived growth factor; TNF-α, tumor necrosis factor; TGF-β, transforming growth factor; IFN-γ, interferon gamma; NS, not significant

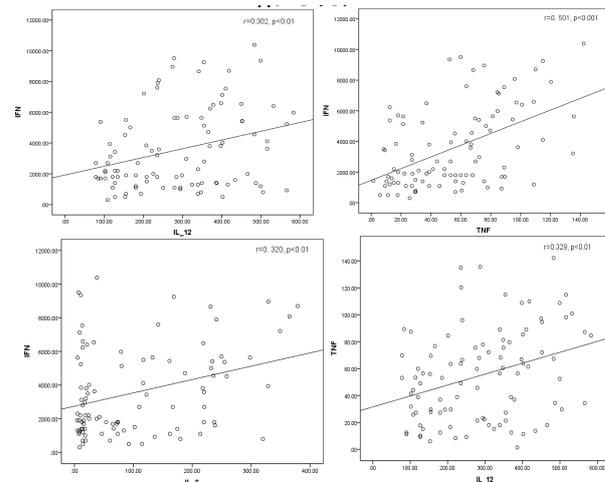
**Figure 2. Correlation between Plasma Level of PDGF and IFN-γ, IL-8 and IL-12 levels**

NHL patients. Demographical and biochemical characters of patient were shown in Table 1. In NHL patients; plasma levels of AST, ALT and LDH showed a significant increase ($p<0.05$, $p<0.01$, $p<0.01$; respectively) in the NHL +ve HCV group compared to the negative group.

Plasma levels of measured cytokines in NHL patients

Mean plasma levels of the measured cytokines for NHL-HCV positive and NHL-HCV negative groups are shown in Table 2. Plasma levels of VEGF, PDGF and IFN-γ showed a significant decrease ($p<0.001$, $p<0.01$, $p<0.05$; respectively) in NHL +ve HCV group than negative ones. In contrast, there was a significant increase ($p<0.05$, $p<0.001$) in plasma levels of IL-12 and IL-8; respectively, in the positive HCV group.

As illustrated in Figure 1, plasma level of VEGF

**Figure 3. Correlation between Plasma Level of IFN-γ and IL-6, IL-12 and TNF-α and between Levels of TNF-α and IL-12**

showed a direct correlation with the PDGF level ($r=0.485$, $p<0.001$) and indirect correlation with IL-12 ($r=-0.429$, $p<0.001$), IFN-γ ($r=-0.238$, $p<0.01$) and TNF-α ($r=-0.238$, $p<0.05$). Plasma levels of PDGF showed indirect correlation with IL-12 ($r=-0.458$, $p<0.001$), IL-8 ($r=-0.275$, $p<0.01$) and IFN-γ ($r=-0.227$, $p<0.05$) levels (Figure 2). A direct correlation was recorded between IFN-γ levels and IL-6 ($r=0.320$, $p<0.01$), IL-12 ($r=0.302$, $p<0.01$) and TNF-α ($r=0.501$, $p<0.001$) and between levels of TNF-α and IL-12 ($r=0.329$, $p<0.01$) (Figure 3).

In the light of biochemical parameters, IL-8 was directly correlated with AST ($r=0.207$, $p<0.05$), ALT ($r=0.234$, $p<0.05$) and bilirubin ($r=0.349$, $p<0.001$) in NHL patients. Indirect correlation ($r=-0.211$, $p<0.05$) between plasma levels of PDGF and AST was demonstrated

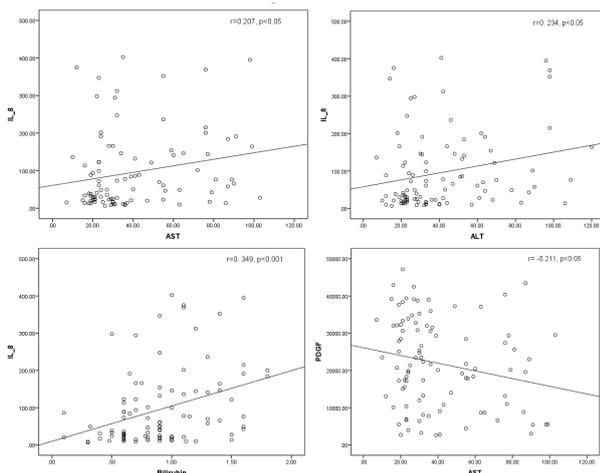


Figure 4. Correlation between Biochemical Parameter Levels and Plasma Levels of IL-8 and PDGF

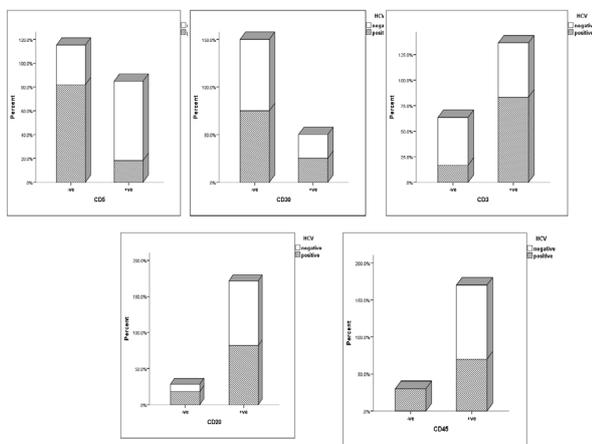


Figure 5. Collective Relationships Between CD Markers and HCV Infection in NHL Patients

(Figure 4).

CD markers in NHL-HCV patients

In this study, we compare the presence of CD5, CD30, CD3, CD20 and CD45 in both groups of NHL patients. Among NHL cases, there was a significant reduction ($p<0.05$) in NHL+ve HCV patients in the prevalence of CD5. In NHL-HCV positive group, CD5+ represent 18.2%, while CD5- represented by 81.8%. On the other hand, in NHL-ve HCV group, the percentage is 66.7% for CD5+ and 33.3% for CD5-. In the NHL -ve HCV group, CD45+ represent 100%, while in NHL +ve HCV group CD45+ and CD45- represented by 70% and 30%; respectively, indicating to a significant ($p<0.05$) reduction in CD45 in NHL with HCV infection. Although it is not statistically significant, a noticeable reduction in CD20 in NHL-HCV positive patient was demonstrated. CD3 and CD30 did not show any statistical significance between both groups.

A significant correlation ($r=-0.480$, $p<0.05$) between the presence of CD5 and HCV infection in NHL patients also with CD45 ($r=-0.471$, $p<0.01$). A direct correlation ($r=0.302$, $p=0.033$) between HCV infection and CD3 was recorded. Figure 5. shows a collective relationship between CD markers and HCV in NHL patients. A highly significant ($p=0.801$, $p<0.001$) correlation recorded

between CD45 and CD20.

CD markers with plasma levels of cytokines and biochemical parameters

In the whole patients' CD45 and CD20 showed an inversely significant correlation ($r=-0.425$, $p<0.05$ and $r=-0.340$, $p<0.01$) with plasma levels of IL-6 and IL-8; respectively. With biochemical data; CD45 correlated directly ($r=0.436$, $p<0.05$) with the number of white blood cells. CD3 showed a direct correlation ($r=0.343$, $p<0.05$) with Hemoglobin levels. In the view of liver enzymes, CD5 and CD45 showed an indirect correlation ($r=-0.412$, $p<0.05$ and $r=-0.403$, $p<0.05$; respectively) with ALT plasma levels.

Discussion

HCV is well-recognized as a cause of chronic liver diseases and hepatocellular carcinoma. The spectrum of HCV infection with hematological manifestations such as NHL was recorded (Sung et al., 2003). Over the last decade, the role of HCV in the development of B-cell NHL is becoming better understood by the significant progress in this research field as the number of patients diagnosed with HCV and NHL rises gradually (Hartridge-Lambert et al., 2012). In our study, the prevalence of HCV infection is about 37.11% of total cases; several studies have reported a high prevalence of HCV infection in patients with B-cell NHL with different association rates (9% to 32%) (Ferri et al., 1994; Mazzaro et al., 1996; Silvestri et al., 1996; Izumi et al., 1997; Murakami and Watanabe, 1997; Yoshikawa et al., 1997; Zuckerman et al., 1997; Harakati et al., 2000; Mizorogi et al., 2000). The study of Cowgill et al. (2004) on the Egyptian population recorded that 42% of subjects had HCV infection, supporting the hypothesis that NHL is a malignant outcome of chronic HCV infection (Cowgill et al., 2004). Rastin et al. (2013) demonstrated that the prevalence of HCV virus has increased in NHL samples supposing that these differences in HCV rate might be due to the environmental factors, race, and the presence of different genotypes and mutations (Rastin et al., 2013)

In the context of B-cell proliferation, it has been shown that HCV plays a pathogenic role in inducing B-NHL (Silvestri et al., 1996). HCV carries the risk to develop the B-NHL, possibly because viral antigens stimulate the host's inflammatory response via extracellular pattern recognition receptors (Dolganiuc et al., 2004). The strong association between HCV infection and B-NHLs has led to search for molecular signatures that can predict patients' characteristics, enhance understanding of biological mechanisms of lymphomagenesis, and might have diagnostic/clinical utility (De Re et al., 2012).

An involvement of the immune system in the pathogenesis of HCV-related lymphoproliferative disorders are probably happening (Libra et al., 2006). Cytokines play important roles in B-cell activation, proliferation, and apoptosis thence may be etiologically associated with risk of B-NHL (Gu et al., 2010). However, how cytokines might be involved in the development of B-NHL in patients chronically infected of HCV still remains obscure (De Re et al., 2012). Increasing evidence

indicates that cytokines play an important role in liver metabolism and in the immune response to viral agents and elevated intrahepatic levels of IL-2, IL-6 and IL-8 was demonstrated in patients with cirrhosis (Napoli et al., 1994; Talaat et al., 2014).

The association between plasma levels of cytokines and NHL with HCV infection has not been prospectively studied. Our study recorded a significant decrease in levels of VEGF, PDGF and IFN- γ in NHL Patients with HCV infection. In the other hand, a significant increase in levels of IL-12 and IL-8 was demonstrated. This up-regulated production might play a role in lymphomagenesis in HCV+ve patients seems consistent with the augmented blood levels of IL-8 found in HCV associated NHL patients.

There are some studies on this context in vitro. Dolganiuc et al. (2004) found induction of levels of IL-6, IL-8, and TNF- α in peripheral blood monocytes isolated from HCV-infected NHL individuals (Dolganiuc et al., 2004). Additionally, Feldmann et al. (2006) study recorded elevation in IL-6 and IL-8 serum levels supposed that the virus core induced production of these cytokines in CD14+ cells which lead to increased B-cell proliferation in vitro (Feldmann et al., 2006).

In our study, IL-6, IL-4 and TNF- α did not show any statistical significance. In contrast to our data, the study of De Re et al. (2012) recorded a higher production of Th2 cytokines (IL-6, IL-4, IL-10, and TNF- α) in NHL patients with HCV infection, suggesting that the Th2 immune response, by means of T-cell-dependent B-cell stimulation, may promote B-NHL (De Re et al., 2012).

Immunohistochemistry is widely used to improve characterization of a number of tumors and to detect occult metastases, especially in lymph nodes. implementation in lymphoma fundamentally associate to providing information on the classification of lymphomas in diagnostic primary tissue (Talaulikar et al., 2008). In the current study, CD markers prevalence showed a variation in their expression in accordance to the HCV infection. CD5 and CD45 showed a negative correlation with HCV infection in NHL patient. In agreement with these results, Palmeira et al. (2012) demonstrated that the malignant B-lymphocytes in NHL were negative CD5 and CD45, but positive CD20 (Palmeira et al., 2012). The study of Gervasi et al. (2004) approved that most of cells in most of the cases had a positive CD5, CD20, and CD45 (Gervasi et al., 2004). In contrast to our study, Hamdy et al. (2015) showed that 90 % of patients had positive CD20 and CD19 in 30 case had NHL with HCV infection (Hamdy et al., 2015). In this work, CD3 showed a positive correlation with HCV infection in NHL patients. In accordance with our data, the results of Pawełczyk et al. (2013) indicate that CD3+ cells are a dominant site for extrahepatic HCV replication (Pawełczyk et al., 2013). In the current results, we found that plasma levels of AST, ALT showed a significant increase in NHL +ve HCV group compared to negative group. On contrary, the study of Marignani et al. (2010) showed no correlation between viral load and ALT levels (Marignani et al., 2010).

In conclusions, Taken together, our study points that HCV is highly prevalent in Egyptian NHL patients. The

presented data suggest that the decrease in at least two of the pro-angiogenic mediators (VEGF, PDGF) and non-significant change in TNF- α , TGF- β and IL-6 from NHL patients with HCV infection may reflect an ongoing anti-angiogenesis response. In the sight of CD markers, a reduction in CD5, CD20 and CD45 and increase in CD3 was present in HCV infected NHL patients. This data might be indicative of the loss of professionally of both B and T cells through a reduction in their CD markers. Therefore, further investigation is warranted by the development of new strategies for treating HCV-positive lymphoma taking in consideration the change in cytokine levels and CD expression. Moreover, treatment of HCV associated NHL should be performed as organized work between hepatologist, hematologist and immunologist.

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