Chronic Hepatitis C Virus Infection and B-cell clonality

Marwa I. Abdelgawad[1], Ebtesam M. El Gezawy [2] and Hanan Sharaf El-Deen Mohammed[3]

[1]Clinical Oncology Department, [2]Clinical Pathology Department, [3]Internal Medicine Department, Faculty of Medicine, Assiut University

Abstract: Background and Aim: An association between non-Hodgkin`s lymphoma (NHL) and hepatitis C virus (HCV) has been evidenced since few years. Expansions of B-cell clonality have been detected in peripheral blood and bone marrow in HCV patients. Lymphoid aggregates are found in the liver of chronically HCV-infected patients. Studying the nature of the aggregates in the molecular level is a guide to find out the prevalence of B-cell clonality. In this work we aimed to study B-cell infiltration of the liver and type of expansion whether monoclonal or polyclonal in chronically HCV patients and factors affecting it. **Subjects and Methods:** Liver biopsies of 120 patients with chronic hepatitis C viral infection were examined for presence of lymphoid aggregation. Clonality of lymphoid B-cells tested by polymerase chain reaction (PCR) to identify immunoglobulin heavy chain gene (IgH) rearrangements. The product is monoclonal if only one discrete band was detected. The correlation between clonality on one side and HCV RNA viral load, stage of fibrosis, grade of inflammation, levels of transaminases, alpha fetoprotein (AFP) and lactic dehydrogenase (LDH) was studied. **Results:** Sixteen patients (13.33%) expressed monoclonal B-lymphocytic (MBL) expansion and 18 patients (15%) showed polyclonal B-lymphocytosis (PCBL). No significant difference in the stage of fibrosis, the degree of inflammation and AFP levels between MBL positive and negative groups was detected in the current study. MBL positive patients had significant much higher viral load (p=0.0001), AST(p =0.0001), ALT(p =0.006) and LDH(p =0.04) compared to MBL negative ones. On the other hand positive polyclonal B-lymphocytes (PCBL) had significant higher stage of fibrosis (p =0.011), grade of inflammation (p =0.026) and liver enzymes; AST and ALT (p =0.0001 for each) serum LDH (p =0.016) and lower viral load (p =0.0001) compared to negative PCBL patients, but no significant difference between PCBL positive patients and PCBL negative patient regarding AFP levels. Correlation between occurrence of clonal *IgH* with laboratory variables revealed significant positive correlation between the occurrence of clonal *IgH* and liver enzymes (AST, ALT), LDH, fibrotic stage and grade of inflammation. **Conclusion:** B-lymphocytic infiltration of the liver occurs in 28.3% in HCV chronically infected patients. We can expect lymphoproliferative infilteration in the liver using noninvasive laboratory investigation as AST, ALT and LDH. We could not depend on viral load to predict lymphoproliferative infilteration in the liver. Rearrangements of clonal *IgH* have significant positive correlation with liver enzymes (AST, ALT), LDH, fibrotic stage and grade of inflammation this suggest that these markers provides a convenient way to study the early stages of neoplastic transformation.

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1. Introduction:

The number of viruses associated with lymphoma has increased over the last 20 years, includes Epstein–Barr virus (EBV), human T-cell lymphotropic virus 1 (HTLV1), human immunodeficiency virus (HIV1 and 2) and human herpesvirus 8 (HHV8). HCV has only relatively recently been recognized as a potential cause of B-cell lymphoma (Turner *et al.,* 2003).

HCV is a lymphotropic and hepatotropic virus and a causative association between HCV and NHL was postulated relatively recently and has been the subject both of intense investigation and of some debate. HCV infection affects the B -cell compartment. Interaction between HCV virus and lymphocytes may occur through binding human CD81 expressed on B lymphocytes in the viral envelope protein E2, this binding could activate a lymphocyte proliferation (Fazi *et al.,* 2010).

In HCV patients, B-cell clonal expansions have been detected in peripheral blood, bone marrow and a high prevalence of B-cell non-Hodgkin`s lymphomas has been documented. Emerging biological investigations on epidemiological data in HCV is involved in pathogenesis in a proportion of patients with NHL (Fazi *et al.,* 2010).

HCV frequently causes extrahepatic manifestations, the most common and severe of which is mixed cryoglobulinemia (MC), a systemic vasculitis affecting small- and medium- sized arteries and veins. MC is characterized by the deposition of immune complexes containing rheumatoid factor (RF), immunoglobulin G (IgG), HCV RNA, and complement on endothelial surfaces, eliciting vascular inflammation through poorly understood mechanisms.

It is clear, however, that HCV MC is a B cell lymphoproliferative disorder (LPD). It is

unknown how B cells activate, clonally expand and differentiate to produce pathologic quantities of self-reactive RF during chronic HCV infection (Charles and Dustin, 2009).

Various subtypes of lymphomas appear to be associated with HCV mainly diffuse large B-cell lymphoma, small lymphocytic lymphoma/ chronic lymphocytic leukaemia (CLL) and marginal zone lymphoma are particularly represented among HCV-positive patients. The prevalence of monoclonal B-cell lymphocytosis reportedly ranges from 3-5% in the general population over the age of 50 years. On the other hand, Nieto *et al.* (2010) and Guidicelli *et al.* (2012) showed that non-CLL-like MBL are also detected in the general population and they show clonality that can be associated with marginal zone lymphoma, lympoplasmacytic lymphoma and mantle cell lymphoma (Nieto *et al.*, 2010) and (Guidicelli *et al.*, 2012).

HCV infection is related intimately to B-cell clonality in lymphoma especially diffuse Large Cell Lymphoma subtype despite its aggressive nature. Clinical presentation of HCV-associated DLBCL has been reported to differ from the HCV-negative counterpart (Fognani *et al.*, 2013).

There is a specific role of HCV virus in maintaining B-cell proliferation, although the exact mechanism remains unknown. This association, together with the histopathology, tolerance to standard-of-care chemo-immunotherapy (R-CHOP or CHOP-like regimens) and final outcome of HCV-positive lymphoma are still matter of debate. HCV infection may influence the short-term outcome of B-NHL because of the emergence of severe hepatic toxicity (HT) during immunochemotherapy. Furthermore, the long term outcome of HCV-related liver disease and patients' quality of life will possibly be affected by rituximab maintenance, multiple-lines of toxicity during chemotherapy and hematopoietic stem cell transplantation (Cox *et al.*, 2013).Moreover, rituximab enhances viral replication but with low probability of hepatic complications. HCV viral load in this setting is not essentially directly associated with liver damage. Therefore a multidisciplinary team with hepatologists and hematologists with close monitoring of liver functions for management of those patients is required. Unless contraindicated by adverse clinical conditions, patients should be treated with standard immuno-chemotherapy. Antiviral treatment should be considered after the end of immuno-chemotherapy, when lymphoma remission has been achieved (De Vita *et al.*, 2012).

Aim of the work

In this work we aimed to study the presence of B-cell infiltration of the liver of chronically HCV infected patients and the type of expansion whether monoclonal or polyclonal in Assiut University (Clinical Oncology, Internal Medicine departments and Virology outpatient clinic) from August 2011 to December 2014. We also studied the factors affecting the B-lymphocyte expansion being monoclonal or polyclonal.

2. Subjects and Methods

One hundred twenty patients from the virology outpatient clinic and Internal Medicine outpatient clinic were recruited in this study. Those patients were diagnosed to have chronic HCV infection both by the detection of HCV-Ab version 3 using ABOTT AXSYM automated immunoassay analyzer and by positivity for circulating HCV-RNA for more than 6 months by Quantitative Real Time PCR for the detection of HCV RNA and viral load using (Applied Biosystems 7500 Fast Real-Time PCR System, CA, USA).They were HBsAg and HIV negative.

A liver panel, which included platelets count (Plt) (using Cell Dyne 3500 automated cell counter), international normalized ratio(INR) (using Thromboril S kit) and levels of total and direct bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin and total proteins was obtained in all patients using BM Hitachi 911 Chemistry Analyzer. LDH was measured by Kinetics spectrophotometrica method. AFP was measured by Chemiluminescence using immulite 1000.

Liver specimens:

For each patient, liver biopsy was taken after making sure that there are no contraindications for biopsy. Liver specimens were washed with PBS solution. Hemorrhagic liver biopsy specimens were eliminated from this study to avoid contamination of liver cells by circulating B lymphocytes. Specimens were stored at -70◦C. The DNA was then isolated from frozen liver tissue.

The histological features of liver biopsy specimens were analyzed according to the Metavir scoring system according to (*Bedossa and Poynard,* 1996). Liver biopsy specimens (> 10mm in length) were fixed, paraffin-embedded, and stained with either hematoxylin-eosin-saffron. The stage of fibrosis and grade of activity were established for each case. Liver fibrosis was staged on a scale of F0-F4, where F0=no fibrosis, F1= portal fibrosis without septa, F2=few septa, F3= numerous septa without cirrhosis, and F4=cirrhosis. The grading of activity, which evaluates the intensity of necro-inflammation, was performed using a scale of A0-A3 where A0 =no histologic activity, A1= mild activity, A2= moderate activity, and A3= severe activity (*Bedossa and Poynard* , 1996).

Polymerase chain reaction of IgH rearrangements:

DNA was extracted from liver tissue using QIAamp® DNA mini kit (QIAGEN Cat No.51304), according to the manufacturer's protocol. Amplification was done to the hypervariable complementary region (CDR-II and CDR-III), included between the third and joining regions (FR-III and JH) of *IgH* genes, with the following primers, using a semi-nested protocol.

The primers were:

- Primer 1: upstream primer of FR3: 5'-CTGTCGACACGGCCGTGTATTACTG-3'
- Primer 2: outer downstream primer, directed to an outer conserved sequence at the 3' end of the IgH J region: 5'-AACTGCAGAGGAGACGGTGACC-3'
- Primer 3: Inner downstream primer, directed to an inner conserved sequence of the IgH J region: 5'-GTGACCAGGGTNCCTTGGCCCCAG-3'.

For semi-nested PCR we use PuReTaq Ready-To-Go™ PCR Beads (GE Healthcare, Munich, Germany) (Lot Number: 389310) in a 25-µl reaction mixture .In the first-round PCR the PCR mixture was as follow (1ul of each primer 1 and 2 and 10 ul of DNA and 13 ul water). The amplification profile was as follows: initial denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 2 min for 30 cycles and a final elongation at 72°C for ten min. In the second -round PCR the PCR mixture was as follow (1ul of each primer 1 and 3 and 1ul o f amplified product in the first run and 22 ul water).Before each round the PCR reaction was heated at 95°C for 7 minutes. In parallel with the samples, negative controls containing no DNA were run. Ten microlitres of the PCR products were analyzed by electrophoresis on 3% agarose gels, stained by ethidium bromide, and viewed under UV light. PCR products were considered to be monoclonal if only one discrete band within the expected size range (100-150 bp) was observed and polyclonal if a diffuse smear pattern or multiple faint indistinct bands were observed on the gel after electrophoresis.

All the involved patients underwent history and clinical examination. A written informed consent was obtained from patients involved in the study.

Statistical analysis:

This study is a cross-sectional study. All the quantitative data were expressed in mean ± Standard deviation (SD). We used two-tailed student T test for calculating the means and SD. An independent-sample test was used to compare difference in means. The Spearman test was used to test correlation between two parameters. Categorical variables were expressed as absolute numbers and percentages.

The results were considered significant when *P*-value is less than 0.05%. All data were analyzed using the SPSS software version 13.

3. Results:

One hundred twenty male patients having HCV infection were included in this study. All of them were discovered after routine testing for the HCV as a prerequisite for working abroad. None of them was suffering from any symptoms and clinical examination was completely free. Absolute lymphocytic count was normal. Sixteen patients had monoclonal B-lymphocytes detected in the liver biopsy (13.33%) and 18 patients had polyclonal B-lymphocytes (15%). According to Metavir scoring system, 9 PCBL cases exhibit fibrotic stage (F4) compared to 3 cases in MBL patients and 10 PCBL cases exhibit activity grade (A3) compared to 5 cases only in MBL (Table 1).

Table (2) shows a comparison between patients who were positive for MBL and those who were negative.

Patients who were positive for clonal B-lymphocytes were not significantly different in age, INR and platelet count from those who were negative for clonal B-lymphocytes. However, serum level of Liver enzymes AST & ALT and LDH were significantly higher in MBL positive patients compared to those who were negative. Serum AFP showed insignificant differences between MBL positive and MBL negative patients. Viral load of HCV RNA was significantly higher in MBL positive compared to MBL negative patients. There was no significant difference in the grade of inflammation or stage of fibrosis between the two groups.

Table (3) showed a comparison between patients who expressed PCBL in their livers and those who did not. Patients who were positive for clonal B-lymphocytes were not significantly different in age, INR and platelet count from those who were negative for clonal B-lymphocytes. Patients who were positive for PCBL had significantly higher levels of AST, ALT, LDH compared to PCBL negative patients. Serum AFP showed insignificant differences between PCBL positive and PCBL negative patients. PCBL positive patients had a significantly higher stage of fibrosis and grade of inflammation compared to PCBL negative patients. Patients with PCBL had significantly lower levels of HCV RNA viral load compared to those with negative PCBL.

Table (4) showed the correlation between the presence of clonal *IgH* rearrangements with laboratory variables where there was significant positive correlation between the occurrence of clonal *IgH* and liver enzymes (AST, ALT), LDH, fibrotic stage and grade of inflammation.

Table (1): Chronic hepatitis C virus patients according to clonality and Metavir score

Parameters	Chronic hepatitis c male patients (N&%): 120 (100%)	
	Monoclonal 16 (13.33%)	Polyclonal 18 (15%)
Stage of fibrosis (N)		
F1	5	2
F2	4	3
F3	4	4
F4	3	9
Grade of inflammation (N)		
A1	6	3
A2	5	5
A3	5	10

Table (2) Characteristics of monoclonal B-lymphocytosis (MBL) positive patients compared to MBL negative patients

	MBL positive patients	MBL negative patients	Significance of difference
Age (years)	45.5 ± 2.4	45.3 ± 4.43	NS
International normalized ratio(INR)	1.07±0.09	1.06 ± 0.07	NS
Aspartat transaminaza (AST) (IU/L)	49 ± 12.2	29.5 ± 9.7	P=0.0001
Alanine transaminase (ALT) (IU/L)	60.22 ± 15.41	24 ± 13.78	P=0.006
Platelet count (X 10^9/ L	202 ± 51	225.77 ± 68.83	NS
Lactate dehydrogenase (LDH) u/l	560±20	200±40	P=0.04
Alpha feto protein(AFP) ng/ml	8.1±1.7	7.9±0.5	NS
Viral load (IU/L)	2965,000 ±484,000	1237,000±501,000	P= 0.0001
Stage of fibrosis	2.01 ± 0.78	1.5± 0.53	NS
Grade of inflammation	2.1 ± 0.78	1.6 ± 0.52	NS

Table (3) Characteristics of polyclonal B-lymphocytosis (PCBL) positive patients compared to PCBL negative patients

	PCBL positive patients	PCBL negative patients	Significance of difference
Age (years)	44.00 ± 2.1	45.85 ± 2.4	NS
International normalized ratio(INR)	1.09 ± 0.37	1.08 ± 0.35	NS
Aspartat transaminaza (AST) (IU/L)	64.000 ± 35.27	19.84 ± 13.7	*P* =0.007
Alanine transaminase (ALT) (IU/L)	56.5 ± 28.33	29.38 ± 19. 9	*P* = 0.007
Platelet count (X 10^9/ L	211,000 ± 1.07	223 ± 66.9	NS
Lactate dehydrogenase (LDH) u/l	575±30	190±10	*P* =0.016
Alpha feto protein(AFP) ng/ml	6.4±1.9	7.31±1.4	NS
Viral load (IU/L)	54,000 ± 51,314	1351,610± 1384,914	*P* = 0.0001
Stage of fibrosis	2.5 ± 1.53	1.85 ± 0.78	*P* = 0.011
Grade of inflammation	2.5 ± 0.53	1.85± 0.69	*P* = 0.026

Table (4) Correlation between clonal IgH rearrangement with other parameters

IgH rearrangement		
Parameters	r	*P* value
Age (years)	-0.211	0.197
International normalized ratio(INR)	0.011	0.77
Aspartat transaminaza (AST) (IU/L)	0.293	0.04
Alanine transaminase (ALT) (IU/L)	0.399	0.032
Platelet count (X 10^9/ L	0.078	0.67
Lactate dehydrogenase (LDH) u/l	0.311	0.043
Alpha feto protein(AFP) ng/ml	-0.274	0.068
Viral load (IU/L)	0.189	0.214
Stage of fibrosis	0.393	0.047
Grade of inflammation	0.489	0.014

4. Discussion:

Presence of B-cell expansion and infiltration of the liver in patients with chronic HCV was studied in this study. B-cell expansion was detected in 28.3% of our HCV patient group. Using the molecular analysis of IgH gene rearrangement, the monoclonal B-cell infiltration was detected in 13.33% of our patients. The PCBL were detected in 15%. Fan et al., (2009) found that MBL to be 10% in HCV-positive patients but Vallat *et al.* (2004) and Fazi *et al.* (2010) reported monoclonal B lymphocytes in HCV patients around 30%. However, Vallat *et al.* (2004) considered clonal B cell infilteration in every sample in which B cell population could be detected including single minor clonal population, several minor clonal population or a dominant clone with other minor clones. In our study only those with single clone were considered monoclonal.

Vallat *et al.*, (2004) reported that only one case (3.3%) among 30 liver biopsies from non-HCV control group showed MBL compared to (30%) in chronic hepatitis C patients, this supports the concept that HCV is strongly associated with the development of lymphoproliferative disorders (Vallat *et al.*, 2004).

Inokuchi *et al.* (2009) suggested that HCV RNA is an independent factor associated with the presence of clonal B lymphocytes among other markers for lymphoproliferation. Also Visco *et al.*, (2012) found Lymphoid follicles more often in chronic hepatitis C (about 55%) than in chronic hepatitis B (about 31%) (Visco *et al.*, 2012).

Matsuo *et al.*, (2004) demonstrated that 2.5% of patients infected with HCV were at risk of developing NHL within a 15-year period follow up and during the same period following antiviral therapy, none of the patients who cleared the virus developed NHL (Matsuo *et al.*, 2004).

In the current study stage of fibrosis and degree of inflammation was higher in MBL positive patients than MBL negative patients but the difference was insignificant. On the other hand, PCBL positive patients had significantly higher grade of inflammation and stage of fibrosis compared to PCBL negative patients. De Re *et al.* (2012) denied the association between the presence of clonal B cell expansion and liver histology , but Vallat *et al.* (2004) said that patients with clonal B cell expansion occurs in liver disease that was more severe as shown by the higher Metavir activity score (Vallat *et al.*, 2004).

Zielecka *et al.* (2008) analyzed the composition of inflammatory infiltrate and its correlation with HBV/HCV antigen expression they found that B-lymphocytes (CD20) made up to 10% -33% of the infiltrate and the number of B lymphocytes increased parallel to inflammatory activity (Zielecka *et al.*, 2008).

patients with positive MBL and PCBL had significant much higher liver enzymes (AST and ALT) compared to clonal negative ones in contrast to De Re *et al.* (2012) who denied the association between the presence of clonal B cell expansion and liver enzymes (De Re *et al.*, 2012).

In the current study, the most striking finding is the significant difference in the HCV RNA load in MBL and PCBL patients where positive MBL patients had much higher load compared with MBL negative ones in spite of that the reverse was true regarding PCBL where viral load was significantly lower in PCBL positive patients which make a dilemma as how viral load exhibit diverse presentation in MBL and PCBL?. This is might be due to the relationship between the severity of liver cell damage and viral count is weak because the technique of viral load test is very variable and the results are not reliable moreover, different times of assay almost always produce different results. Moreover, FDA (Food and Drug Administration) has not yet approved viral count as a diagnostic procedure for degree of liver damage and it is only used for research purposes (Monsour, 2001). Supporting that, Liu et al., (2009) concluded that serum HCV-RNA titer could not reflect the degree of liver histological damage (Liu *et al.*, 2009).

Vallat *et al.* (2004) said that patients with B cell expansion more frequently had a high HCV load compared with those who had not, however, accordance to their results and our results clear cut correlation was not reported between viral load and clonality of B-cell or IgH rearrangements (Vallat *et al.*, 2004).

Matsuo *et al.* (2004) tried to give evidence for the correlation between HCV RNA load and the lymphotropic effect after treating 9 patients with splenic lymphoma, villous lymphocytes (SLVL) and HCV infection with interferon alfa-2b alone or with ribavirin. Of the 9 IFN-treated patients, 7 achieved a complete haematological remission, defined by the absence of abnormal lymphocytosis and the resolution of spleneomegaly, after the HCV RNA load became undetectable (Hermine *et al.*, 2002), but the number of their cases was small that we cannot depend on their results.

Similar results were reported by Saadoun *et al.* (2005) and Batch *et al.* (2010) who demonstrated a causal relationship between HCV replication and lymphogenesis in SLVL. They added that among patients with marginal zone lymphoma of MALT or splenic subtypes, alpha-IFN and ribavirin yielded a 60% response rate, which was correlated to virologic response in most cases. (Batch *et al.*, 2010 and Saadoun *et al.*, 2005), but their results could be due to the effect of interferon itself.

In the current study patients with positive MCBL and PCBL had higher levels of LDH than

clonal negative patients supporting this finding, De Renzo *et al.* (2008) reported that, patients with HCV related diffuse large B-cell lymphoma (DLBCL) have been described to have more frequent extra nodal involvement (especially spleen and liver) and elevated lactate dehydrogenase (LDH) compared to their HCV-negative counterparts (De Renzo *et al.*, 2008).

It is possible to regard monitoring serum LDH even if it is produced in various organs (Koukourakis *et al.*, 2006) as it showed significant positive correlation with IgH rearrangements

In spite of LDH is potentially biased in HCV-positive patients, as it is influenced not only by lymphoma but also by chronic HCV infection, the diagnostic usefulness of this enzyme by itself and determination of the iso enzymes that comprise the LDH better to be considered as Yang *et al.* (2009) concluded that levels of LDH is valuable for clinical stage, prognosis and evaluation of therapeutic response in patients with NHL (Yang *et al.*, 2009).

In the current study patients with positive and negative clonal expansion whether MCBL or PCBL have insignificant differences in AFP serum levels so AFP could not be considered as a good test to suspect possibility of lymphoma occurrence in chronic hepatitis C infection.

Correlation between the presence of clonal *IgH* rearrangements with laboratory variables revealed significant positive correlation between the occurrence of clonal *IgH* and liver enzymes (AST, ALT), LDH, fibrotic stage and grade of inflammation.

Conclusion

We can expect lymphoproliferative infilteration in the liver using noninvasive laboratory investigation as AST, ALT and LDH.

We could not depend on viral load to predict lymphoproliferative infilteration in the liver.

Rearrangements of clonal *IgH* have significant positive correlation with liver enzymes (AST, ALT), LDH, fibrotic stage and grade of inflammation this suggest that these markers provides a convenient way to study the early stages of neoplastic transformation.

Recommendations:

Future studies may be needed for detecting the effect of antiviral treatment on the prevalence of B- cell clonality in chronic HCV patients.

Lymphoproliferative infilteration should be considered during treatment of chronic HCV patients.

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