Original Article

B-cell monoclonal lymphocytosis in chronic hepatitis C virus infection

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Summary

Background: *Clinical data document the connection between hepatitis C virus (HCV) and B cell proliferative. Monoclonal B lymphocytosis (MBL) is an asymptomatic condition characterized by the presence in the peripheral blood of a clonal B–cell population which might evolve into malignant B cell lymphoproliferative disease, like chronic lymphocytic leukemia or indulant B cell lymphoma. The underlying association between HCV and MBL is not yet understood.* ***Aim of the work:*** *To evaluate the presence of monoclonal B lymphocytosis in patients with chronic hepatitis C with various activity and severity according to METAVIR Scoring System.* ***Methods****: The study included one hundred patients with chronic HCV infection and forty healthy controls. Liver biopsy was done for patient's group and analyzed according to METAVIR Scoring System. Flow cytometric analysis for B-cell monoclonal lymphocytosis was done for both patients and control groups.* ***Results****: MBL were identified in 24/100 (24%) in patients with chronic HCV at significantly higher frequency versus in the control group 3/40 (7.5%). No significant difference regarding MBL was found between early (F1-F2) and late (F3-F4) fibrosis subgroups; however, MBL expression was significantly higher in A1 when compared to A2 and A3 activity grads. Concerning laboratory data there was no significant difference between patients with MBL and patients without MBL apart from significant increase in total leucocyte count in patients without MBL. Also the age was significantly increased in MBL positive patients versus MBL negative patients.* ***Conclusions****: B-cell monoclonal lymphocytosis showed significant increase in HCV infected patients more than in the general population. No significant differences in MBL expression between early and advanced fibrosis stage however, MBL expression was significantly higher in A1 versus A2 and A3activety grads.*

Keywords: *B-cell monoclonal lymphocytosis; chronic hepatitis C infection; Liver biopsy; Liver fibrosis*

Introduction

Hepatitis C virus (HCV) infection is a global health problem which affects a significant proportion of the world[1]. About 80% of newly infected patients progress to develop chronic infection. Cirrhosis develops in about 10% to 20% of persons with chronic infection, and liver cancer develops in 1% to 5% of persons with chronic infection over a period of 20 to 30 years[2]. Besides being a hepatotropic virus, HCV is also lymphotropic and its infection affects the B lymphocytes compartments, with the occurrence of B cell proliferative disorders. HCV infection is strongly associated with mixed cryglobulinemia (MC)[3]. In addition, HCV has been suggested to play a role in the pathogenesis of B cell non –Hodgkin lymphomas (NHL) outside the context of MC[4]. Several distinct types of NHL can be associated to HCV infection, including diffuse large B cell-lymphoma (DLBCL)[5], marginal zone lymphoma (splenic-SMZL, nodal and extranodal)[6], chronic lymphocytic leukemia (CLL)[7], and lymphoplasmacytic lymphoma. Monoclonal B lymphocytosis (MBL) is an asymptomatic condition characterized by the presence in the peripheral blood of a clonal B–cell population that might evolve in to malignant B cell lymphoproliferative disease, like chronic lymphocytic leukemia (CLL) or indulant B

cell lymphoma[8]. According to revised guidelines developed at the 2008 international workshop on chronic lymphocytic leukemia, MBL involves fewer than 5×109 /L in the peripheral blood, in the absence of clinical signs or symptoms of a B –cell lymphoproliferative disorders (B-LPD)[9]. Most MBL clonal B cells express an immunopheno type similar to that observed in chronic lymphocytic leukemia[10]. The absolute B-cell count (B-ALC) threshold distinguishes this CLL-like MBL from CLL. However, progression to leukemia requiring CLL- directed therapy occurs in approximately 1% to 2% of cases per year[11]. Studies have reported a wide range in MBL prevalence, dependent largely on the characteristics of the population exa-mined and the detection methods utilized for MBL identification. Prevalence in the general adult population (not including those with a family history of CLL) ranges between 0.1% to 14% according to a systematic review of MBL population studies[12]. Clinical data support the relationship between HCV and B cell proliferative disorders[13] as also demonstrated by the fact that antiviral therapy may lead to regression of at least a portion of HCV-related lymphomas[14]. HCV affects more generally the B cell compartment in infected individuals, leading to the appearance of monoclonal B lymphocytes in the blood, bone marrow, and liver as revealed by the detection of monoclonal immunoglobulin heavy chain (IGH) gene rearrangements in all these tissues[15]. This study aims to analyse the presence of monoclonal B lymphocytosis in patients with chronic hepatitis C with various activity and severity which may help in the future for further define the relation between chronic HCV infection and the risk of development of clinically relevant B cell lymphproliferation.

Patients and Methods

The present work included one hundred patients with chronic HCV infection and forty healthy controls. The studied population were divided into two groups; the first group is the patient group included 100 patients with chronic HCV infection [60 males (60%) and 40 females (40%)] with their mean age is 44.2 years; the second group is the control group included 40 healthy subjects, [23 males (57.5%) and 17 females (42.5%)]. They were age and sex matched and living in the same area and environment. The patients were collected from outpatient's clinics of Tropical Medicine Department in Mansoura University Hospital. All patients and control groups gave written informed consent to their particippation in this study, and the local ethics committee gave their prior approval to the study. **1. Inclusion criteria**: * Age group from 18 to 60 years. * Chronic hepatitis C virus infection. **2. Exclusion criteria of** cases: other causes of chronic liver disease including; chronic HBV infection, co-infection with hepatitis B (positive HBs Ag), hepatocellular carcinoma, alcoholic related liver disease, metabolic liver diseases and autoimmune liver disease. Also, other causes of lymph proliferative disease include; Mantle cell lymphoma, prolymphocytic leukemia, hairy cell leukemia follicular lymphoma, marginal zone lymphoma and history of Childhood CLL. All patients will undergo the following; complete history taking, full medical examination. **3. Laboratory investigations**: including, complete blood picture, liver function tests (serum albumin, serum bilirubin, SGPT, SGOT, PT, INR, α fetoprotein), serum creatinine, viral markers (Anti HCV, HBsAg, Anti HIV) and PCR for HCV RNA. **4. Abdominal ultrasound (US)**. **5. Percutaneous Liver Biopsy** (Ultrasound guided). * The patient lies supine and rests the right hand above the head. A local anesthesia can be used at the site of insertion of the needle biopsy. * Make a small incision in the right side of the abdomen of the patient at the upper border of the lower rib. * Insert the needle biopsy. * Ask the patient to exhale and hold his breathing while inserting the needle and quickly removes a sample of liver tissue. * Place a bandage over the incision site. * The patient has to lie on his right side up for 2 hours to reduce the risk of bleeding, with medical monitoring of signs of bleeding for 2 to 4 more hours[16].

METAVIR Scoring System

According to the Metavir Score System, fibrosis is scored as F0 (abscent), F1 (portal fibrosis), F2 (portal fibrosis with few septa), F3 (septal fibrosis) and F4 (cirrhosis). Both F1 and F2 are considered as early fibrosis and F3 &F4 are considered as late fibrosis. In addition, necroinflammation activity (A) is graded as A0 (abscent), A1 (mild), A2 (moderate) and A3 (severe)[17].

Flow cytometric analysis for B-cell monoclonal lymphocytosis

It was carried out at flow cytometry laboratory of Oncology Center, Clinical Pathology Department, Mansoura University, Mansoura, Egypt.

(1) *Preparation of peripheral blood film*

Thin blood film is prepared by spreading 10ul whole blood on glass slide (Fig1).

(2) Flowcytometry

Cells were stained with the specific monoclonal antibodies and then acquired on FACS caliber TM (BD bioscience, San Jose, CA) and analyzed using flow software (BD Bioscience).

(3) Staining protocol for flowcytometry

Human whole blood are the samples commonly used for this assay .The number of T cells& malignant lymphocyte clone cells expressing PD1 and PDL1 will be assessed by flowcytometry in all studied individual according to the stain and then -lyse -and -wash protocol.

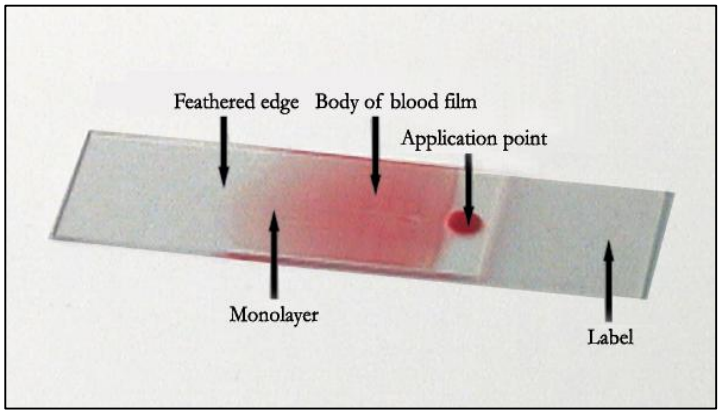

Figure (1) showing peripheral blood film slide[18].

Statistical Methods

All statistical analyses were carried out using the SPSS version 17.0 software (SPSS Inc., Chicago, Illinois, USA). Continuous variables were described as mean ± SD and categorical variables were described a sa perce-ntage (frequency). Wilcoxon rank sum tests and Student's t-tests were used for continuous factors; Fisher's exact and Pearson's $\chi 2$-tests were used for categorical factors. The ROC Curve (receiver operating characteristic) provides a useful way to evaluate the sensitivity and specificity for quantitative diagnostic measures that categorize cases into one of two groups. The optimum cut off point was defined as that which maximized the AUC value. The area under the ROC curve (AUC) results were considered excellent for AUC values between 0.9-1, good for AUC values between 0.8-0.9, fair for AUC values between 0.7-0.8, poor for AUC values between 0.6-0.7 and failed for AUC values between 0.5-0.6.A P value of less than0.05 was considered significant.

Results

The study included one hundred patients with chronic HCV infection and forty healthy controls with cross matching as regard the age and the gender. Moreover, patients with chronic HCV sub grouped according to stage of fibrosis into; early fibrosis (F1-F2) were 49 patient and advanced fibrosis (F3-F4) were 51 patients with no significant difference as regard the age and the sex, tab. (1). Table (2) shows a comparison of B-cell monoclonal lymphocytosis between studied groups. There was significant increase in B-cell monoclonal lymphocytosis in HCV infected patients when compared to control group while, no significant difference was found between early and late fibrosis subgroups. Moreover, MBL expression was significantly higher in A1 when compared to A2 and A3, fig. (2). Table (3) shows comparison between patients with MBL and patients without MBL regarding the demographic data with no significant difference as regard the sex but, with significant increase in the age in chronic HCV patients with MBL. Concerning laboratory data there was no significant difference between patients

with MBL and patients without MBL apart from significant increase in total lecocytic count in patients without MBL and AST which was more in patients with MBL than in patients without MBL, tab. (4).

Receiver operating characteristic (ROC) curve analysis of MBL expression as a predictor for progression of liver fibrosis from early to advanced fibrosis.

The area under the ROC (AUC) and performance characteristics of MBL expression failed to predict the stage of liver fibrosis and also cannot differentiate between early and advanced fibrosis; (AUC =.542, 95% CI=.428-.656; p=.467). At MBL best cut off value of 4, sensitivity was 52.9%, specificity was 53.1%, tab. (5) and fig (3).

Table (1) Demographic data in control and HCV patients according to fibrosis stage.

Studied groups		Control group (N=40)		Patient group (N=100)		Patients with Fibrosis (Number=100)				P[1]	P[2]
						Patients with Early fibrosis (N=49)		Patients with Advanced fibrosis (N=51)			
		Mean±SD		mean±SD		mean±SD		mean±SD			
Age (in years)		42.40±6.332		44.19±7.176		44.27±7.812		44.12±6.584		0.872	0.919
		N	%	N	%	N	%	N	%	-	-
Sex	Male	23	57.5	60	60	33	67.3	27	52.9	0.786	0.142
	Female	17	42.5	40	40	16	32.7	24	47.1	-	-

***P1**, comparison between patients and control groups; p2: comparison between early fibrosis (F1-F2) and advanced fibrosis (F3-F4).*

Table (2) Comparison of B-cell monoclonal lymphocytosis between studied groups.

Studied groups	Control (N=40)		All Patients (N=100)		Fibrosis (N=100)				P[1]	P[2]
					Patients with Early fibrosis		Patients with Advanced fibrosis			
	MBL +ve	MBL -ve	MBL +ve	MBL -ve	MBL +ve	MBL -ve	MBL +ve	MBL -ve		
B-cell monoclonal lymphocytosis	3 (7.5%)	37 (92.5%)	24 (24%)	76 (76%)	11 (22.5%)	38 (77.5%)	13 (25.5%)	38 (74.5%)	<0.001	0.467

***P1**, comparison between HCV infected cases and controls; p2, comparison between early fibrosis (F1-F2) and advanced fibrosis (F3-F4). Man Whitney test was used for comparisons.*

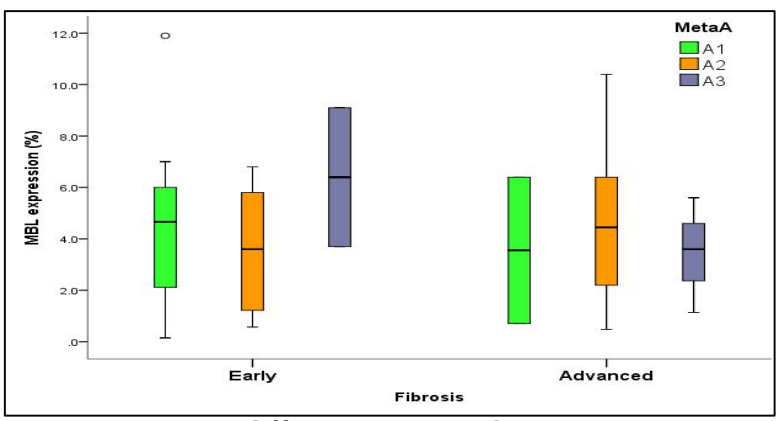

Figure (2) MBL expression in different activity grades.

Table (3) Comparison between patients with MBL and patients without MBL regarding the demographic data among chronic HCV infection.

Studied groups		MBL +ve (N=24)	MBL-ve (N=76)	P value
Age (Years)		47.6 ± 5.6	43.1 ± 7.2	0.007
Sex	Male	14 (58.3 %)	48 (63.2 %)	0.25
	Female	10 (41.7 %)	28 (36.8 %)	

Table (4) Comparison between chronic HCV patients with and without MBL regarding laboratory results.

Laboratory tests	MBL+Ve	MBL-Ve	P Value
HB level (gm/dL)	12.2 ± 1.4	12.6 ± 1.3	0.29
RBCs	3.6 ± 0.2	3.62 ± 0.25	0.65
TLC ($X10^9$/L)	6.5 ± 2.2	7.2 ± 2.1	0.006*
Platelet ($X10^9$/L)	233.3 ± 51.9	210.6 ± 48.6	0.052
ALT (IU/L)	103.6 ± 53.3	81.1 ± 46.8	0.052
AST(IU/L)	95.3 ± 53.7	65.5 ± 49.7	0.04
Bilirubin (mg/dL)	0.89 ± 0.24	0.84 ± 0.23	0.36
Albumin(mg/dL)	3.7 ± 0.7	3.9 ± 0.5	0.18
AFP	11.9 ± 10.2	11.5 ± 11.1	0.87

Table (5) AUC and performance characteristics of MBL expression as a predictor for progression of liver fibrosis from early to advanced fibrosis.

AUC	.542
95% CI	.428-.656
P	.467
Cut off	4
Sensitivity (%)	52.9
Specificity (%)	53.1
PPV (%)	54
NPV (%)	52
Accuracy (%)	53

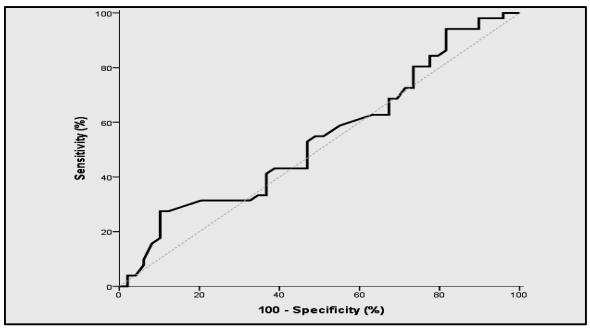

Figure (3) ROC curve of MBL expression for discrimination between early and advanced fibrosis

Discussion

Infection with hepatitis C virus (HCV), a major viral cause of chronic liver disease, frequently progresses to steatosis and cirrhosis, which can lead to hepatocellular carcinoma[19]. HCV infects not only hepatocytes but also extra hepatic cells[20]. HCV can infect the B lymphocytes, leading to malignant lymphoma by a multi step pathonegetic pathway: first, HCV induces an oligoclonal proliferation of the infected B-cells, and then the presence of HCV in lymphocytes could initiate growth dysregulation and predispose the lymphocyte to development of further molecular changes, leading eventually to malignant lymphoma. The HCVE2 envelope protein has been identified as a potential antigen that may drive the development of lymphoma[21]. The prevalence of HCV infection is higher in patients with B-cell non-Hodgkin lymphoma than in the general population, studies reporting controversial results depending on the geographical area. The most frequent association is with lymphoplasmacytoid lymphoma, other histological types associated with HCV are: follicular, lymphocytic, marginal zone and diffuse large B-cell non-Hodgkin lymphoma[22,23]. Monoclonal B lymphocytosis (MBL) is an asymptomatic condition characterized by the presence in the peripheral blood of a clonal B-cell population which might evolve into malignant B-cell lymphoproliferative disease, like chronic lymphocytic leukemia (CLL) or indolent B-cell lymphoma[24]. Such a monoclonal B-cell population can be detected in approximately 3.5% of healthy individuals, with higher frequency in males and in elderly people. In HCV-infected patients MBL can be identified at a higher frequency than in the general population[25]. There are only a few studies that investigated the prevalence of MBL in patients with HCV infection, with different results. The present study aims to determine the prevalence of MBL in HCV patients and to determine its clinical histological and laboratory associations. This study demonstrated that, the expression of MBL was significantly increased in HCV positive patients versus that reported in the general populations. It was well known that HCV was found to induce lymphocytosis[26]. Moreover, clinical data support the relationship between HCV and B cell prolife rative disorders[27,28] as demonstrated by the fact that antiviral therapy may lead to regression of at least a portion of HCV-related lymphomas[29]. Also, it has been reported that, monoclonal B lymp-hocytes can be frequently found circulating in the peripheral blood of otherwise healthy individuals (3.5–12%), increasing with age and irrespective of HCV infection[30]. In agreement with our result, Tőrők-Vistai et al[31] found that, the prevalence of MBL in patients with HCV infection is significantly higher than that reported in the literature in the general population. Also, Fazi et al showed that, MBL are more in HCV patients than in the general populations[32]. In our study, we found that there was no significant difference in expression of MBL between males and females. This was in agreement with other studies that demonstrated that, there was no statistically significant difference concerning the gender in comparing patients with or without MBL, and found that the frequency was the same in males and females[31,32]). In this study we found that, the prevalence of MBL in control group was 7.5%, this in agreement with the result of Rashwan et al[33], who aimed to study the frequency of MBL among healthy elderly adults who were chosen from general practice, ophthalmology, gynaecology, cardiology, dermatology, and orthopedic preoperative patients. He found that the frequency of MBL among the studied samples was 5.5% without significant difference between males and females. The detected frequency found may be related to the sensitivity of the method used. Higher frequentcies may be detected if more colours were used to detect more antigens. In addition, other Spanish study done by Neito et al[30], studied healthy volunteers from the primary health care and found that, MBL positive people was 14.3% with male predominance, but without statistical significance. On the other hand, an Italian study done by Dagklis et al[34], examined healthy volunteers in a geographically isolated rural area and found MBL positive people was 7.4% with male predominance. In this study, age was significantly increased in MBL positive patients than in MBL negative. This was in agreement with Fazi et al[32] who found MBL clones in HCV positive patients were present in 24.4% of subjects below 65 years of age, and the frequency increased to 37.8% in the individuals >65 years . Shim et al[35] showed that the prevalence of MBL increased with age in both genders. Also, Rawstron et al., (2002) found that, MBL is more prevalent in old age patients 40–59 years (2.1%), ≥60 years (5.0%). On the other hand, Tőrők-Vistai et al[31], found that there was no a statistically significant difference in expression of MBL concerning

the age. In addition, Rashwan et al[33] found non-significant increase in the expression of MBL in old age. This may be related to lower number of patients included in that study. Laboratory data differed only in terms of the leucocyte count which was significantly lower in patients with MBL than in patients without. This was in agreement with Tőrők-Vistai et al[31], who showed patients with MBL have lower leucocytic count than patients without MBL. On the other side, Fazi et al[32] found that all patients with MBL have normal total leucocytic count as patients without. This study did not find significant difference in expression of MBL between early and advanced fibrosis, which means that the stage of the liver disease does not affect the expression of MBL, this was against the result of Tőrők-Vistai et al[31], who found a higher prevalence of MBL in patients with cirrhosis compared to those with chronic hepatitis (31% versus 18%). Also, these findings are not concomitant with Fazi et al[32] who found the MBL frequency appeared to correlate with advanced hepatic disease as it was significantly higher in individuals with cirrhosis as compared with those with chronic hepatitis. Thereby, suggesting that the persistency of the viral infection may be critical for the onset of single B cell clones.

Conclusion

B-cell monoclonal lymphocytosis showed significant increase in HCV infected patients more than in the general population. No significant differences in MBL expression between early and advanced fibrosis stage.

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