Hepatitis C-Associated Chronic Lymphoproliferative Disorders: A Single Center Experience

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Abstract- Hepatitis C virus (HCV) infection is a major public health concern in Egypt with its unique genotype. Besides liver disease, HCV is a lymphotropic virus involved in the pathogenesis of various extrahepatic diseases. A causative role of HCV in the generation of chronic lymphoproliferative diseases as well as its impact on disease behavior in HCV chronic carriers are not fully understood. We investigated the prevalence of HCV among cohort of Egyptian patients (*n*:84) with chronic lymphoproliferative diseases, the relation between HCV infection and the immunological state of this population and also their clinical and laboratory characteristics. High prevalence of HCV (40%) among Egyptian patients with chronic lymphoproliferative diseases with following subtype frequency was revealed; CLL (38.2%) followed by DLBCL (20.6%), and LPL (17.6%). We found significant correlation between HCV and platelet count *(P=0.014)*, Albumin *(P=0.02)*, LDH *(P=0.014)* and B2M *(P=0.05)*. Otherwise, there were no significant correlations with other parameters especially the immunological assessment; serum immunoglobulins, Coombs test, and cryoglobulinemia. HCV prevalence among patients with chronic lymphoproliferative diseases is higher than that estimated in the general population. Those patients should be tested for HCV during the assessment. Our observations suggest that HCV may have an oncogenic role in Egyptian patients with chronic lymphoproliferative diseases and it may affect the prognostic markers in those populations.

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Introduction

Hepatitis C virus (HCV) infection is a major public health problem with more than 170 million people chronically infected worldwide. HCV is a small, enveloped, single standard RNA virus of the family *Flaviviridae* (1).

In Egypt, the prevalence rate of HCV infected individuals was 872,000 (15% of the population) in 2013, with an estimated incidence of newly infected 125,000 HCV individuals each year (2), the rate which is considered as one of the highest prevalence rates of HCV worldwide (3) and it is worth noting that Genotype 4 is most common in central Africa, Egypt and Middle East (4).

Besides liver disease, HCV is a lymphotropic virus involved in the pathogenesis of various extrahepatic diseases. Many of the extrahepatic outcomes of HCV infection are linked to the deregulation of B cells (5). It is well acknowledged that HCV represents a major etiologic agent of mixed cryoglobulinemia (MC) type II, which is characterized by a low-grade B cell clonal lymphoproliferative disorder, initially confined to the bone marrow but then evolving into a more aggressive malignant lymphoma in about 10 % to 20 % of patients several years after diagnosis (6).

A causative role of HCV in the generation of chronic lymphoproliferative diseases in HCV chronic carriers is strongly supported by numerous epidemiological and clinical observations and experimental data initiated with an Italian report of the unexpected high rate of HCV ongoing infection in patients with 'idiopathic' B-NHL (7). There is a suggestion that some B-cell non-Hodgkin lymphoma (NHL) associated with HCV arise from clonal expansion of B-cells with particular immunoglobulin gene rearrangements specific for the

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E2 protein of the HCV envelope (8).

This association showed a geographical heterogeneity similarly to that observed for other HCV-associated diseases, especially the MCs (9). This epidemiological feature may reflect the multifactorial etiopathogenesis, including both genetic background and environmental cofactors, of this heterogeneous group of chronic lymphoproliferative diseases.

So our objectives in this study was to evaluate the prevalence of HCV among cohort of Egyptian patients with chronic lymphoproliferative diseases, the relation between HCV infection and the immunological state of those population and also to study the clinical and laboratory characteristics of HCV positive chronic lymphoproliferative population especially in a population with high prevalence of HCV like Egypt.

Materials and Methods

A total of 84 Egyptian subjects with de novo chronic lymphoproliferative disease (33 women, 51 men, mean age 54.54±11.92 years) were enrolled in the study; all were consecutively recruited from the clinical hematology unit of the Kasr Al-Ainy teaching hospital, Cairo University where they diagnosed and followed up prospectively between October 2014 and December 2016. The study complied with good clinical practice protocols and with the ethical rules stated in the Declaration of Helsinki (as revised in Tokyo 2004). The study has been approved by the local Ethics Committee, and all patients gave their written informed consent prior to recruitment.

Diagnosis of B-lymphoproliferative disorders (B-LDL)

For chronic lymphocytic leukemia (CLL), according to the National Cancer Institute (NCI)-lymphocytosis> 5 x 109/L in peripheral blood and confirmation of the immunophenotype by flow-cytometry (10). For non-Hodgkin's lymphoma (NHL) – by assessing the immunophenotype of the malignant lymphoid population by immunohistochemistry, using modified REAL and WHO classifications (11).

Methods

A total of 84 subjects with chronic lymphoproliferative diseases were subjected to full history taking, thorough clinical examination (B symptoms; fever, weight loss and night sweats and lymphoid organ assessment), and laboratory investigations which included complete blood count (CBC), erythrocyte sedimentation rate (ESR), Lactate dehydrogenase (LDH), Beta 2-microglobulin (B2M), liver functions in the form of; Serum Albumin, total bilirubin and prothrombin concentration (PC), serum immunoglobulins IgG, IgM, IgA levels, cryoglobulin and coombs test (direct and indirect).

Cryoglobulin assessment

Clotted blood samples in the second tube were centrifuged at 37° C for 10 min at 2000 rpm. Blood samples were obtained and kept at 37° C for 30 min before separation. The serum obtained (10 mL) was transferred to a 15-mL glass-graduated conical tube and incubated at 4° C for 5 days. The cryoprecipitate was detected according to the following: If the serum remained completely clear for 5 days, it was reported as negative. If a specimen became definitely cloudy (with visible precipitate), the tube was placed in 37° C water bath for about 1-4 h. If it cleared, the tube was placed back at 4° C. If precipitate reformed, it was reported as positive.

Diagnosis of hepatitis infections

Serum HCV antibodies were detected by ELISA (Dia Sorin, Torino, Italy), while HCV genotyping was detected by INNO-LiPA HCV II (Bayer HealthCare, Eragny, France). RT-PCR (real time polymerase chain reaction) for detection and quantitation of HCV RNA: RNA was extracted from serum and PBMC using The QIAamp DSP Virus Kit (QIAGEN, Catalogue #60704), total RNA extraction reagent according to the manufacturer's protocol. RNA was reverse-transcribed, amplified and analyzed with artus HCV RG RT-PCR Kit (QIAGEN GmbH, Germany, Catalogue #210212) on Rotor-Gene Q Instruments. The analytical detection limit of the artus HCV RG RT-PCR Kit is 0.19 IU/µl. Results were interrupted as following Negative <0.19 IU/µl, Mild viremia up to 1x 10^5 IU/µl, Moderate viremia 1x 10^5 -1x 10^6 IU/µl and High viremia more than 1x 10^6 IU/µl.

Statistical analysis

Data were coded and entered using the statistical package SPSS (Statistical Package for the Social Sciences) version 23. Data were summarized using mean, standard deviation, median, minimum, and maximum in quantitative data and using frequency (count) and relative frequency (percentage) for categorical data. Comparisons between quantitative variables were done using the non-parametric Kruskal-Wallis and Mann-Whitney tests. For comparing

categorical data, Chi square ($\chi 2$) test was performed. The exact test was used instead when the expected frequency is less than 5. *P* less than 0.05 were considered as statistically significant.

Results

Patients included in our study were 33 females (39.3%) and 51 males (60.7%), their age ranged between (18-76 years) with median age of 56 years (mean age 54.54±11.92 years) at the hematological diagnosis. Subtypes of chronic lymphoproliferative diseases were as follow; 39 patients had CLL (46.4%), 16 patients had follicular lymphoma (FL) (19.0%), 16 patients had diffuse large B cell lymphoma (DLBCL) (19.0%), 6 patients had splenic marginal zone lymphoma (SMZL) (7.1%), 1 patient had MALT lymphoma (1.2%) and 6 patients had Lymphoplasmacytic lymphoma (LPL) (7.1%), paramedic and non-parametric features are summarized in table 1-2.

Table 1. Parametric data of patients with chronic lymphoproliferative disease

Parameters	Mean±Standard Deviation	Median	Range
Age (years)	54.54±11.92	56	(18-76)
Spleen (cm)	17.11±2.27	17	(13-23)
Viral loads (IU/Ml)	$1.67X10^8$±$8.89X10^8$	$1.87X10^8$	(0-$7.00X10^9$)
TLC (cells x 10^3/mm^3)	41.35±70.29	9.15	(1.2-347)
ANC(cells x 10^3/mm^3)	4.97±5.02	3.53	(0.35-33)
Lymphocyte (cells x 10^3/mm^3)	37.29±67.64	5.64	(0.12-338.1)
HB% (gm/dl)	9.88±2.63	9.4	(3.3-16)
PLT (cells x 10^3/mm^3)	144.69±94.63	126	(10-567)
LDH (U/L)	553.23±293.62	515	(146-1712)
B2M (ug/ml)	4.02±1.37	3.90	(1.6-8)
Serum albumin (g/dl)	4.63±8.12	3.85	(1.7-78)
T.BIL (mg/dl)	0.77±0.66	0.5	(0.1-3.5)
PC %	80.63±13.75	82	(35-100)
IgM (mg/dl)	410.43±980.71	140.9	(9.7-5700)
IgG (mg/dl)	1274.61±597.1	1252	(211-4210)
IgA (mg/dl)	208.02±135.96	190	(14.2-797.1)

In studied patients, positive HCV Ab was detected in 34 out of 84 (40.5%) patients, and serum HCV RNA detection results were positive in 32 out of 84 (38.1%) patients with viral load as follow; 12 patients had high viremia (14.3%), 6 patients had moderate viremia (7.1%), and 12 patients had low viremia (14.3%), HCV genotyping results showed that all HCV patients were genotype 4.

In all studied patients Immunological status were assessed and revealed that mean IgG was 1274.61 (normal reference 700-1600 mg/dl) and 11 patients (13.09%) had decreased IgG level 8 of them were CLL, mean IgM was 410.43 (normal reference 40-230 mg/dl) and 20 patients (23.08%) had decreased IgM all of them were CLL patients and meant IgA was 208.02 (normal reference 70-400 mg/dl) and 9 patients (10.7%) had decreased IgA all of them also were CLL patients and 4 patients (4.76%) had poly hypogammaglobinemia, Cryoglobulinemia was positive in 6 patients (7.1%) 4 of them had Lymphoplasmacytic lymphoma and 2 patients had CLL and coombs tests were positive in only 2 patients (2.4%) who were CLL patients.

Patients were sub grouped into 2 groups according to HCV Ab status; group I (HCV Ab Positive) and group II (HCV Ab negative) and they were compared regarding clinical, and laboratory features and data are summarized in table 3-4; there was significant correlation between HCV status and direct platelet count (*P 0.014*), LDH *(P 0.013)*, B2 microglobulin *(P 0.005)*, serum albumin *(P 0.002)*, prothrombin concentration *(P 0.001)* and chronic lymphoproliferative diseases subtypes *(P 0.015)* otherwise there are no significant correlations with other parameters especially the immunological assessment; serum immunoglobulins, coombs test and cryoglobulinemia.

Table 2. Non-parametric data of patients with chronic lymphoproliferative disease

Parameters		Count	%
Sex	**Male**	51	60.7%
	Female	33	39.3%
Subtypes	**Follicular lymphoma**	16	19.0%
	DLBCL	16	19.0%
	LPL	6	7.1%
	SMZL	6	7.1%
	MALT	1	1.2%
	CLL	39	46.4%
LNs groups	**0**	11	13.1%
	1	10	11.9%
	2	27	32.1%
	3	19	22.6%
	4	9	10.7%
	5	8	9.5%
B- symptoms	**Positive**	58	69.0%
	Negative	26	31.0%
Ann Arbor Stage *	**I**	11	24%
	II	15	33%
	III	13	28.8%
	IV	6	13.3%
International prognostic index (IPI) *	**Low Risk**	19	42%
	Intermediate low	11	24%
	Intermediate high	15	33%
	High	0	0
HCV Ab	**Positive**	34	40.5%
	Negative	50	59.5%
PCR for HCV	**Positive**	32	38.1%
	Negative	52	61.9%
viral load	**Negative**	54	64.3%
	Mild viremia	12	14.3%
	Moderate viremia	6	7.1%
	High viremia	12	14.3%
Cryoglobulinemia	**Positive**	6	7.1%
	Negative	78	92.9%
Coombs	**Positive**	2	2.4%
	Negative	82	97.6%

* for patients with NHL only n:45

Table 3. Clinical and laboratory comparison between two studied groups

Parameter	HCV status				*P*
	Positive		Negative		
	Mean±SD	Range	Mean±SD	Range	
Age (years)	55.21±11.47	(32-75)	54.08±12.30	(18.0-76.0)	0.55
Spleen (cm)	17.47±2.49	(14-22)	16.86±2.09	(13-23)	0.251
TLC (cells x $10^3/mm^3$)	34.37±66.15	(1.5-345)	46.09±73.25	(1.2-347)	0.242
ANC (cells x $10^3/mm^3$)	4.34±3.66	(0.75-16)	5.40±5.76	(0.35-33)	0.529
Lymphocyte (cells x $10^3/mm^3$)	32.96±66.18	(0.12-338.1)	40.23±69.12	(0.45-333.12)	0.5
HB% (gm/dl)	9.90±2.39	(6.3-15.6)	9.86±2.80	(3.3-16)	0.877
PLT (cells x $10^3/mm^3$)	114.76±57.55	(29-265)	165.04±109.06	(10-567)	0.014*
LDH (U/L)	645.32±310.21	(146-1270)	490.6±267.1	(160-1712)	0.013*
B2M (ug/ml)	4.38±1.11	(1.9-6.8)	3.78±1.49	(1.6-8)	0.005*
Serum albumin (g/dl)	5.74±1.2	(2.6-7.8)	3.88±0.54	(1.7-4.9)	0.002*
T.BIL (mg/dl)	.91±.79	(0.22-3.5)	0.68±0.54	(0.1-2.8)	0.067
PC %	72.90±16.50	(35-96)	85.88±8.19	(70-100)	<0.001*
IgM (mg/dl)	669.861263.27	(13.2-5700)	234.03±689.88	(9.7-4940)	0.066
IgG (mg/dl)	1344.59±520	(436-3090)	1227.02±645.06	(211-4210)	0.303
IgA (mg/dl)	218.55±121.67	(58-582)	200.85±145.65	(14.2-797.1)	0.316

*Significantly different at $P < 0.05$

Table 4. Comparison between two studied groups (non-parametric data)

Parameters		HCV				*P*
		Positive		Negative		
		Count	%	Count	%	
Sex	Male	20	58.8%	31	62.0%	0.770
	Female	14	41.2%	19	38.0%	
Subtypes	Follicular lymphoma	4	11.8%	12	24.0%	0.015*
	DLBCL	7	20.6%	9	18.0%	
	LPL	6	17.6%	0	.0%	
	SMZL	3	8.8%	3	6.0%	
	MALT	1	2.9%	0	.0%	
	CLL	13	38.2%	26	52.0%	
LNs groups	0	5	14.7%	6	12.0%	0.925
	1	3	8.8%	7	14.0%	
	2	13	38.2%	14	28.0%	
	3	7	20.6%	12	24.0%	
	4	3	8.8%	6	12.0%	
	5	3	8.8%	5	10.0%	
B- symptoms	Positive	25	73.5%	33	66.0%	0.464
	Negative	9	26.5%	17	34.0%	
Ann Arbor Stage**	I	5	23.8%	6	25%	0.432
	II	7	33.3%	8	33.3%	
	III	6	28.5%	7	29%	
	IV	3	14%	3	12.5%	
International prognostic index (IPI)**	Low Risk	9	42%	10	41%	0.446
	Intermediate low	5	23%	6	25%	
	Intermediate high	7	33.3%	8	33.3%	
	High risk	0	0	0	0	
Cryoglobulinemia	Positive	4	11.8%	2	4.0%	0.216
	Negative	30	88.2%	48	96.0%	
Coombs	Positive	0	.0%	2	4.0%	0.512
	Negative	34	100.0%	48	96.0%	

Significantly different at *P* of < 0.05, ** for patients with NHL only n:45

Table 5. HCV associated chronic lymphoproliferative diseases in comparable studies

Studies	Median Age (years)	M:F Ratio	Cases N	HCV Prevalence in cases	Control N	HCV Prevalencein control
Ciufu C et al., 2013 Romania [33]	60.35	1:0.68	42	52.3%	ND	ND
Mousa SM. 2014 Egypt [13]	42.9	1:1	100	42%	ND	ND
Cowgill K at al., 2004 Egypt [15]	48.2	1.24:1	227	42%	ND	ND
Abu-Taleb F et al., 2013 Egypt [14]	ND	ND	57	42%	ND	ND
Youssef S et al., 2012 Egypt [34]	54.8	1.08:1	50	26%	50	4%
Talamini R et al., 2004 Italy [35]	59	ND	225	19.6%	1005	3.5%
Salem A, 2009 Yemeni [36]	41.3	2.2:1	192	15.1%	12274	4%
Caviglia G et al., 2015 Italy [37]	61.5	0.7:1	1313	9.2%	ND	ND
Onyekwere C et al., 2016 Nigeria [38]	95.6	ND	33	9.1 %	405	0.7%
Rastin M et al., 2013 Iran [39]	ND	ND	54	7.4%	ND	ND

Continuance of Table 5

Engels E et al., 2004 USA [40]	57	1.17:1	813	3.6%	684	2.1%
Sonmez M et al., 2007 Turkey [41]	58.1	1:1.4	109	2.8%	551	5.1%
Spinelli J et al., 2008 Canada [42]	ND	1:1.4	795	2.4%	697	0.9%
Schöllkopf C et al., 2008 Sweden and Denmark [43]	ND	ND	2,819	2%	3,187	0.1-0.5%
Park S et al., 2008 Korea [44]	48	1.4:1	235	2%	235	1.7%
Varma S et al., 2011 India [45]	48.7	1.8:1	57	1.75%	171	1.17%

ND; no data

Discussion

HCV infections represent an important public health problem, because of the increasing prevalence, evolution to chronic disease, cirrhosis, and hepatocellular carcinoma, and also because of their association to autoimmune diseases and chronic lymphoproliferative disorders. Being a country with a high incidence of HCV infection (12), we aimed to focus on studying the clinical and laboratory features of HCV associated with chronic lymphoproliferative disorders with special focus on the immunological features.

Regarding the prevalence of HCV among chronic lymphoproliferative disorders, we found that 40.5% of our patients had HCV infection which matches some Egyptian studies done like *Mousa S et al., Abu-Taleb F et al., and Cowgill K at al*., whom nearly had matched results (13-15).

The prevalence of chronic lymphoproliferative disorders attributable to HCV varies greatly by country and may be increased more than 20% in areas where HCV prevalence is high. Various studies as summarized in table 5 showed that there is a greater association of HCV with chronic lymphoproliferative disorders in countries, like Egypt and Italy. However, there are studies which have shown negative association like Canada and Turkey. The striking geographic variations in this relation could be explained by that genetic and/or environmental factors might involve in the pathogenesis of this disorder, the duration of persistent infection of HCV may have an influence on the carcinogenesis of lymphoid cells and also studies which conducted in low prevalence countries might not have included enough patients to detect the association. However, meta analyses indicated a significant association between HCV and B-NHL and CLL as showed by *Gisbert et al.*, in a systematic review and meta-analysis included 5542 patients and stated that HCV prevalence in patients with B-NHL is approximately 15%, higher than that reported in general population (1.5%) and concluded that HCV might have a role in the pathogenesis of B cell NHL (16), and a recent meta-analysis by *Pozzato G et al.*, included 9038 patients with NHL stated that people infected by HCV from Japan and from the Mediterranean basin show a relative risk of NHL from 2 to 4 times higher than people of Northern Europe (3).

In our study, all HCV positive patients were having genotype 4 of HCV. This result confirms the data reported by *Miller FD and Abu-Raddad LJ* as they stated that HCV genotype 4 predominates in Egypt (17).

Regarding subtype frequency, among HCV positive group in our study, the most frequent subtype was CLL (38.2%) followed by DLBCL (20.6%) and LPL (17.6%) subsequently. An answer to a question of which subtypes of chronic lymphoproliferative diseases are most closely associated to HCV? Still remains a matter of debate.

Torres H et al., showed that DLBCL (62%) is the most common HCV related B-NHLs, followed by follicular lymphoma (13%) and MZL (11%) (18), *Goldman et al.*, stated that the most common subtypes were of DLBCL (54.9%), followed by chronic lymphocytic leukemia (11.9%), follicular lymphoma (6.3%), T cell lymphoma, and mantle cell lymphoma (6.6%) (19), *Libra M et al.*, showed that marginal zone lymphomas (MZL), in particular splenic marginal zone lymphomas (SMZL), lymphoplasmacytic lymphoma (LPL), and diffuse large B-cell lymphoma (DLBC) are the most frequently B-NHL subtypes described as being associated with HCV (20). One meta-analysis published by *Dal Maso L and Franceschi S* did not find any subtype-specific association which may be attributable to a lack in the number of well-matched cases and controls (21). In a large European multicenter case-control study (Epilymph), DLBCL, MZL, and LPL were identified as most closely related to HCV infection and

however cases samples size was of 1807 cases, those subgroups consisted of relatively few cases (22). In our study, the sample size of patients was not sufficient to explore deeply all lymphomas subgroups.

Regarding immunological assessments in our studied patients only 7.1% patients had cryoglobulinemia with no significant correlation with HCV status *(P 0.216)* which strangely didn't match a bundle of studies like *Ferri et al.,* who published the first report indicating an association between HCV and cryoglobulinemia and found that the prevalence of cryoglobulinemia is particularly high in hepatitis C viral infection (23). Also studies by *Lunel et al., Kayali et al., and Stefanova-Petrova et al.* found that cryoglobulinemia is about 40 % in HCV positive patients (24-26). On the other hand, one Egyptian study matched our results as they found only 11 patients had cryoglobinemia among 90 NHL patients (27), the controversy of the different studies may be related to the differences in races and hepatitis C genotypes. In our study, we didn't found also significant correlation between HCV status and serum immunoglobulins' level, and we noticed that most patients with low immunoglobulins' level in our study were CLL patients and the same observation was with coombs tests which match the disease nature itself.

We found significant correlation between HCV status and platelet count (*P 0.014*) which is supported by a systematic review of 27 studies reported a 24% prevalence of thrombocytopenia in chronic HCV infected patients in more than half of the studies with a wide range of 0.2-45% depending on the definition of thrombocytopenia (28). The pathogenesis of thrombocytopenia among chronic HCV-infected patients is multifactorial. Possible causes include sequestration because of hypersplenism secondary to portal hypertension and splenomegaly, bone marrow suppression either by HCV directly or by antiviral treatment, immune-mediated platelet destruction, and impaired thrombopoietin production resulting from hepatocellular damage (29). However, the exact cause of thrombocytopenia is not properly investigated in our study.

Also, we found significant correlation between HCV status and prognostic markers like LDH and B2microglobulin which were higher in HCV positive patients (*P 0.013, 0.005* respectively) that finding goes with *Huckans et al.,* who found that B2M levels higher in in adults with HCV (n=39) than those without (n=40) (*P*<0.001**)** (30), this findings could be explained by; the link of HCV to clonal expansion of B cells, the temporary intracellular virus replication with damage of B-cells (31) and therefore the tumor aggressiveness, however, since an active replication of HCV in human B or T lymphocytes *in vivo* has never been demonstrated, a direct oncogenic effect by HCV inside B cells is unlikely. In addition, viral proteins, indicative of active replication, could never be demonstrated in the neoplastic lymphoid tissue of the HCV-NHL (32).

Finally, we want to conclude that HCV prevalence among patients with chronic lymphoproliferative diseases is higher than that estimated in the general population. Those patients should be tested for HCV during initial disease assessment. Our observations suggest that HCV may have an oncogenic role in Egyptian patients with chronic lymphoproliferative diseases and it may affect the prognostic markers in those populations. However more laboratory research is required to provide a better understanding of the etiopathogenesis of HCV associated NHL. Ultimately, this may lead to more prevention of NHL by allowing for the targeting and treatment of high-risk HCV-infected individuals, and it could help inform treatment strategies for HCV-associated NHL with more coming studies.

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