Molecular Study for detection of Hepatitis C virus among patients with Diffuse Large B-Cell Lymphoma (DLBCL)

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Abstract
Background: Diffuse large B-cell lymphoma (DLBCL), is the most common type of lymphoma, and is discreditable for its heterogeneity, with aggressive nature, and the common development may rise by some specific pathogens. For declaration these problems, this study need to place on researching the molecular origins of DLBCL to understand more explanations. the influence of hepatitis C virus (HCV) is also associated with extra-hepatic immune indices and B-cell non-Hodgkin lymphoma (NHL), particularly marginal zone lymphoma, or transformed diffuse large B cell lymphoma, Hepatitis C virus (HCV) act a non-retroviral oncogenic RNA virus, commonly related with hepatocellular carcinoma (HCC). HCV linked DLBCL require unusual features related to their HCV negative complements, signifying a probable effect of the virus meanwhile the actual primary stages of lymphomagenesis, still essential to be extra clarified for a link between HCV and lymphoproliferative disorders. Studies solitary involved small records of HCV-positive patients and not all studies concerning HCV infection and DLBCL exhibited dependable results. So, this detail need more approving studies confirm that HCV infection performance one of serious risk factor in triggering DLBCL disease. Aim of study: this study approving study search for confirm that HCV infection presentation as a one of thoughtful risk factor in triggering DLBCL disease. Patients and methods: a retrospective study performed on 50 formalin-fixed paraffin-embedded tissue specimens of patients serologically positive for HCV and diagnosed as having DLBCL obtained from the archives of the National Cancer of medical laboratories/ city of medical city, Baghdad, from January to September 2022. All collected samples from serologically positive cases with adequate paraffin-embedded tissue material were included in the study. All the 50 patients with DLBCL with previous Hepatitis C virus infection, and 25 patients with appearance healthy as control without DLBCL (negative results) were included with previous HCV infection. Tested with molecular method PCR and RT-PCR, for detecting HCV virus within their biopsy samples. Results: the HCV-positive DLBCL patients reported to take altered features, with raised Alanine aminotransferase (51.8+7.7), but not in Aspartate aminotransferase and g-Glutamyl transpeptidase, Age and Sex: mostly were in mean of age (43.2+8) in further in male gender (31/50) and female (19/50), and HCV RNA load in DLBCL patients were highly significant (<0.001) more than in control patients. Also, the results Genomic and Anti-genomic DLBCL patients comparison with control patients were highly significant of (<0.001), and some of positive results in control patients(200*105) copies/mg total RNA among DLBCL, while were (3.04 * 102) in control patients, but reversible result in Anti-genomic results, were highly occur in control patients. Conclusions: The patients with chronic HCV infection are at increased risk to develop DLBCL dieease. HCV-RNA replication in B-lymphocytes has action of oncogenic effect recognized by intracellular HCV proteins. The probable role of viral lymphotropism in the pathogenesis of HCV-associated Lymphoprelfirative Diseases, especially with progressive DLBCL. Genotypes tests play a critical role in evaluating treatment choices and methods for prepare the suitable drugs, as it exposed the prognosis of disease, severity, and response to type of therapy according to genotypes.
Keywords: Molecular and Genetic Study, Hepatitis C virus, virulence genes, Diffuse Large B-Cell Lymphoma (DLBCL)

1. Introduction

Diffuse large B-cell lymphoma (DLBCL), is the most common type of lymphoma, and is discreditable for its heterogeneity, with aggressive nature, and the common development may rise by some specific pathogens. For declaration these problems, this study need to place on researching the molecular origins of DLBCL to understand more explanations. the influence of hepatitis C virus (HCV) is also associated with extra-hepatic immune indices and B-cell non- Hodgkin lymphoma (NHL), particularly marginal zone lymphoma, or transformed diffuse large B cell lymphoma, Hepatitis C virus (HCV) act a non-retroviral oncogenic RNA virus, commonly related with hepatocellular carcinoma (HCC) [1], and B-cell lymphomas, and numerous latest surveying studies have described that patients with HCV linked DLBCL require unusual features related to their HCV negative complements, signifying a probable effect

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of the virus meanwhile the actual primary stages of lymphomagenesis, still essential to be extra clarified for a link between HCV and lymphoproliferative disorders [2]. The HCV-related lymphomas has a limited immunoglobulin variable- region gene range, so as to the lymphoma B cell receptors act as soluble immunoglobulin Gs and membrane IgMs do not attach to the HCV antigens. It directs the majority of lymphomas that do not arise from B- cells that involving in viral salvage [3]. Another theory states that HCV replication in B lymphocytes has oncogenic effect mediated by intracellular HCV proteins [4]. HCV infected patients did not catch any supposed mutation, thus the researchers decided that HCV does not mostly prompt mutations in the genes elaborate in oncogenesis, for example TP53, CTNNB1, and BCL6 in B-lymphocytes [5]. Furthermore, it was establish that the cytotoxic T lymphocyte antigen- 4 (CTLA -4) + 49 A/G - polymorphism is related with a greater risk of BCNHL incidence. The discovery of HCV was unique in that the presence of the virus was established using molecular cloning techniques relatively than direct biological methods [6]. Then the viral sequence was first found by extracting all the nucleic acid from the serum of a non-A non-B hepatitis from infected chimpanzee, producing cDNA clones and detecting the clone consistent to the HCV genome, by using these methods, diverse strains of HCV were recognized and characterized into at least six major genotypes founded on nucleic acid sequence alone [7]. The genotypes stand approximately (65%) identical through the entire HCV genome. B-cell derived Non-Hodgkin's Lymphoma is the most common lymphoma associated with HCV infection. The reflection of the effect of viral extinction using antiviral agents intensely supports the etiopathogenetic link between HCV infection and lymphomagenesis [8]. These contain a key role played by the constant antigenic stimulation of the B-cell compartment, and the role of viral lymphotropism with viral proteins, chromosomal deviations, cytokines, and microRNAs. There are growing evidence of supportive role of microRNA (miRNA) deregulation the pathogenesis in chronic HCV infection. MiRNAs are small RNA molecules (19–22- noncoding RNAs) capable to prompt translational inhibition of objective genes by limited improper complementarity to the target mRNA 3_UTR. Also, liver-specific miRNA (miR-122), has been exposed to be essential for HCV replication [9]. HCV infection is also related with B-cell non-Hodgkin lymphoma (NHL) with diverse clinical presentation and result, especially in diffuse large B-cell lymphoma (DLBCL) in current epidemiological studies [10]. Because of the detail that direct anti-HCV therapy prompts hematologic response in lethargic NHL patients with HCV infection, HCV infection has been related to NHL, and front-line treatment of asymptomatic indolent NHLs is optional [11]. In DLBCL, the record common NHL, is an aggressive type of NHL with specific genetic characters and clinical performances [12]. Although HCV-positive DLBCLs exhibition specific clinical features and consequences, the optimum management and timing of anti-HCV therapy in aggressive lymphoma is quiet indeterminate [13]. Prior reports presented that HCV-positive patients with DLBCL showed poorer overall survival (OS), and the occurrence of severe hepatic toxicity in patients of HCV-positive was considerably higher than that of HCV-negative patients. Nevertheless, all these studies solitary involved small records of HCV-positive patients and not all studies concerning infection with HCV and DLBCL exhibited dependable results [14]. So, this detail need more approving studies confirm that HCV infection performance one of serious risk factor in triggering DLBCL disease.

2. Patients and Methods

Patients and Samples

The study was conducting as a retrospective study performed on 50 formalin-fixed paraffin-embedded tissue specimens of patients serologically positive for HCV and diagnosed as having DLBCL obtained from the archives of the National Cancer of medical laboratories/ city of medical city, Baghdad, from January to September 2022. All collected samples from serologically positive cases with adequate paraffin-embedded tissue material were included in the study. All the 50 patients with DLBCL with previous Hepatitis C virus infection, and 25 patients with appearance healthy as control without DLBCL (negative results) were included with previous HCV infection. Up to (50) samples of biopsies were used from DLBCL patients were previously infected with HCV virus and have chronic infection or cured. Paraffin wax–embedded biopsy pieces (thickness of 4 mm) were de-waxed in xylene and were rehydrated concluded an ethanol successions.

3. Method

In this study used molecular method for viral genetic material extraction, by use of the SV Total RNA Isolation kit (Promega), and then detection of gene by Real-time PCR was run in a Light Cycler (Roche Molecular Biochemicals) with 2 mL of cDNA in a final volume of 20 mL, with use of the Light Cycler Fast Start DNA Master SYBR Green I kit (Roche Molecular Biochemicals) [15].

Preparation of sample

1. Paraffin wax–embedded biopsy pieces (thickness of 4 mm) were de-waxed in xylene and were rehydrated concluded an ethanol successions.
2. The biopsy sections were digested with proteinase K (1 mg/ mL) at 37C for 20 min, were post-fixed in a newly ready solution of 4% formaldehyde, and were acetylated in 0.1 mol/ L solution of triethanolamine with acetic anhydride for 20 min.
3. The sections were rinsed in 2 standard saline

citrate (SSC; 20 SSC: 3 mol/L NaCl and 0.3 mol/L sodium citrate) and were rapidly dehydrated in ethanol.

4. Hybridization was made in a solution containing 50% deionized formamide, 0.1 mol/ L phosphate buffer, 2 SSC, 250 mg/mL yeast tRNA, and 100 mg/mL dextran sulfate containing 5 ng of the corresponding heat-denatured probe per section.

5. The sections were incubated at 50 C for 16 h. After hybridization, the sections were washed in 3 SSC at 50 C for 1 h, were digested with RNase A (20 mg/ mL) and were rinsed in 1.5 SSC and 0.75 SSC at 50C for 1 h each. The digoxigenin-labeled hybrids were detected with a digoxigenin antibody alkaline phosphatase conjugate and an enzyme substrate chromogen (nitroblue tetrazolium/ 5-bromo-4-chloro-3-indolyl phosphate), according to the instructions of the manufacturer of the Dig Nucleic Acid Detection kit (Roche Molecular Biochemicals).

6. Detection of genomic HCV

7. Detection of genomic HCV RNA strands by quantitative, strand-specific, real-time reverse-transcription polymerase chain reaction (RT-PCR).

8. Total RNA was isolated from tissue specimens, by use of the SV Total RNA Isolation kit (Promega). After precipitation, the pellet was dissolved in diethyl pyrocarbonate–treated distilled water.

9. The total RNA concentration from the samples was determined using spectrophotometry.

10. Quantification of the 5noncoding region of the genomic HCV RNA strands was done using a strandspecific Real time RT-PCR, with use of the thermostable enzyme Tth for the synthesis of cDNA at a high temperature.

11. Thus, for the amplification of the genomic HCV RNA strand, cDNA was generated in 20 mL of reaction mixture that contained the total RNA extracted from 200 mL of serum or 0.5 mg of total RNA from liver specimens or PBMC samples, 50 pmol/L antisense primer UTRLC2 (5-CAAGCACCCTATCAGGCAGT-3), 1 mmol/L MnCl2, 200 mmol/L each deoxynucleotide triphosphate, 1 RT buffer (Applied Biosystems), and 5 U of the (Applied Biosystems).

12. After 20 min at 65 C, the RT activity was inactivated by Mn2+ chelation with 8 mL of the 10-chelating buffer (Applied Biosystems), followed by heating at 95 C for 30 min.

13. For amplification of antigenomic HCV RNA, cDNA was synthesized under the same conditions by the addition of 50 pmol/L sense primer UTRLC1 (5-CTTCACGCAGAAAGCGTCTA-3).

14. Real-time PCR was run in a LightCycler (Roche Molecular Biochemicals) with 2 mL of cDNA in a final volume of 20 mL, with use of the LightCycler FastStart DNA Master SYBR Green I kit (Roche Molecular Biochemicals).

15. The reaction mixture contained 4 mmol/L MgCl2, 0.5 mmol/L primers UTRLC1 and UTRLC2, and 2 mL of SYBR Green Master mix.

16. Amplification was performed as follows: initial denaturation and activation of enzyme were performed at 95C for 10 min; followed by 60 cycles at 95C for 1 s, at 60C for 5 s, and at 72C for 10 s; and then followed by a final step of fluorescence acquisition performed at 89C for 5 s. Two standard curves constructed with 10-fold dilutions of synthetic genomic and antigenomic HCV RNA (from 3.2108 to 0.322 replicas) used for quantify both of HCV RNA-stands.

17. The specificity of this assay for detection of the HCV RNA anti-genomic strand was evaluated by performance RT with the sense primer and sequential dilutions of the synthetic genomic HCV RNA as template. Linearity of the quantification assay ranged from (3.2 to 3.2108) copies of genomic or anti-genomic HCV RNA strand per reaction.

4. Statistical Analysis

Data were statistically analysed using SPSS program (version 18 and statistically, the data have been evaluated through the use of Microsoft Program, also the Specific group differences have been defined through the use mean, standard deviation, percentiles, P value-chi square, standard deveasion, of least significant differences as indicated via [16].

5. Result

HCV detection results

Comparison between patients with DLBCL had previous hepatitis C virus positive with control through some biochemical tests such as Alanine aminotransferase, Aspartate aminotransferase and g-Glutamyl transpeptidase

Table (1): Comparison of DLBCL patients had previous hepatitis C virus positive with control through some biochemical tests

Biochemical tests	Patients No: 50		mean of Age /years	Laboratory values	
				DLBCL Patients	25 of control who had hepatitis C with no DLBCL
	male	female	mean ± SD	mean ± SD	
Alanine aminotransferase*	31	19	43.2 + 8	51.8 + 7.7	31.9 + 9.6
Aspartate aminotransferase*				22.9 + 8.6	38.4 + 8.7
g-Glutamyl transpeptidase#				24.2 + 7.2	31.6 + 5.4
*Normal value: 43 IU/L/ # Normal value: 45 IU/L.					

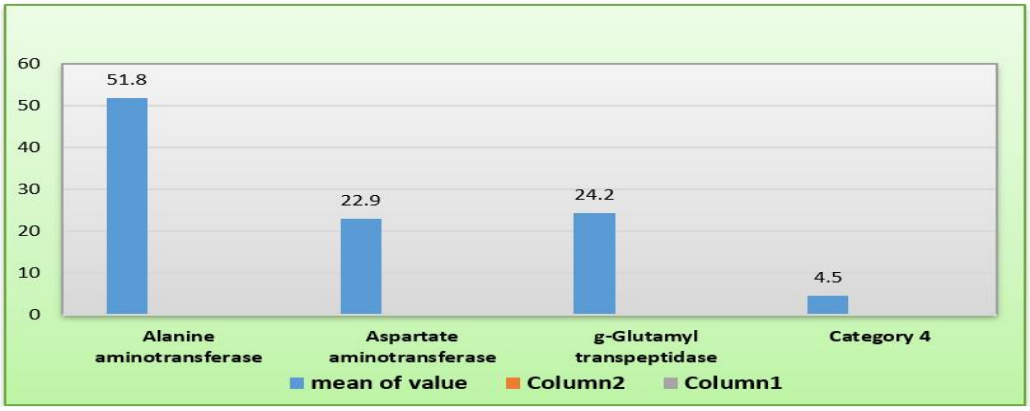

Figure (1): Comparison of DLBCL patients had previous hepatitis C virus positive with control through some biochemical tests

Table (2): HCV RNA load in DLBCL biopsy samples, copies/mg with total RNA						
	HCV RNA load in all biopsy samples copies/mg total RNA		HCV RNA load in DLBCL biopsy samples, copies/mg total RNA		HCV RNA load in biopsy of *control samples copies/mg total RNA	
	Genomic	Antigenomic	Genomic	Antigenomic	Genomic	Antigenomic
1	$1.50*10^5$	8.50 * 104	Negative	Negative	Negative	$1.10*10^3$
2	$1.60*10^5$	$2.45*10^4$	$4.35*10^5$	$2.30*10^5$	Negative	Negative
3	$2.60*10^5$	$8.80*10^4$	5.10*105	Negative	$1.52*10^2$	$1.22*10^4$
4	$1.55*10^5$	$8.56*10^4$	Negative	Negative	Negative	Negative
5	$3.40*10^5$	$2.19*10^5$	$5.10*10^5$	$4.30*10^4$	Negative	$2.00 * 10^4$
6	$9.90*10^4$	$6.00 * 10^4$	$200*10^5$	$1.11*10^5$	$3.04 * 10^2$	$4.11 * 10^4$
*control: Chronic HCV patients without DLBCL						

Table (3): Significance of HCV RNA load in DLBCL samples comparison with control					
	HCV RNA load in DLBCL biopsy samples, copies/mg total RNA		HCV RNA load in biopsy of *control samples copies/mg total RNA		P value
	Genomic	Antigenomic	Genomic	Antigenomic	
1	Negative	Negative	Negative	$1.10*10^3$	
2	$4.35*10^5$	$2.30*10^5$	Negative	Negative	0.0001 *(HS)
3	$5.10*10^5$	Negative	$1.52*10^2$	$1.22*10^4$	0.005 *(HS)
4	Negative	Negative	Negative	Negative	
5	$5.18*10^5$	$4.30*10^4$	Negative	$2.00 * 10^4$	0.001 *(HS)
6	$200*10^5$	$1.11*10^5$	$3.04 * 10^2$	$4.11 * 10^4$	0.006 *(HS)
*(HS): High Significant					

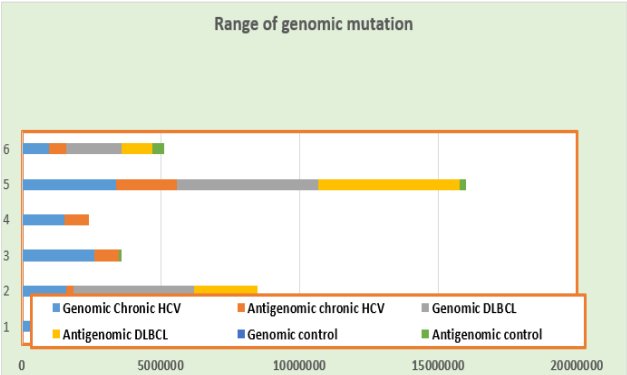

Figure (2): HCV RNA load in DLBCL biopsy samples, chronic and control as copies/mg with total RNA

6. Discussion

HCV infection is highly related with lymphoma, especially non-Hodgkin lymphoma of diffuse large B-cell (DLBCL) and lethargic B cell non-Hodgkin lymphomas.

Patients sample with DLBCL had previous hepatitis C virus positive and control samples who were without DLBCL, all were taken after HCV suppression by anti-HCV treatment, "lethargic B-cell in non-Hodgkin lymphoma relapse, particularly in marginal zone lymphoma, that is occasionally detected, suggestive of a relation between HCV and lymphoma development" [17].

Comparison of DLBCL patients with control through some biochemical tests

In compared with the non-HCV positive asces of patients with diffuse large B-cell lymphoma (DLBCL), the HCV-positive DLBCL patients reported to take altered features, with raised Alanine aminotransferase (51.8+7.7), but not in Aspartate aminotransferase and g-Glutamyl transpeptidase,

Age and Sex

Mean of age 43.2+8, this age may refer to be as a risk factor in progression of this infection and lead to more development events within active immune system, astudy support that said considering the age dissemination, with the prevalence of HCV infection that presented a progressively increased before (60) years old and the occurrence of HCV infection presented a decrease with the age after (60) years old and that may because of the decline in physiological flexibility and economic power that result in reduction the number of elderly

investigation [18].

Male (31) and female (19), so the most number was in males, and that is mean the occurrence of many HCV-RNA developed disease detection was more predominant among males than females, in this study and this supported by some studies said that HCV infection more predominant in male between ages 60 and 69, and they has the equal proportions among them after this old, and the number of HCV infection presented a rise with year [19].

The old age consider from high risk of progression of this desease, with High viral load of HCV-RNA is related with a poorer prognosis in patients with diffuse large B-cell lymphoma (DLBCL) (Tasleem S, Sood GK. 2015) and HCV infection known by causing liver cirrhosis, and hence hepatocellular carcinoma, therefore, the appropriate treatment is very essential, and throughout typical treatments with rituximab, doxorubicin, cyclophosphamide, vincristine and prednisolone: (R-CHOP) for cure DLBCL, in a study, about total of 20% of patients expert degree of hepatic toxicity [20]. So, the DLBCL patients with HCV infection managed with (R-CHOP) might need suspicious checking for hepatic toxicity in addition to correct managing, equally, that is needing for many studies to evaluate hepatitis C flares through treatment period with (R-CHOP) [21].

HCV RNA load in DLBCL patients and control

The results Genomic and Antigenomic DLBCL patients' comparison with control patients were highly significant of (<0.001), and this is refer to

But also, there are many patients in control with previous infection at risk of develop into DLBCL desease, as there are a risk of progressing liver cirrhosis, and/or liver cancer after HCV infection. "It was revealed that HCV is correspondingly a lymphotropic virus. As a significance of the lymphatic infection, several lymphoproliferative disorders (LPDs) have been related with this virus" [22].

It was hypothesized that HCV may be elaborate in the pathogenesis of NHL as well. And this hypothesis was validated by several annotations, including the significantly high occurrence of HCV infection in NHL patients in a number of studies [23].

Many data are currently accessible screening, in most cases, a significant link with B-cell NHL, even with a clear gradient and relating with different histopathological types of lymphoma the most firmly associated being the marginal zone, lymphoplasmacytic, and diffuse large B-cell lymphoma [24].

HCV-positive in DLBCL confirmation to be causative agents

Genomic and Antigenomic results

The results of HCV-RNA presence in patient's biopsies confirm that this viral infection induce transformation of immune B cells with proliferation and progression to DLBCL disease ($200*10^5$) copies/mg total RNA among DLBCL, while were ($3.04 * 10^2$) in control patients, but reversible result in Anti-genomic results, were highly occur in control patients. and this may because of the detail that direct anti-HCV therapy prompts hematologic response in lethargic NHL patients with HCV infection, HCV infection has been related to NHL, and front-line treatment of asymptomatic indolent NHLs is optional [7] Also antigenomic were present in both groups DLBCL and control patients, with more presence in control group, and this maybe due to affect the B cells and prevent or impaired its differentiation to plasma cells and then stopping producing the antibodies, that for imparing and modulating the binding process of antibodies with their specific epitops or conducters in the site of mutated genes within B cells themselves, Although HCV-positive DLBCLs exhibition specific clinical features and consequences, the optimum management and timing of anti-HCV therapy in aggressive lymphoma is quiet indeterminate [13]. Prior reports presented that HCV-positive patients with DLBCL showed poorer overall survival (OS), and the occurrence of severe hepatic toxicity in patients of HCV-positive was considerably higher than that of HCV-negative patients and this hepatic toxicity may result in that HCV is involving in an significant initiation of a reactive oxygen species [6] and this can cause stable B-lymphocyte damage, by means of DNA mutations of a tumor suppressor genes [8]. Nevertheless, many studies solitary involved small records of HCV-positive patients and not all studies concerning HCV infection and DLBCL exhibited dependable results [12].

Because of HCV-associated lymphomas use a restricted immunoglobulin variable region gene selection, so that the lymphoma B-cell receptors expressed as soluble immunoglobulin, and the membrane IgMs do not bind to the HCV antigens. So that follows the majority of lymphomas do not occur from B cells that are elaborate in viral clearance [12]. And this lead to focusing the target of therapy of DLBCL patients with previously infected with HCV, on this pathway.

7. Conclusion

a. The patients with chronic HCV infection are at increased risk to develop DLBCL dieease.

b. HCV-RNA replication in B-lymphocytes has action of oncogenic effect recognized by intracellular HCV proteins.

c. The probable role of viral lymphotropism in the pathogenesis of HCV-associated Lymphoprelfirative Diseases, especially with progressive DLBCL.

d. Genotypes tests play a critical role in evaluating treatment choices and methods for prepare the suitable drugs, as it exposed the prognosis of disease, severity, and response to type of therapy according to genotypes.

8. Recommendation

Post-retardation of HCV eradication, after effective immuno-chemotherapy of DLBCL might be deliberated to avoid relapse.

9. Acknowledgment

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