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Possible Role of Lymphotoxin α, β and their Receptor (TLβRs) in Promoting Liver Carcinogenesis during Infection with Hepatitis C Virus

Areej A Hussein[1] *BSc PhD*, **Ghazi R Auda**[2] *BVM PhD*

[1]Dept. of Microbiology, College of Medicine, Dayala University, Dayala, Iraq, [2]Division of Virology, National Institute for Biological Standards and control. UK

Abstract

Background Lymphotoxin α, β and their receptor play an important role in the control of lymphoid organ development and support of immune responses against pathogens.

Objective To investigate expression of the lymphotoxin α, β and their receptor TLβRs using immunohistochemistry technique in patients with chronic active hepatitis and hepatocellular carcinoma.

Methods Thirty five formalin fixed, paraffin embedded liver tissues, obtained from Liver and Digestive System Technical Hospital and private laboratories in Baghdad, were studied. In addition, thirteen apparently normal liver autopsies were collected from the Forensic Medicine Institute Archives after permission and used as control group.

Liver tissue sections were cut at 4 µm and placed on positively-charged slides, used for the detection of lymphotoxin α, β and receptor TLβRs by immunohistochemistry technique.

Results The expressions of lymphotoxin α, β and receptor TLβRs were detected in most patients infected with HCV, 88%, 84%, 76% respectively in patient with chronic active hepatitis and 80%, 70%, 90% respectively in patients with hepatocellular carcinoma while low level of expression of these markers was observed in healthy control group.

Conclusion Lymphotoxin α, β and their receptor TLβRs may play an important role in the development and progression of HCV associated liver pathology.

Key words lymphotoxin, TLR, carcinogenesis hepatocellular carcinoma.

Introduction

Inflammation is a defensive process initiated by innate and specific cellular and humoral immune component in response to an insulting agent, which in most instances an infectious agent [1,2]. Initiation of the inflammatory process is triggered by activation of the immune component through the release of vasoactive and chemotactic substances elicited due to trauma or infection [3].

Tumor necrosis factor (TNF) superfamily comprises several cytokines including, but not limited to, lymphotoxin (LT) α, β and their tumor necrosis factor receptor (TLRs). These factors are known to play a role in the induction of necrotizing activity of neoplastic cells [4].

Lymphotoxins α and β are known to be responsible for organogenesis and lymphoid tissue maintenance [5]. They are generally produced, under normal physiological circumstances, by activated T, B and NK

lymphocytes and other lymphoid tissue components [6].

The most common cause of chronic hepatitis is infection with HBV and HCV [7]. Persistent infections with these viruses are frequently associated with the development of hepatocellular carcinoma [8]. The role of these infections in the induction of neoplastic changes in liver tissues is still to be elucidated. The core protein of hepatitis C virus is known to have multifunctional features, including binding to the death domain of the tumor necrosis factor receptor type 1 (TNFR 1). It also known to bind to the cytoplasmic tail lymphotoxin β receptor, reflecting a possible involvement in the signaling pathways of apoptosis [9].

The over expression of certain cytotoxic cytokines has been implicated as a possible inducing factor for the progression towards hepatocellular carcinoma (HCC) [10].

This study aims to determine the extent of expression of lymphotoxin α, β and their receptor (TLRs) using immunohistochemistry techniques in patients with chronic HCV infection and hepatocellular carcinoma. The study also aims to elucidate the correlation between these cytokines expression and different clinicopathological variables such as age, gender, histopathological activity index (HAI), stage and grade.

Methods

Study population. Thirty five formalin-fixed, paraffin embedded liver tissue blocks were obtained from patients with confirmed cases of chronic HCV infection and hepatocellular carcinoma. The age of patients was ranged from 17 to 65 years. The histopathological types of hepatocellular carcinoma included in this study were moderately differentiated adenocarcinoma (4 cases) and poorly differentiated adeno-carcinoma (6 cases). All patients had positive test for anti-HCV antibodies (third-generation enzyme linked immunosorbent assay (ELISA). The patients' samples were collected during the period from January 2010 till December 2011 from the archives of histopathology laboratories of liver and digestive system technical hospital and private laboratories in Baghdad, but this research perform during 23, March 2012 till 20, September 2012.

Normal liver specimens were obtained from thirteen persons were collected from the Forensic Medicine Institute Archives.

Formalin-fixed, paraffin embedded tissue blocks were sectioned (4 μm) thickness, one section was stained with Haematoxylin and Eosin, and four sections were mounted on positively charged slides to be used for immunohistochemistry technique for the detection of lymphotoxin α, β and their receptor TLRs.

The histopathological diagnosis of the tissue blocks used in this study was primarily based on that obtained from histopathological records of liver biopsy samples and hospital laboratory records. Confirmatory histopathological re-evaluation of each obtained tissue blocks was done.

Immunohistochemical staining. Was carried out using mouse anti-human lymphotoxin alpha (US Biological- USA Cat. Number L2610-03B), mouse anti-human lymphotoxin beta (abcom-UK Cat. Number ab89568), mouse anti-human lymphotoxin beta receptor (US Biological- USA Cat. Number L8015-03L) and immune-histochemistry detection kit (US Biological/USA Cat. Number 17506).

The slides were deparaffinized by immersion two times in xylene for 5 minutes each time, and they were then rehydrated in serial alcohols in the following order: 100%, 95%, 70% and water for 5 minute each. Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide for 30 minutes. Slides were then washed with distilled water followed by two times in phosphate-buffered saline for 5 minutes.

All of the slides were treated with 1% normal serum and incubated for 30 minutes at room temperature. Excess normal serum was tipped off slides before adding the primary antibody, dilution 1:250 for each lymphotoxin α and TLβRs, dilution 1:500 for lymphotoxin β, as recommended by manufacturer's instructions.

Slides were then incubated overnight at room temperature. In the next day the slides were rinsed gently two times with phosphate-buffer saline for 5 minutes and the slides were incubated with anti-mouse IgG biotin for 30 minutes at R.T then washed two times in phosphate-buffered saline for 5 minutes. Detection solution was added for 30 minutes at room temperature, and then slides were washed two times with phosphate-buffered saline for 5 minutes followed by the addition of the diluted liquid DAB for 20 minutes at room temperature. After soaking the tissue in water, it was counterstained with Hematoxylin for 30 sec. Slides washed well in running tap water for 30 sec, then dehydrated by serial alcohols 70%, 95%, 100%, 100% for 3 minutes each time and two times xylene for 5 minutes then mounted with permanent-mounted medium (DPX) and examined under light microscope at 400 magnification**.** The intensity of reactivity was graded as follows: 0 (absent), + (weak), ++ (moderate), +++ (intense) [11]. The Statistical analysis was performed using Fisher exact test.

Results

Patient's details: Thirty five cases were obtained from patients with chronic HCV infection 15 males (60%) and 10 females (40%) and hepatocellular carcinoma 10 males (100%). The mean age of patients with HCV infection was (37.6 ± 13.3 years) and patients with hepatocellular carcinoma was (44.5 ± 7.8 years). Histopathological typing for hepatocellular carcinoma revealed that 4 cases (40%) had moderately differentiated adenocarcinoma and 6 cases (60%) had poorly differentiated adenocarcinoma. Normal liver specimens were obtained from thirteen persons 8 males (61.53%) and 5 female (38.46%). The mean age was (55.2 ± 9.2 years).

Immunohistochemical staining: The current results revealed a significant increased in the cellular expression of lymphotoxin α and their receptor (TLRs) while non significant increased in expression of lymphotoxin β among the 35 investigated diseased liver samples as showed in Tables 1-3 and fig. 1- 3. On the other hand, there was low positive result among control groups.

Table 1. The Expression of LT- α in studied groups

Result of Immunohistochemistry		LT- α Expression	*P* value
Patients with Chronic HCV infection	Positive Negative Total	22 (88%) 3 (12%) 25 (100%)	< 0.001
HCC	Positive Negative Total	8 (80%) 2 (20%) 10 (100%)	0.001
Control Group	Positive Negative Total	1 (7.69%) 12 (92.30%) 13 (100%)	

Table 2. The Expression of LT-β in studied groups.

Result of Immunohistochemistry		LT-β Expression	*P* value
Patients with Chronic HCV infection	Positive Negative Total	21 (84%) 4 (16%) 25 (100%)	< 0.062
HCC	Positive Negative Total	7 (70%) 3 (30%) 10 (100%)	0.669
Control Group	Positive Negative Total	7 (53.84%) 6 (46.15%) 13 (100%)	

Table 3. The Expression of LTR in studied groups

Result of Immunohistochemistry		LTR Expression	*P* value
Patients with Chronic HCV infection	Positive Negative Total	19 (76%) 6 (24%) 25 (100%)	< 0.001
HCC	Positive Negative Total	9 (90%) 1 (10%) 10 (100%)	< 0.001
Control Group	Positive Negative Total	0 (0%) 13 (100%) 13 (100%)	

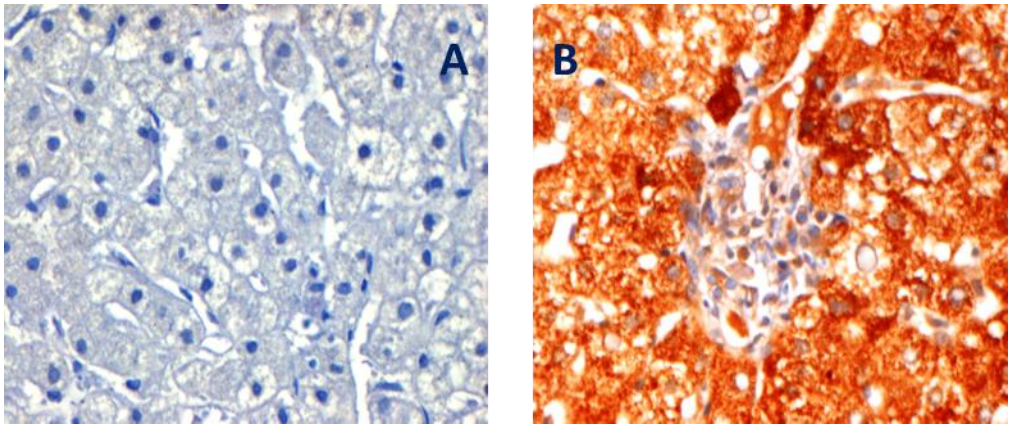

Fig. 1. Immunohistochemistry for LT-α in liver section with chronic HCV infection section, stained by DAB chromogen and counter stained with heamatoxylin. A: Negative expression, B: LT-α positive expression (400X).

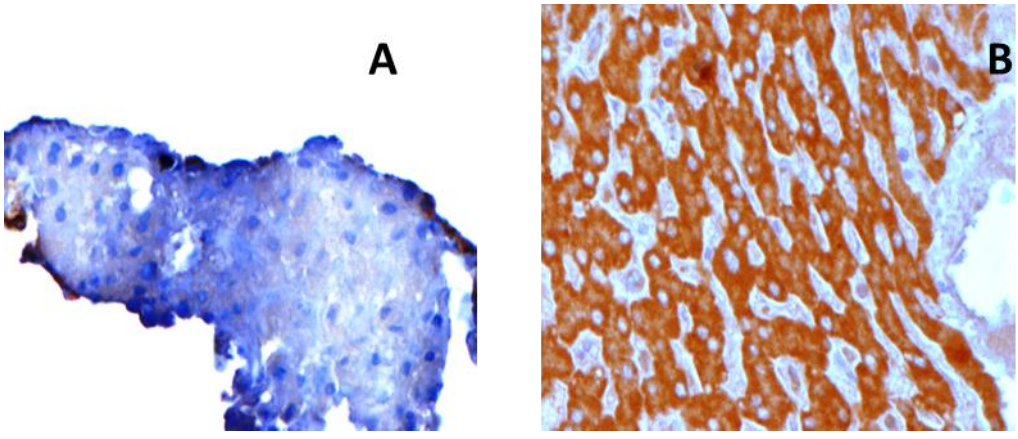

Fig. 2. Immunohistochemistry for LT- β in hepatocellar carcinoma infected section, stained by DAB chromogen and counter stained with heamatoxylin. A: Negative expression, B: LT- β positive expression (400X)

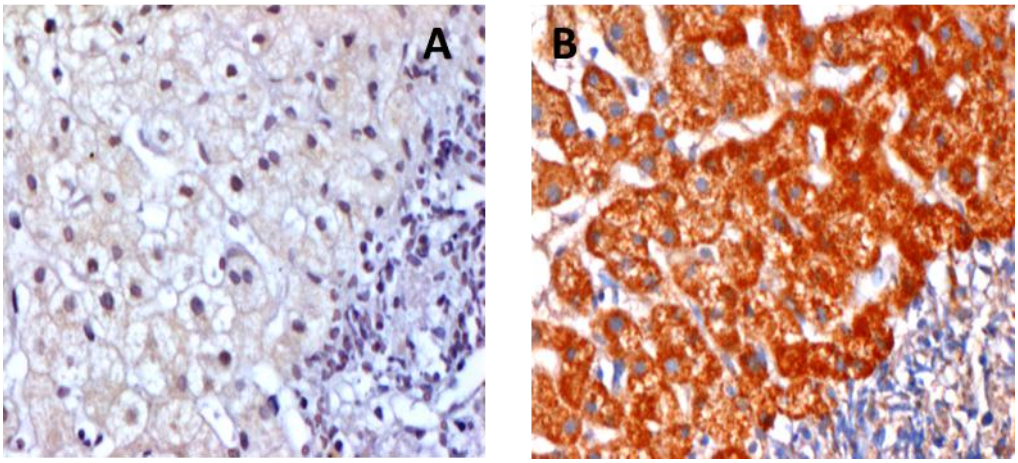

Fig. 3. Immunohistochemistry for LTβR in liver section with chronic HCV infection section stained by DAB chromogen and counter stained with heamatoxylin. A: Negative expression, B: LTβR positive expression (400X)

Tables 4-9 demonstrate correlation between expression of lymphotoxin α, β and their receptor (TLRs) with different variables. The results showed that there were no significant differences between expression of each lymphotoxin β with age, gender, grade and other while significant correlation between lymphotoxin α with age and receptor (TLRs) with disease stage of fibrosis. There was no correlation between hepatocellular carcinoma and the variables, which may be due to the limited sample size.

Table 4. Expression of LT- α in patients with chronic HCV infection and healthy control group

Variables		Expression of LT-α				*P* value
		Neg.	Low	Intermediate	High	
Age	≤ 40	0	2 (9.09%)	0	14 (63.63%)	0.037
	> 40	3 (100%)	2 (9.09%)	1 (4.54%)	3 (13.63%)	
Gender	Male	2 (66.6%)	2 (9.09%)	1 (4.54%)	10 (45.45%)	1.000
	Female	1 (33.3%)	2 (9.09%)	0	7 (31.81%)	
HAI	3/18	1 (33.3%)	0	0	1 (4.54%)	0.896
	4/18	1 (33.3%)	1 (4.54%)	0	5 (22.72%)	
	5/18	1 (33.3%)	2 (9.09%)	1 (4.54%)	6 (27.27%)	
	6/18	0	0	0	1 (4.54%)	
	7/18	0	0	0	1 (4.54%)	
	8/18	0	1 (4.54%)	0	2 (9.09%)	
	9/18	0	0	0	1 (4.54%)	
Stage	0	1 (33.3%)	0	0	2 (9.09%)	0.715
	1/6	0	2 (9.09%)	1 (4.54%)	3 (13.63%)	
	2/6	1 (33.3%)	0	0	3 (13.63%)	
	3/6	1 (33.3%)	1 (4.54%)	0	4 (18.18%)	
	4/6	0	0	0	1 (4.54%)	
	5/6	0	1 (4.54%)	0	1 (4.54%)	
	6/6	0	0	0	3 (13.63%)	
Control		12 (92.30%)	0	0	1 (7.69%)	

Table 5. Expression of LT- α in patients with hepatocellar carcinoma and healthy control group

Variables		Expression of LT-α			
		Negative	Low	Intermediate	High
Age	≤ 40	1 (50%)	1 (12.5%)	2 (25%)	1 (12.5%)
	> 40	1 (50%)	1 (12.5%)	2 (25%)	1 (12.5%)
Gender	Male	2 (100%)	2 (25%)	4 (50%)	2 (25%)
	Female	0	0	0	0
Grade	I	0	0	0	0
	II	0	2 (25%)	0	2 (25%)
	III	2 (100%)	0	4 (50%)	0
Control		12 (92.30%)	0	0	1 (7.69%)	

Table 6. Expression of LT-β in patients with chronic HCV infection and healthy control group

Variables		Expression of LT-β				*P* value
		Negative	Low	Intermediate	High	
Age	≤ 40	1 (25%)	1 (4.76%)	2 (9.52%)	12 (57.14%)	0.116
	> 40	3 (75%)	1 (4.76%)	2 (9.52%)	3 (14.28%)	
Gender	Male	4 (100%)	1 (4.76%)	2 (9.52%)	8 (38.09%)	0.125
	Female	0	1 (4.76%)	2 (9.52%)	7 (33.33%)	
HAI	3/18	1 (25%)	0	0	1 (4.76%)	0.452
	4/18	0	1 (4.76%)	0	6 (28.57%)	
	5/18	3 (75%)	0	2 (9.52%)	5 (23.80%)	
	6/18	0	0	1 (4.76%)	0	
	7/18	0	0	0	1 (4.76%)	
	8/18	0	1 (4.76%)	1 (4.76%)	1 (4.76%)	
	9/18	0	0	0	1 (4.76%)	
Stage	0	1 (25%)	0	0	2 (9.52%)	0.715
	1/6	1 (25%)	0	2 (9.52%)	3 (14.28%)	
	2/6	0	1 (4.54%)	1 (4.76%)	2 (9.52%)	
	3/6	2 (50%)	0	1.4.76%)	3 (14.28%)	
	4/6	0	0	0	1 (4.54%)	
	5/6	0	0	0	2 (9.52%)	
	6/6	0	1 (4.76%)	0	2 (9.52%)	
Control		6(46.15%)	5(38.46%)	2(15.38%)	0	

Table 7. Expression of LT-β in patients with hepatocellar carcinoma and healthy control group

Variables		Expression of LT-α			
		Negative	Low	Intermediate	High
Age	≤ 40	2 (66.6%)	0	1 (14.28%)	2 (28.57%)
	> 40	1 (33.3%)	2 (28.57%)	1 (14.28%)	1 (14.28%)
Gender	Male	3 (100%)	2 (28.57%)	2 (28.57%)	3 (42.85%)
	Female	0	0	0	0
Grade	I	0	0	0	0
	II	2 (66.6%)	0	2 (28.57%)	0
	III	1 (33.3%)	2	0	3 (42.85%)
Control		6 (46.15%)	5 (38.46%)	2 (15.38%)	0	

Table 8. Expression of LTβR in patients with chronic HCV infection and healthy control group

Variables		Expression of LT-βR				*P* value
		Negative	Low	Intermediate	High	
Age	≤ 40	4 (66.66%)	3 (15.78%)	3 (15.78%)	6 (31.57%)	1.00
	> 40	2 (33.33%)	2 (10.52%)	1 (5.26%)	4 (21.05%)	
Gender	Male	3 (50%)	2 (10.52%)	3 (15.78%)	7 (36.84%)	0.653
	Female	3 (50%)	3 (15.78%)	1 (5.26%)	3 (15.78%)	
HAI	3/18	0	0	0	2 (10.52%)	0.634
	4/18	1 (16.66%)	2 (10.52%)	1 (5.26%)	3 (15.78%)	
	5/18	4 (66.66%)	1 (5.26%)	2	3 (15.78%)	
	6/18	0	0	0	1 (5.26%)	
	7/18	0	0	0	1 (5.26%)	
	8/18	1 (16.66%)	1 (5.26%)	1 (5.26%)	0	
	9/18	0	1 (5.26%)	0	0	
Stage	0	0	0	0	3 (15.78%)	0.715
	1/6	5 (83.33%)	1 (5.26%)	0	0	
	2/6	0	1 (5.26%)	1 (5.26%)	2 (10.52%)	
	3/6	0	1 (5.26%)	2 (10.52%)	3 (15.78%)	
	4/6	0	0	0	1 (5.26%)	
	5/6	1 (16.66%)	1 (5.26%)	0	0	
	6/6	0	1 (5.26%)	1 (5.26%)	1 (5.26%)	
Control		13 (100%)	0	0	0	

Table 9. Expression of LTβR in patients with hepatocellar carcinoma and healthy control group

Variables		Expression of LT-βR			
		Negative	Low	Intermediate	High
Age	≤ 40	0	2 (22.22%)	1 (11.11%)	2 (22.22%)
	> 40	1 (100%)	2 (22.22%)	0	2 (22.22%)
Gender	Male	1 (100%)	4 (44.44%)	1 (11.11%)	4 (44.44%)
	Female	0	0	0	0
Grade	I	0	0	0	0
	II	0	0	0	4 (44.44%)
	III	1 (100%)	4 (44.44%)	1 (11.11%)	0
Control	13 (100%)	0	0	0	

Discussion

It has been established that signaling pathways of lymphotoxins can induce both canonical and noncanonical nuclear factor kappa B (NF-kB) cell survival system, whose role in controlling hepatic neoplasia remains controversial [10,12].

The current study had demonstrated that lymphotoxin α and β was over expressed in patients with chronic HCV infection and hepatocellular carcinoma (Table 1). This was in agreement with the finding of Haybaeck *et al* (2009) [14] who reported the role of persistent lymphotoxin pathways in the development of hepatocellular carcinoma. It has been shown, after studying the expression of lymphotoxin α, β and their receptor in HCV infected transgenic mice that overexpressing these proteins in

hepatocytes are more likely to develop chronic hepatitis followed by hepatocellular carcinoma.

Lymphotoxins are implicated indirectly in the induction of endothelogenesis, lymph-angiogenesis and inflammation by their direct action on NK cells to activate stromal cells and the production of Vascular endothelial growth factor A and C (VEGF-A, C) which are a crucial inflammatory mediator [14,15]. In addition, the production of chemokines and adhesion molecules by LT α could be implicated in the recruitment of macrophages which produce VEGF-C. On the other hand, tumor necrosis factor has been shown to up-regulate the expression of VEGF-C by macrophages [16].

Many investigations have documented the role of LTα in host defense mechanisms and reaction to infections, as mice deficient of this cytokine increases their susceptibility to infection with *Staphylococcus aureus* [17]. It was also reported that LTα is required for the granuloma formation and resistance to infection by *Mycobacterium*, *Leishmania*, *Plasmodium and Toxoplasma gondii* infections in mice [18-21]. In a study on transgenic mice it has been proposed that LTα plays a smaller role in the maintenance of lymphoid organs and has no direct involvement in the regulation of TNF [22].

In vivo and *in vitro* studies have indicated that infection with HBV or HCV leads to an increase in the LT expression in hepatocytes [23,24]. Another study performed *in vitro*, revealed that components of LTβR signaling pathway are required for HCV replication [25].

This study has revealed that there is no correlation between lymphotoxin α and the gender of patients, HAI or the stage of the disease. On the other hand, a significant correlation exists with that of the age of patients. This age related increase of incidence of hepatic neoplasia in HCV infected subjects appears to be a attributed to the inherent decline of the immune system and macrophage surveillance in old patients in addition to the increasing incidence of mutations of HCV infected hepatocytes [26].

Hepatocellular carcinoma is considered as the most common primary liver malignancy where the average age at diagnosis ranges from 60 to 80 years. During infection with HCV hepatic cirrhosis develops replacing injured liver cells. Formation of regenerative nodules is one of the healing processes that are usually happening in cases of hepatic cirrhosis and adenomatous hyperplasia. Development of the neoplasm is believed to evolve following cellular mutations that happen at the regenerative nodules which are then transform into malignancy [27].

The results of lymphotoxin β staining, shown in table 2, this result revealed increase in the expression of lymphotoxin β among studied group. This agrees with many authors who indicate that lymphotoxin β is expressed in chronic liver injury [25,28], and with Heliken-Walder *et al.*, (2005) [29] who indicate high expressed LTα and -β in the liver during analysis of two transgenic mouse lines. On the other hand this study did not reveal significant correlation between the positive signals of lymphotoxin β and different clinicopathological variable.

Lymphotoxin α is recognized by the same receptor of tumor necrosis factor and lymphotoxin beta is recognized by its receptor [30].

The current result demonstrated a significant increase in the cellular expression of lymphotoxin β receptor (TLβRs), among patients with chronic HCV infection and those with hepatocellular carcinoma. Previous studies demonstrated the role of LT-α1β2/LT-βR in the transduction of both apoptotic and non-apoptotic signaling pathways [31,32]. Moreover, it has also been reported that activation of LT-β receptor can induce inflammation through the production of chemokines and endothelial adhesion molecules necessary for recruitment of lymphocytes to sites of insult [33-35].

The work by Ruddell and colleagues demonstrated that LT β R signaling regulates hepatic stellate cell function and hepatic wound healing as well as controlling liver homeostasis in both health and disease [36].

Several reports point towards an interaction of the HCV core protein with the LT α R, leading to the modulation of the LT αR-signaling pathway [37-40]. The main finding of this study is that HCV infection activates the production of lymphotoxin α, β and their receptor TLRs in human liver tissues as evidenced by immunohistochemical studies.

Our result revealed there is no significant correlation between positive LTβR signaling with age of patients, grade and HAI, while significant correlation with stage of fibrosis of patients with chronic HCV infection, this may be related with activation of TLR-mediated signaling pathways initiating an early inflammatory response are indispensable for protecting the host against pathogenic organisms, an excessive and/or prolonged activation may lead to both acute and chronic inflammatory diseases. Therefore, the intensity and duration of TLR responses must be tightly regulated. Down regulation of TLR signaling, called TLR tolerance, as well as cross-tolerance among various TLR ligands might have been developed to prevent excessive inflammatory damage to the host [43].

Expression of LTβ-R on human fibroblasts and human carcinoma cell lines maintained in vitro has also been described, and stimulation of these cells through LTβ-R can produce growth stimulation, growth arrest, or cytokine production, depending on the cell type [42-44].

The expression of lymphotoxin α and β and their receptor TLRs has been reported by several researchers. The differences between the results of the these researchers and even with the results of present study could be related to many factors, like type of the tissue whether human or mice, sample size, stage of fibrosis, grade of the tumor, the methodology and affinity of the antibody, the duration of incubation, the sensitivity of detection system and lack of standardized technique because these factors also affect the expression of these markers. In conclusion, three members of tumor necrosis factor super family were over expressed in liver tissues and may be have critical role in the liver injures. Further studies are needed with large sample size to indicate the same results.

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Correspondence to Dr. Areej A Hussein
E-mail: Dr.Areej.Hussein@medicine.uodiyala.equ.iq
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