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Role of Tgf-β1and Gremlin-1in the Pathogenesis of Chronic Hcv Infection and Hepatocellular Carcinoma

KEYWORDS: HCV, gremlin-1, transforming growth factor beta-1, immunohistochemistry technique

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ABSTRACT *Background: Hepatitis C virus (HCV) has evolved various strategies to counteract the host immune response and to establish persistent infection. Transforming growth factor beta (TGF)-β1 and related factors are multifunctional cytokines that regulate growth, differentiation, adhesion and apoptosis of various cells plays critical roles in morphogenesis of various tissues in early embryonic development. Gremlin, secreted by fibroblasts, inhibited bone morphogenic protein (BMP), which mediates stem cell maturation into adult functioning hepatocytes.*

Objective: To elucidate the role of transforming growth factor beta-1 and gremlin-1 in chronic HCV infection and hepatocellular carcinoma pathogenesis.

Methods: Thirty five formalin-fixed of paraffin-embedded liver samples obtained from Liver and Digestive System Hospital and private laboratories in Baghdad were included in this study. In addition, thirteen apparently normal liver autopsies were used as control group. Liver tissue sections were cut at 4 µm and placed on positively-charged slides, for the detection of transforming growth factor beta (TGF-β1) and gremlin-1 using immunohistochemistry technique (IHC).

Results: In the present study, the expression of transforming growth factor-β1in tissues patients with chronic hepatitis C was 72.72% (16 out of 22) and gremlin 1 was 81.81% (18 out of 22), while in patient with hepatocellular carcinoma was 80% (8 out of 10), 90% (9 out of 10) respectively. There were no significant differences between TGF-β1and gremlin-1 markers with age, gender, histological active index, grade and stage of fibrosis. All control cases were negative for two markers. In conclusion, TGF-β1 and Gremlin-1 participates in the pathogenesis of chronic HCV infection and hepatocellular carcinoma, and they might play a possible role as a bio-diagnostic marker in hepatitis C.

Introduction

Hepatitis C Virus (HCV) constitutes a global health problem with 170 million of people infected worldwide. Of those infected with HCV, 80% develop a chronic infection, which can result in cirrhosis and/or hepatocellular carcinoma [1]. Hepatitis C virus genome consists of a positive-stranded RNA of about 9.6kb that encodes a large polyprote in of 3008-037 amino acids. This polyprote in undergoes proteolytic processing by cellular signalases and viral proteases to yield at least 10 mature viral proteins classified as structural or non-structural (NS) proteins [2].

Transforming growth factor (TGF)-β1has been identified as members of the TGF-β1 superfamily consists of more than 30 proteins which includes TGF-βs, activins and bone morphogenetic proteins (BMPs). Activins, BMPs and TGF-β-1 might play important roles in early Embryogenesis [3]. The TGF beta family includes TGF-β1, TGF-β2, and TGF-β3. Like the BMPs, TGF betas are involved in embryogenesis and cell differentiation, but they are also involved in apoptosis, as well as other functions. They bind to TGF-beta receptor type-2 (TGFBR2) [4]. TGF-β1playsa crucial role relatively in the late stage of adult tissues development and it acts as a potent growth inhibitor for most types of cells, including epithelial cells, endothelial cells, hematopoietic cells and lymphocytes [5]. In addition, TGF-β-1functionsas a fibrogenic factor and is responsible for tissue sclerosis of liver, kidney, lung, skin and other tissues.

Gremlin-1 (grem-1) is an inhibitor factor in the TGF-beta signaling pathway, also known as Drm, is a highly conserved 20.7-kDa, 184 amino acid glycoprotein part of the DAN family and is a cysteine knot-secreted protein [6]. Gremlin-1 is known for its antagonistic reaction with bone morphogenetic proteins in the TGF-β1 signaling pathway. Grem-1 inhibits BMP-2, -4, and -7. Inhibition by grem-1 of BMPs in mice allow the expression of fibroblast growth factors (FGFs) 4 and 8 and Sonic hedgehog (SHH) which are necessary for proper limb development [7]. In this study, the objectives were to elucidate the role of transforming growth factor beta-1 and gremlin-1 in the chronic HCV infection and hepatocellular carcinoma pathogenesis, and to elucidate the correlation between these cytokines expression and age, gender and histopathological activity index (HAI), and its stage.

Materials and Methods

Patients and Samples:

Twenty two liver biopsy specimens that diagnosed with chronic HCV infection in 13 males and 9 female and ten (males) specimens from patients who diagnosed with hepatocellular carcinoma were randomly selected from the pathology archive at the Liver and Digestive System Technical Hospital in Baghdad during the period from January 2010 to December 2011. Patient age ranged from 14 to 65 years-old with a mean age of 37.4 years. The histopathological of hepatocellular carcinoma was classified into moderately differentiated adenocarcinoma (Grade II) and poorly differentiated adenocarcinoma. All patients had positive test for anti-HCV antibodies (third-generation ELISA). Normal liver specimens were obtained from 13 persons (8 males and 5 female) with mean age 55.2 years-old as control group. From each tissue block, a series of 4µm sections were cut and stained with hematoxylin and eosin for pathological evaluation and immune histochemistry.

Antibodies and Immunohistochemical Staining:

The antibodies used in this study included anti-TGF-β1 antibody and anti-Gremlin-1 antibody (Cambridge Science Park. England). Immunodetection for TGF-β1 and Gremlin-1was performed according to manufacture instruction of Cambridge Science Company. Sections were dewaxed and subjected to antigen heat retrieval. Endogenous peroxidase

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activity and non-specific binding were blocked by incubation with 3% hydrogen peroxide and protein block, respectively. Slides were then incubated sequentially with primary antibodies for 1hour at 37°C and secondary antibody for 10 minutes at room temperature followed by incubation with the Streptavidine-HRP antibodies for 10 minutes at 37oC. Diaminobenzidinehydrochloride (DAB) was used as the chromogen to visualize peroxidase activity. Sections were counterstained with hematoxylin and overlaid with cover slips.

Evaluation of Immunohistochemistry Results:
Positive reading was indicated when the cells display a brown cytoplasmic pigmentation of immunostaining staining, while negative reading was indicated for absence of immunostaining.

Assessment of TGF-β1 and Gremlin-1 Immunoreactivity:
The scoring system of immunostaining antibodies was evaluated the positively stained cells, which counted at 5 representative random fields (40X) using light microscope, the scoring of the antibodies with the criteria combined intensity with the rate of positive cell. The percentage of positive cells for the protein of interest were scored as1= (0–25%), 2= (26–50%), 3 = (51-75%) and 4= (76-100%)[8]. The scoring was performed using a semi quantitative scale described previously [9]. The intensity of staining and the distribution as an approximate percentage of positive cells were evaluated.

Diagnosis and Staging of Liver Fibrosis
Histological diagnosis by biopsy: In order to objectively evaluate the stage of fibrosis, liver biopsy to diagnose the liver inflammation grade and also the stage of the fibrosis. The most commonly used scoring system, which stages the fibrosis from 0 to 5. Stage 0: normal; Stage 1: portal expansion with fibrosis (<1/3 tracts with wisps of bridging); Stage 2: bridging fibrosis; Stage 3: marked bridging fibrosis or early cirrhosis (with thin septa fibrosis); Stage 4: definite cirrhosis with <50% of biopsy fibrosis; Stage 5: definite cirrhosis with >50% of biopsy fibrosis

Statistical Analysis
The statistical analysis was performed using the t-test with Fischer exact test for quantitative parameters such as age of patient, also used chi-Squared test for qualitative parameters such as gender, histological grade and stage of fibrosis.

Results
Patients details:
The results of this study showed that chronic HCV infection percentage of males was more than females but statistical analysis did not show the significant differences at P>0.05, where 13 males (59.09%) and 9 females (40.90%) out of total cases. The mean age of patients with HCV infection was 37.4 years-old compared with control group with a significant differences at P<0.05. The mean age of patients with HCV infection was 37.6 years-old and patients with hepatocellular carcinoma was 44.5years-old. Hepatocellular carcinoma was 10 cases in males (Table 1). The hepatocellular carcinoma histopathological typing revealed that 4 cases had moderately differentiated adenocarcinoma and 6 cases had poorly differentiated adenocarcinoma.

Immunohistochemical (IHC) staining:
The interested results of this study showed strongly cellular expression of TGF-b1 in 16 cases out of 22 cases were positive of chronic HCV infection with a significant increased at P<0.005, while 8 cases out of 10 cases of hepatocellular carcinoma were strongly positive with a significant increased at P<0.001 (Table 2) and Figure (1). On the other hand there was no positive result among control group.

Table 1: Mean age distribution among the chronic HCV infections and hepatocellular carcinoma

Studied groups	Number		Age/ years		(t-test)/ P-value*
		Mean	Mini	Maxi	
Control	13	55.2	38	68	P<0.0005
Chronic HCV	22	37.4	14	65	
HCC	10	44.5	35	60	

*Significant

Table 2: The Expression of TGF-b1 amongthe chronic HCV infections and hepatocellular carcinoma

Immunohisto-chemistry	Positive	Negative	Total Comparison of Significance p-value
Chronic HCV infection	16 (72.7%)	6 (27.3%)	22 (100%) P<0.005*
HCC	8(80%)	2(20%)	10 (100%) P<0.001*
Control	0	13 (100%)	13 (100%)

*Significant

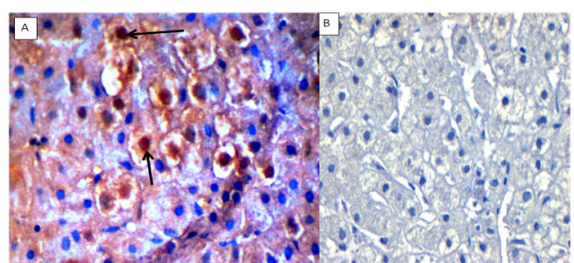

Figure 1: Human liver tissue, Immunohistochemistry for TGF-β–1 in chronic HCV infection section, stained by DAB chromogen is shown as brown in positive cases (arrows), A- positive expression and counter stained with heamatoxylin B- Negative expression X 400.

The result of this study showed strong brown staining was observed in most cells with positive gremlin-1 expression by IHC demonstrated in 18 out of 22 cases (81.8%) of chronic HCV infection cases. Within hepatocellular carcinoma 9 cases (90%) showed positive signal of gremlin-1. There was a highly significant statistically differences found between the patients and healthy control group at p<0.01 (Table 3 and Figure 2).

Table (3): The Expression of Gremlin-1 amongthe chronic HCV infections and hepatocellular carcinoma

Immunohisto-chemistry	Positive	Negative	Total Comparison of Significance p-value
Chronic HCV infection	18(81.8%)	4(18.2%)	22 (100%) P<0.0005*
HCC	9(90%)	1(10%)	10(100%) P<0.001*
Control	0	13 (100%)	13 (100%)

*Significant

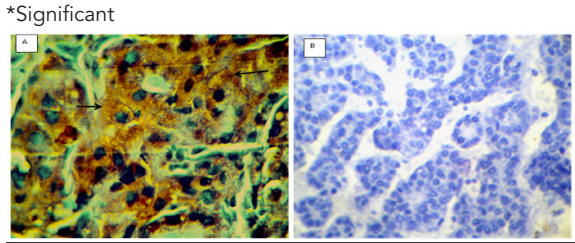

Figure 2: Human liver tissue, Immunohistochemistry for Gremlin-1 in HCC section, stained by DAB chromogen and counter stained with heamatoxylin is shown as brown in positive cases (arrows) expression, A - positive expression and B- Negative X400.

The results showed patients with positive TGF-b1 according to the age group <= 40years was 11 cases, while age group > 40 was 5 cases out of total cases. However there was no

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statistical significant differences noticed at P>0.05. Positive results showed 9 cases in males, while 7 in females out of 22, and the majority of the cases was in score 3, but with no revealed significant differences. The histopathological active index showed the majority of positive cases in score 3 that occurred within 5/18, the statistical analysis revealed significant differences between expressions of TGF-β-1 with each one, while no revealed significant correlation with stage of fibrosis, which showed majority of cases in score 3 (Table 4). Hepatocellular carcinoma showed 8 cases positive of TGF-b1 out of total HCC majority of these cases are scored 3 and grade II (Table 5).

Table 4: The correlation between expressions of TGF-b1 with different variables Expression of TGF-b1 in patients with chronic HCV infection and apparently healthy control group

Variables		Expression of TGF-β1				Significance	
		Neg.	Score 1	Score 2	Score 3	p-value	Sig.
Age	<= 40	3 (21.4%)	1 (7.1%)	3 (21.4%)	7 (50%)	0.405	NS
	> 40	3 (37.5%)	0	0	5 (62.5%)		
Gender	Male	4 (30.8%)	0	2 (15.4%)	7 (53.8%)	0.646	NS
	Female	2 (22.2%)	1 (11.1%)	1 (11.1%)	5 (55.6%)		
HAI	3/18	1 (100%)	0	0	0	0.038	Sig.
	4/18	1 (16.7%)	1 (16.7%)	0	4 (66.7%)		
	5/18	4 (44.4%)	0	0	5(55.6%)		
	6/18	0	0	0	1(100%)		
	7/18	0	0	0	1 (100%)		
	8/18	0	0	3 (100%)	0		
	9/18	0	0	0	1 (100%)		
Stage of fibrosis	0	1 (50%)	0	0	1 (50%)	0.101	NS
	1/6	1 (20%)	1 (20%)	1 (20%)	2 (40%)		
	2/6	3 (75%)	0	0	1 (25%)		
	3/6	1 (16.7%)	0	0	5 (83.3%)		
	4/6	0	0	0	1 (100%)		
	5/6	0	0	0	2 (100%)		
	6/6	0	0	2 (100%)	0		
Control		13(100%)					

Table 5: Expression of TGF-b1 in patients with hepatocellar carcinoma and apparently healthy control group

Variables		Expression of TGF-b1			
Neg.		Score 1	Score 2	Score 3	
Age	<= 40	1(50%)	1(12.5%)	1(12.5%)	2(25%)
	> 40	1(50%)	1(12.5%)	1(12.5%)	2(25%)
Gender	Male	2(100%)	2(25%)	2(25%)	4(50%)
	Female	0	0	0	0
Grade	I	0	0	0	0
	II	0	2(25%)	2(25%)	4(50%)
	III	2(100%)	0	0	0
Control		13(100%)	0	0	0

In the present study, the results of frequency distribution of Gremlin1 scoring did not show significant differences between each score and any of patient's criteria such as age, gender, HAI and stage of fibrosis, the majority of cases showed in score 2 in the HAI and fibrosis (Table 6). Within hepatocellular carcinoma most positive occur in male in age group more 40 years and most positive cases had grade III at score 3 (Table 7).

Table (6): Expression of Gremlin1in patients with chronic HCV infection and apparently healthy control group

Variables		Expression of Gremlin 1				Comparison of Significance	
		Neg.	Score 1	Score 2	Score 3	p-value	Sig.
Age	<= 40	2 (14.3%)	3 (21.4%)	6 (42.9%)	3 (21.4%)	0.5114	NS
	> 40	2 (25%)	2 (25%)	1 (12.5%)	3 (37.5%)		
Gender	Male	4 (30.8%)	2 (15.4%)	3 (30.8%)	3 (23.1%)	0.291	NS
	Female	0	3 (33.3%)	3 (33.3%)	3 (33.3%)		
HAI	3/18	1 (100%)	0	0	0	0.540	NS
	4/18	0	2 (33.3%)	2 (33.3%)	2 (33.3%)		
	5/18	2 (22.2%)	1 (11.1%)	3 (33.3%)	3 (33.3%)		
	6/18	0	1 (100%)	0	0		
	7/18	0	1 (100%)	0	0		
	8/18	1 (33.3%)	0	1 (33.3%)	1 (33.3%)		
	9/18	0	0	1 (100%)	0		
Stage of fibrosis	0	1 (50%)	0	1 (50%)	0	0.126	NS
	1/6	0	0	1 (20%)	4 (80%)		
	2/6	1 (25%)	2(50%)	1 (25%)	0		
	3/6	1 (16.7%)	3 (50%)	0	2 (33.3%)		
	4/6	0	0	1 (100%)	0		
	5/6	0	0	2 (100%)	0		
	6/6	1 (50%)	0	1 (50%)	0		
Control		13(100%)					

Table (7): Expression of Gremlin-1 in patients with hepatocellar carcinoma and apparently healthy control group

Variables		Expression of Gremlin1			
Neg.		Score 1	Score 2	Score 3	
Age	<= 40	0	1(11.11%)	2(22.22%)	2(22.22%)
	> 40	1(100%)	0	0	4(44.44%)
Gender	Male	1(100%)	1(11.11%)	2(22.22%)	6(66.66%)
	Female	0	0	0	0
Grade	I	0	0	0	0
	II	0	0	0	4(44.44%)
	III	1(100%)	1(11.11%)	2(22.22%)	2(22.22%)
Control		13(100%)	0	0	0

Discussion

In the current study, the immunohistochemical evaluation of TGF-β1 expression revealed that sixteen out of 22 cases (72.72%) were positive in tissue of patients with chronic hepatitis C. This result was in agreement with the finding of Miyazono et al. [5] who indicated that TGF-β acts as a potent growth inhibitor for most types of cells, including epithelial cells, endothelial cells, hematopoietic cells and lymphocytes. In addition, TGF-β functions as a fibrogenic factor and is responsible for tissue sclerosis of liver, kidney, lung, skin and other tissues. TGF-β and related factors are produced as dimeric precursors, in which the C-terminal portions form active ligands following proteolytic processing [10]. The secreted TGF-b-like factors bind to two different types of serine/threonine kinase receptor of type I and type II [11].

Regarding the TGF-β1 expression, the positive results revealed that the prevalence of TGF–β1 was higher in age group <= 40 than others as recorded. This may be related to the high incidence of blood contamination at different age group.Concerning the gender, the results revealed that the positive rate of TGF-β1 was higher in male than female, but statistical analysis not revealed significant correlation. While in hepatocellular all cases belong to males.This has previously been explained, in part, by the fact that males have a greater incidence of viral hepatitis and alcoholic cirrhosis [12] also a recent meta-analysis showed a more than multiplicative interaction between HCV infection and cigarette smoking [13]. Some studies have associated increased hepatocellular carcinoma risk with high levels of testosterone [14].

According to histopathological active index statistical analysis revealed significant differences of TGF-b1expressions, this related with the risk of developing HCC for a patient with HCV-related cirrhosis is approximately 2-6% per year [15]. HCC risk increases to 17-fold in HCV-infected patients compared to HCV-negative subjects [16]. Generally, HCC develops only after two or more decades of HCV infection and the increased risk is restricted largely to patients with cirrhosis or advanced fibrosis [17]. It is well known that the incidence of HCC in patients with HCV correlates with the progression of liver fibrosis. The degree of liver fibrosis and the time of acquisition of the infection modify the risk of HCC occurrence and are different in all HCV patients, HCC without cirrhosis in HCV-infected patients, though rare, have been reported. The relation of the virus to the development of HCC is through chronic hepatitis and cirrhosis [18].

Bone morphogenetic proteins (BMP) induce the differentiation of cells of the osteoblastic lineage and enhance the function of the osteoblast. Growth factor activity is regulated by binding proteins. The results of Renata et al. [19] showed that BMPs induce noggin, a glycoprotein that binds and blocks BMP action, additional BMP antagonists such as gremlin. The formalin-fixed, paraffin-embedded archival tissue blocks with chronic active hepatitis C to a recent generation of IHC technique to detect the Gremlin-1, which appeared at 18 out of 22 cases (81.8%) of chronic HCV infection were positive for Gremlin-1. These findings agreed with [20], who found that gremlin 1 was expressed at higher levels in chronic hepatitis infection cases. Also, the present study revealed that 9 out of 10 cases (90%) of hepatocellular carcinoma were positive for

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Gremlin-1. This is in agreement with pervious report revealed that Gremlin mRNA (198 bp) was expressed in 24/35 (68.7%) hepatitis cases and 35/35 (100%) hepatocellular cases [21].

In the present study, the two groups of patients expressed gremlin-1 that was significantly higher in hepatocellular carcinoma cases than hepatitis cases. There are also claims that gremlin possesses an oncogenic role. Data from Sneddon et al. [22] have shown that gremlin is over-expressed in various human malignancies including carcinomas of the cervix, lung, ovary, kidney, breast, colon and pancreas as well as sarcomas. The source of gremlin in highly cellular HCCs is Kupffer cells [23]. Gremlin is produced by fibrotic tissue and inhibits BMP function [24,25]. This production of gremlin may cause maturation arrest of stem cells and their proliferation in an immature state predisposes HCC development. We found that gremlin-1 was expressed at higher levels in chronic hepatitis infection cases, compared with those of normal cases. Gremlin 1 expression correlated with the grade and stage of the disease. These findings are consistent with data from [26, 27]. Gremlin antagonizes BMP function under several disease conditions including idiopathic pulmonary fibrosis [28], proliferative vitreoretinopathy [29] and renal fibrosis [30]. It noncovalently binds to BMP family members to prevent interactions with their cognate receptors, thus altering the effective concentration of active signaling molecule. There are also claims that gremlin possesses an oncogenic role [31].

There is might association of chronic HCV infection and positive expression of Gremlin 1with age. Notable, no significant association has been shown between Gremlin-1 expression and age of patients, could be explained by the prolonged chance of exposure to HCV infection, which were regarded as an important promoting factors in the development of liver disease. Regarding the study of the gender distribution in positive Gremlin-1 results, it was found that no statistically significant differences between males and females. In these findings may be related with male and female have the same chance for exposure to viral infection but in Australian liver cancer is nearly three times more common in males than females [32]. Not found may search for interpretation of my result, this may be related with some studies have been performed using experimental models of carcinogenesis [33], or a different disease setting (etiologic factors other than HCV such as the studies by Durnez et al. [34], and Kumar et al, [35]. The pathogenetic pathways activated in HCCs complicating HCV may be quite different from those occurred in other settings such as chemical carcinogenesis. In conclusion, TGF-β1 and Gremlin-1 participates in the pathogenesis of chronic HCV infection and hepatocellular carcinoma, and they might play a possible role as a bio-diagnostic marker in hepatitis C.

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