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Assessment of Transforming Growth Factor β_1 and DNA Cell Cycle in Egyptian Patients with Chronic Hepatitis-C Virus and Hepatocellular Carcinoma

Zahran Faten[1]*, El- Emshaty Hoda[2], Gouida Mona[3], Hussien Mohamed[4]

*[1]Department of Chemistry, Zagazig University, Egypt
[2]Gastroenterology Center, Mansoura University, Egypt
[3]Mansoura Children Hospital, Mansoura University, Egypt
[4]Mansoura Chest Hospital, Egypt

ABSTRACT

Hepatocellular carcinoma (HCC) is the end-stage of chronic liver diseases (CLDs). The main aetiologies of CLDs are chronic hepatitis C virus (HCV) infection. The aim of this work was to evaluate diagnostic value of TGF-β1 in patients with chronic liver disease (CLD) Fibrosis, HCC and healthy individuals. The present study included 51 patients with Fibrosis (F2-F4), 30 Hepatocellular Carcinoma (HCC) patients, in addition 40 normal healthy individuals were enrolled in this study as control group. AFP and CEA were estimated in all groups. TGF-β1 and DNA Cell Cycle were estimated using Flow Cytometry technique. Results from this study revealed that there was high significance difference in APRI ratio, FIB-4 ratio between HCC, Fibrosis comparing with Control group ($p<0.05$), also there was high prevalence TGF-β1 in HCC patients comparing with both Fibrosis and healthy control group ($P<0.005$). In our study G2/M phase DNA Cell cycle was increased significantly in both HCC and Fibrosis patients. Based on our observation in this study TGF-β1 has diagnostic value importance in assessment of hepatocellular carcinoma.

Keywords: Hepatocellular Carcinoma (HCC), Transforming growth Factor beta1 TGF-β1, Alpha Feto Protein (AFP), Carcinoembryonic Antigen (CEA)

I. INTRODUCTION

Hepatitis C virus (HCV) infection is a global public health burden. Approximately 170 million people are infected with HCV worldwide, and most of these patients become persistently infected. Furthermore, HCV infection in some patients may progress into chronic liver diseases, such as steatosis, cirrhosis, and hepatocellular carcinoma [1]. In Egypt, hepatocellular carcinoma (HCC) is the second most common cancer in men and the 6th most common cancers in women. Hospital-based studies from Egypt have reported an overall increase in the relative frequency of all liver-related cancers in Egypt, from approximately 4% in 1993 to 7.3% in 2003. This rising incidence [2] may be due to high prevalence of hepatitis C virus (HCV) and its complications and the fact that people born 20 years ago or earlier in Egypt has not been vaccinated against hepatitis B virus (HBV) [3]. Because of the prevalence, HCC is the most studied primary liver cancer. Several different histological subtypes are known such as scirrhous HCC, fibrolamellar carcinoma, combined HCC-Cholangiocarcinoma (HCC-CC), sarcomatoid HCC, undifferentiated carcinoma, lymphoepithelioma-like HCC, clear cell HCC, diffuse cirrhosis-like HCC, steatohepatitic HCC, transitional liver cell tumor, and CAP carcinoma [4]. The lack of useful molecular markers to classify HCC aggressiveness hereby complicates clinical analyses to stage patient's outcomes [5]. Development of liver tumors and their evolution to HCC is a multi-step process where different HCC-etiologies provoke continuous rounds of hepatocytes damage and regeneration. These cycles of damage-death-regeneration lead to collagen accumulation contributing to liver fibrosis. Over an extended time, this triggers a cirrhotic state considered as a pathological state of the liver whose lesions can progress to a pre-malignant state producing dysplastic

nodules. Later, these nodules will evolve to HCC invading the surrounding stroma and occasionally generating metastatic events. Transcriptional analyses of liver tumors revealed alterations of several molecular pathways during cancer development implicated in cell proliferation, cell cycle regulation, apoptosis, angiogenesis, cell signaling, metabolism, and immune response (particularly in HCC with HBV/HCV infection). Investigations in Egypt have shown the increasing importance of HCV infection in the etiology of liver cancer, estimated to account for 40-50% of cases, and the declining influence of HBV and HBV/HCV infection (25% and 15%, respectively). The rising incidence of HCC in Egypt could be also explained through improvements in screening programs and diagnostic tools, as well as the increased survival rate among patients with cirrhosis allowing time for some of them to develop HCC [6]. Oncofetal antigens are proteins produced during fetal life and disappear after birth. In cancer patients, these proteins reappear which demonstrates that certain genes are reactivated as the result of the malignant transformation of cells [7]. Carcinoembryonic antigen (CEA) is expressed in various neoplasms of endodermal origin including HCC. However, serum CEA levels alone are not specific for HCC [8]. Transforming growth factor-β (TGF-β) is a central regulator in chronic liver disease contributing to all stages of disease progression from initial liver injury through inflammation and fibrosis to cirrhosis and hepatocellular carcinoma [9]. The cell cycle itself consists of an ordered set of events, ultimately resulting in cell growth and division to produce daughter cells. The eukaryotic cell cycle can generally be divided into four stages known as G1, S, G2 and M [10].

II. SUBJECTS AND METHODS

This study was conducted on 121 Egyptian individuals classified into three different groups as the following: groups I consisted of 51 Fibrosis (F2-F4) with chronic HCV infection, group II consisted of 30 HCC patients due to chronic HCV infection from Gastroenterology Center, Mansoura University, and group III which consisted of 40 healthy individuals with no liver diseases. All patients recruited from Gastroenterology Center, Mansoura University.

Samples collection and Flow cytometry analysis

Six milliliters of venous blood specimens were collected from all patients and healthy control groups. Laboratory investigations included (AST, ALT) [11], total bilirubin [12], and serum albumin [13]. All were performed on Beckman CX9 autoanalyser.

APRI = (AST / upper limit of normal AST) × 100 / platelet count) [14]

FIB-4 = (AST × age / platelet counts × ALT 1/2) [15]

AFP was determined by the ELISA technique. [16]

CEA was determined by ELISA technique. [17]

Peripheral Blood Mononuclear Cells (PBMC) isolation: Human PBMC are isolated using a density gradient technique. The two most commonly used density gradient solutions are Ficoll-Paque PLUS from (Sigma Aldrich co, USA). DNA Cell Cycle was made using Propedium Iodide (PI) from (Sigma Aldrich co, USA). Flow Cytometry analysis of TGF-β1 from (BD Bioscience co, USA) [18]

Statistical Analysis

A computer software package (SPSS), version 16.0 was used in the analysis. For quantitative variables, mean and standard deviation. Frequency and percentage are presented for qualitative variables. Significance level (p) value was expressed as follows: $p > 0.05$ = Insignificant, $p < 0.05$ = Significant and $p < 0.001$ = highly significance. One-way ANOVA test was used for comparing between different groups.

III. RESULTS

In this study our subjects were divided into Group I which including 51 Fibrosis (F2-F4) patients, 42 males (82.4%) and 9 females (17.6 %), their age ranged from 43-87 years with a mean of 59.9±10.1 years. Group II which including 30 HCC patients, 23 males (76.7%) and 7 females (23.3%), and their age ranged from 43-74 years with a mean of 57±8.81 years. Group III which including 40 unrelated healthy adults with no liver diseases as a control group 28 males (70%) and 12 females (30%), and their age ranged from 20-51 years with a mean of 30.8±7.57 years.

In our study, liver function tests including (AST), (ALT), albumin and total bilirubin, were measured using standard methodologies Routine blood pictures including platelets counting were determined. The AST/ALT ratio, FIB-4 and APRI (AST/platelets count ratio index).

TABLE 1: Individual characters in all studied groups

Groups Characters	Group I N= 51	Group II N= 30	Group III N= 40
Age	59.9 ±10.1	57 ± 8.81	30.8 ±7.57
Males	42 (82.4 %)	23 (76.7 %)	28 (70 %)
Females	9 (17.6 %)	7 (23.3 %)	12 (30 %)

TABLE 2: TGF-β_1 Flow Cytometry in all groups.

Groups TGF-β1 (M1%)	Group I N = 51	Group II N= 30	Group III N = 40	P-Value
Mean ± SD.	55.5 ± 18.0	78.6 ± 9.0	12.07± 1.62	**P<0.005**
Range	24.3 – 87.0	59.9 – 88.6	10.1 – 15.3	

TABLE 3: Biochemical parameters in all groups

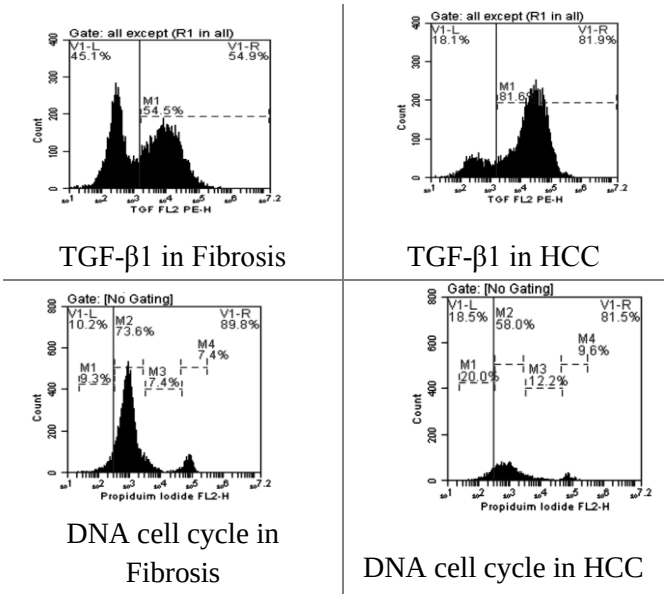
Biochemical parameters	Group I N= 51	Group II N= 30	Group III N= 40	P – value
ALT (U/ml)	**59.9 ± 36.7**	**56.3 ± 35.7**	**12.15 ± 2.6**	**P<0.005**
AST (U/ml)	**73.2 ± 35.6**	**70.1 ± 28.3**	**12.4 ± 2.7**	**P<0.001**
Platelets ($\times 10^9$)	**200.8 ± 47.9**	**153.8 ± 54.5**	**289.3 ± 16.6**	**P=0.000**
Bilirubin(mg/dl)	**4.1 ± 6.3**	**7.4 ± 11.0**	**0.3 ± 0.18**	**P<0.005**
Albumin(g/ dl)	**3.2 ± 0.15**	**2.91 ± 0.25**	**4.3 ± 0.43**	**P<0.001**
AST/ALT ratio	**1.4 ± 0.7**	**1.5 ± 0.58**	**1.07 ± 0.37**	**P<0.005**
APRI ratio	**1.3 ± 1.3**	**0.98 ± 0.61**	**0.1 ± 0.2**	**P<0.001**
FIB-4 ratio	**4.2 ± 3.6**	**3.7 ± 2.3**	**0.38 ± 0.13**	**P<0.005**

TABLE 4: Tumor markers in all groups

Tumor Markers	Group I N =51 Mean ± SD.	Group II N =30 Mean ± SD.	Group III N =40 Mean ± SD.	P Value
AFP (ng/ml)	**25.08 ± 22.2**	**423.3 ± 4.7**	**1.68±0.74**	**P<0.005**
CEA (µg/l)	**2.64 ± 0.66**	**3.61± 0.6**	**1.26±0.13**	**P<0.005**

TABLE 5: DNA Cell Cycle Phases Flow Cytometry in all groups.

Groups / DNA Cell Cycle	Group I N = 51 Mean ± SD.	Group II N = 30 Mean ± SD.	Group III N = 40 Mean ± SD.	P value
Sub G_1	17.7 ± 5.9	9.55 ± 4.72	5.7 ± 3.36	P = 0.000
G_0/G_1	53.4 ± 11.1	62.2 ± 10.02	88.8 ± 3.32	P < 0.001
S – phase	9.75 ± 3.34	15.8 ± 7.6	3.0 ± 0.27	P < 0.005
G_2/M	18.3 ± 11.3	10.2 ± 7.23	2.4 ± 0.81	P <0.005



TGF-β1 in Fibrosis | TGF-β1 in HCC

DNA cell cycle in Fibrosis | DNA cell cycle in HCC

Figure 1: Flow Cytometry of TGF-β1 and DNA Cell Cycle.

IV.DISCUSSION

Chronic liver diseases (CLD) and its end-stages, cirrhosis and hepatocellular carcinoma (HCC), are leading causes of morbidity and mortality worldwide with enormous socioeconomic costs. Patients with liver cirrhosis are at high risk of deadly hepatic failure and well over 80% of HCC develop on a cirrhotic background. HCC ranks as the fifth most common cancer and, with over 600,000 deaths per annum, it constitutes a major global health problem. The main aetiologies of CLDs are chronic hepatitis C virus (HCV) and hepatitis B virus (HBV) infections, alcohol abuse and, as a result of metabolic syndrome reaching

epidemic proportions, an increasing prevalence of non-alcoholic steatohepatitis (NASH) [19].

In this study our subjects were divided into Group I which including 51 Fibrosis (F2-F4) patients, 42 males (82.4%) and 9 females (17.6 %), their age ranged from 43-87 years with a mean of 59.9±10.1 years. Group II which including 30 HCC patients, 23 males (76.7%) and 7 females (23.3%), and their age ranged from 43-74 years with a mean of 57±8.81 years. Group III which including 40 unrelated healthy adults with no liver diseases as a control group 28 males (70%) and 12 females (30%), and their age ranged from 20-51 years with a mean of 30.8±7.57 years. Our data was in agree with **Hussein *et al.*** [20], **Hernandez-Castillo *et al.*** [21], **and Massoud *et al.*** [22], who reported that the mean ages among HCC cases were 53.7 ± 10.1, 57.4 ± 8.7, and 55.2 ± 8 years, respectively.

In the present study ALT was increased in Fibrosis and HCC patients significantly compared with Healthy control group Mean ± SD. were 59.9± 36.7, 56.3± 35.7 and 12.15± 2.6; respectively (P<0.005). Also, AST activity was increased in both Fibrosis and HCC patients comparing with Healthy control group Mean ± SD. were 73.2 ± 35.6, 70.1 ± 28.3 and 12.4 ± 2.7 (U/ml); respectively (p<0.005). Serum bilirubin was increased significantly in both HCC and Fibrosis patients comparing with Health control group with values 7.4 ± 11.0, 4.1 ± 6.3 and 0.3 ± 0.18; respectively (p< 0.005). In our study albumin was decreased significantly in both HCC and Fibrosis patients comparing with Healthy control group with values 2.91 ± 0.25, 3.2 ± 0.15 and 4.3 ± 0.43; respectively (p<0.005). (Table 3). Several investigators detect the levels of biochemical markers (AST, ALT, bilirubins) of liver fibrosis group were higher than healthy individual's groups (all-p < 0.001). Our data agree with **Durazo *et al.*** [23] who reported that the mean value of AST in HCC was 3.5 times the upper limit of normal, and also with **Okonkwo *et al.,*** [24] who found that the serum AST in HCC was 1.39 times the upper limit of normal; the serum ALT level showed a statistically significant difference between the HCC group and the non-HCC group, which was in agreement with **Durazo *et al***. [23]

Alpha-Fetoprotein (AFP) is glycoprotein with a size of 591 amino acids. It is normally synthesized during fetal life, first in the yolk sac and then in fetal liver; its synthesis is normally repressed in adults. [24] High levels of AFP are observed during adulthood only under certain conditions, such as pregnancy, the presence of some neoplasia (e.g. HCC, gastric carcinoma, testicular carcinoma, lung cancer and pancreatic cancer) and some non-neoplastic disorders such as HC and chronic hepatitis.[25] In the present study AFP and (Carcinoembryonic Antigen) CEA were estimated using ELISA technique in Fibrosis, HCC and healthy control the Mean values of AFP were 25.08 ± 22.2, 423.3 ± 4.7 and 1.68±0.74; respectively (p<0.001). The mean values of CEA concentration in Fibrosis, HCC and healthy control patients were 2.64 ± 0.66 and 3.61 ± 0.6 and 1.26±0.13; respectively, this was in agreement with **Hussein *et al***. [20] who showed a significant elevation of serum α-FP in HCC patients. **Durazo *et al.*** [23] found that the serum level of α-FP was significantly higher in HCC patients than in non-HCC patients (P<0.0001).

Transforming growth factor beta-1 (TGF- β1) is a multi-functional cytokine derived from various cell, including leukocytes, Kupffer cells and hepatic lipocytes. It has regulatory roles in growth and differentiation of both normal and transformed cells. TGF-beta-1 seems to be over expressed in HCC compared to that of chronic hepatitis C. [26]. TGF-beta 1 is suggested to play a role in development, growth or progression of hepatocellular carcinoma (HCC) [27]. In our study blood peripheral mononuclear cells TGF-β1 (M1 %) was increased significantly in HCC and Fibrosis patients comparing with Healthy control group with values Mean± SD.78.6 ± 9.0, 55.5 ± 18.0 and 12.07± 1.62 respectively (p<0.005), this agree with number of studies suggest that activation of TGF-β promote HCC development. Transcriptome analysis of human HCC revealed that TGF-β was associated with larger tumors and poor prognosis. [28] In 25% of all early HCC, there is an association between TGF-β signaling and expression of α-fetoprotein (AFP) and EpCAM. [29]

Transcriptional analyses of liver tumors revealed alterations of several molecular pathways during cancer development implicated in cell proliferation, cell cycle regulation, apoptosis, angiogenesis, cell signaling, metabolism, and immune response (particularly in HCC with HBV/HCV infection) [30]. Under normal conditions, hepatocytes are fully differentiated and do not proliferate but are able, upon injury, to exit their quiescent state (G_0), enter the cell cycle, and progress

through the four different cell cycle phases (G_1, S, G_2, M) to finally divide this agree with our study in which blood peripheral mononuclear cells sub G_1 was increased significantly in both HCC and Fibrosis patients with values 9.55 ± 4.72 and 17.7 ± 5.9; respectively comparing with Healthy control group 5.7 ± 3.36 ($p<0.005$). Our data revealed that S-phase was increased significantly in both HCC and Fibrosis patients with values 15.8 ± 7.6 and 9.75 ± 3.34; respectively while in Healthy control group S-phase was 3.0 ± 0.27 ($p<0.005$). In our study G_2/M phase DNA Cell cycle was increased significantly in both HCC and Fibrosis patients with values 10.2 ± 7.23 and 18.3 ± 11.3; respectively while in Healthy control group G_2/M phase was 2.4 ± 0.81 ($p<0.005$). Table (5)

V. CONCLUSION

Based on our observation in this study TGF-β1 and has diagnostic value in assessment of patients with chronic hepatitis-c virus and hepatocellular carcinoma. Hepatocellular damage resulting in change in DNA cell cycle and G2/M phase DNA Cell cycle was increased in both HCC and Fibrosis patients comparing with healthy control.

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